Am. J. Trop. Med. Hyg., 74(6), 2006, pp. 953–959

GENETIC DIVERSITY AND STRUCTURE OF AFRICAN *PLASMODIUM FALCIPARUM* POPULATIONS IN URBAN AND RURAL AREAS

HERVÉ BOGREAU,* FRANÇOIS RENAUD, HOUSEM BOUCHIBA, PATRICK DURAND, SERGE-BRICE ASSI, MARIE-CLAIRE HENRY, ERIC GARNOTEL, BRUNO PRADINES, THIERRY FUSAI, BOUBACAR WADE, ERIC ADEHOSSI, PHILIPPE PAROLA, MOHAMED ALI KAMIL, ODILE PUIJALON, AND CHRISTOPHE ROGIER*

Institut de Médecine Tropicale du Service de Santé des Armées, Unité de Recherche en Biologie et Epidémiologie Parasitaire, Marseille, France; Institut de Recherche pour le Développement, Génétique des Maladies Infectieuses, Montpellier, France; Institut Pierre Richet, Bouaké, Côte d'Ivoire; Centre Muraz, Bobo-Dioulasso, Burkina-Faso; Hôpital d'Instruction des Armées Alphonse Laveran, Service de Biologie Médicale, Marseille, France; Hôpital Principal de Dakar, Dakar, Senegal; Hôpital National de Niamey, Niamey, Niger; Laboratoire de Parasitologie et Mycologie, Institut National de la Santé et de la Recherche Médicale, Unite 399, Institut Fédératif 48, Faculté de Médecine, Marseille, France; Direction et Laboratoire d'Epidémiologie et d'Hygiène Publique, Djibouti Ville, République de Djibouti; Institut Pasteur, Paris, France

Abstract. The genetic variability and population structure of *Plasmodium falciparum* are key factors in malaria control strategies. Studies have suggested no *P. falciparum* population structure although linkage disequilibrium was observed in some African areas. We have assessed length polymorphism at 6–22 microsatellites in four urban and rural sites (Djibouti, Dakar, Niamey, and Zouan-Hounien, n = 240 blood samples). Results have shown a *P. falciparum* population structure in Africa (Fst = 0.17–0.24), lower genetic diversity in Djibouti (He = 0.53) than in the other sites (He = 0.73–0.76), and 3) significant linkage disequilibrium in Djibouti. These results could be related to geographic isolation and low flow of parasites between sites. They also suggest a potential effect of rural suburbs to generate genetic diversity in towns. This could affect the dispersal of selected drug resistance and should be considered when adapting urban malaria control strategies.

INTRODUCTION

Plasmodium falciparum, one of the causes of malaria, still remains a major public health problem. Although many efforts have been made to control this pathogen, between 300 and 500 million clinical cases are still encountered each year. This parasite exhibits developed and complex genetic polymorphism that confers the ability to develop multiple drug resistance[1] or to circumvent vaccines.[2] The emergence and spreading of resistant strains hamper efforts to control malaria and the situation is getting worse in some areas.[3] The spread of drug resistance is due to gene flow and the scale of the *P. falciparum* population structure. A better understanding of *P. falciparum* population genetics is necessary to adjust control measures.

Several studies have investigated *P. falciparum* population structure. Most have analyzed antigen-coding genes or drug resistance–associated genes and such loci are under selective pressure.[4,5] However, analyses are impaired by immunologic effects on selection[6] or drug resistance, which reduces genetic diversity.[7]

Genetic analysis of *P. falciparum* populations with putative neutral microsatellites has been performed in Africa,[8–11] where > 90% of malaria mortality occurs,[3] and where the dynamic and genetics of *P. falciparum* populations remain unclear. Anderson and others have reported that low geographic genetic differentiation and high within-population genetic variability prevail in areas with a high degree of malaria transmission, with little linkage disequilibrium.[8] Such linkage disequilibrium between loci, which suggests non-random genotype distribution, has been observed in Zimbabwe, Congo,[10] and Senegal.[9] Moreover, geographic genetic differentiation exists in Sudan between rural and urban sites.[11] Such findings appear to be inconsistent and new data are necessary to assess the structure and diversity of *P. falciparum* in Africa.

Approximately 25% of the African population lives in cities, and the urbanization rate (2–6% per year) in developing countries remains high.[12] Urbanization impedes malaria transmission and would increase the number of non-immune individuals in urban settings,[13] with most being at risk of contracting potentially severe forms of the disease.[12] Thus, the specificity of the urban epidemiology of malaria must be considered in developing control strategies.[14]

To assess these issues, we conducted a study of *P. falciparum* diversity and structure. Our objectives were to assess the genetic diversity, structure, and differentiation of *P. falciparum* populations according to geographic distances, accessibility, and level of malaria transmission in urban and rural sites. We report the results of genetic analysis and discuss parameters that could explain the observed patterns of diversity and geographic structure.

MATERIALS AND METHODS

The study was conducted in three urban sites in Africa (Dakar, Senegal, Niamey, Niger, and Djibouti City, Republic of Djibouti) and in a rural area (Zouan-Hounien, Danané region, Côte d'Ivoire). *Plasmodium falciparum* populations were characterized by multilocus microsatellite genotyping.

***Plasmodium falciparum* isolates.** Blood samples were collected during the rainy season from *P. falciparum*-infected urban dwellers who came to health centers in Dakar (14°40′N, 17°26′E), Niamey (13°31′N, 2°06′E), and Djibouti (11°35′N, 43°08′E), as well as villagers in the rural area of Zouan-Hounien, Côte d'Ivoire (6°55′N, 8°09′E). Because of their lack of immunity, most of *P. falciparum*-infected urban dwellers were symptomatic.[15,16] Approximately 500 μL of blood were collected from each volunteer after informed consent had been obtained. Samples were frozen and kept at

* Address correspondence to Hervé Bogreau or Christophe Rogier, Institut de Médecine Tropicale du Service de Santé des Armées, Unité de Recherche en Biologie et Epidémiologie Parasitaire, Boulevard Charles Livon, Parc du Pharo, Marseille, France. E-mails: hervebogreau@yahoo.fr or christophe.rogier@wanadoo.fr

–20°C. Ethical clearance was obtained from local ethics committees (Ministries of Health of Djibouti, Senegal, Côte d'Ivoire, and Niger).

Malaria in Dakar is hypo-endemic and transmission is seasonal, remaining less than one infected bite/person/year. The *P. falciparum* prevalence rate is less than 5% in the general population.[13,16,17] Thirty-seven infected blood samples were obtained in September 2002. Malaria in Niamey is mainly hypo-endemic and transmission is estimated to be less than one infected bite/person/year in most of the city, but it may be higher on the Niger River banks. The *P. falciparum* prevalence rate in the general population is generally less than 5%, particularly during the dry season, but may reach 30–50% during the rainy season at a few river banks sites.[18] Forty-three infected blood samples were obtained in December 2001. Malaria in Djibouti is hypo-endemic with local transmission, mostly epidemic, less than one infected bite/person/year.[19] The *P. falciparum* prevalence rate in the general population is less than 5%.[20,21] Forty-two infected blood samples were obtained in September and October 2001.[22] Malaria in the rural area of Zouan-Hounien is holo-endemic, with high transmission (> 300 infected bites/person/year), and perennial. The *P. falciparum* prevalence rate exceeds 80%.[23,24] One hundred eighteen infected blood samples were obtained in July 2001. Malaria endemicity at the four sample sites shows the following pattern: Djibouti < Dakar < Niamey << rural area of Zouan-Hounien.

Genotyping by polymerase chain reaction (PCR). DNA was extracted from blood samples by using the ENZA blood DNA kit according to the manufacturer's recommendations (Biofidal, Vaulx en Velin, France) and eluted in 100 μL of elution buffer per 250 μL of whole blood. DNA repeats (microsatellites) were amplified by PCR with fluorescent end-labeled primers from flanking sequences (Table 1). The primers and PCR conditions are shown in Table 1. All primers were synthesized and purified in the primers production laboratory at the Institute of Tropical Medicine (Marseille, France). The PCR products were subjected to electrophoresis on polyacrylamide gels with internal size standards (Tamra 500; Applied Biosystems, Foster City, CA) using GENSCAN® software (Applied Biosystems).

Molecular markers. Twenty-two microsatellites (Table 1) previously described[25,26] were analyzed. Panel A was composed of six microsatellites (C4M79, Pf2689, TRAP, Pf2802, 7A11, and C4M69) from *P. falciparum* populations from all four studied sites. Panel B was composed of panel A plus 10 additional microsatellites (C4M79, Pf2689, TRAP, Pf2802, 7A11, C4M69, PE14F, 3E7, C3M27, CAL, C3M35, RRR1, SSP, C9M57, MMSA, and Pf9735) from *P. falciparum* populations from Dakar, Djibouti and Niamey. Panel C was composed of six microsatellites (TA40, TA42, TA81, TA87, Pfg377, and 2490), previously used by Anderson and others,[8] were analyzed to compare our results with those of previous *P. falciparum* genetic population studies. All genetic analyses were performed with panels A, B, and C.

Multiplicity of infections. Infected blood samples may contain one or several haploidic clones of *P. falciparum.* In the case of multi-infection, more than one allele can be distinguished at each locus and it is generally impossible to consider the actual multilocus genotype of each clone, i.e., it is unfeasible to match the alleles of distinct loci. However, when several alleles were observed at only one locus and only one allele was observed at each other locus, it was possible to infer reconstruction of multilocus genotypes. For example, if the polymorphic locus exhibited (n) alleles, we scored (n) multilocus genotypes with identical alleles for mono-allelic loci and different alleles corresponding to variant alleles for the only one polymorphic locus. We refer to such data reconstructed multilocus genotypes. The multiplicity of infection, i.e., the number of distinct parasite populations in each isolate, was estimated from the locus that exhibited the highest number of alleles in that given isolate.

We conducted separate analysis considering either mono-infected isolates, i.e., with only one allele shown at a locus, or reconstructed multilocus genotypes from isolates that were multi-allelic at only one locus. The isolates with more than one allele at more than one locus were systematically removed from the analysis.

Measurement of genetic diversity. Genetic diversity was assessed by the number of alleles per locus (A) and Nei's unbiased expected heterozygosity (He)[27] from haploid data using FSTAT version 2.9.4.[28] Differences between sites of the estimated He at each locus were tested by the Wilcoxon signed rank test using STATA 7 software (Stata Corporation, College Station, TX).

Population genetic structure. Population genetic structure was investigated using Wright's F-statistics.[29] The index Fst measures the genetic differentiation between samples. This parameter was computed with FSTAT version 2.9.4.[28,30]

Canonical correspondence analysis (CCA) was carried out to illustrate measures of population structure using CANOCO® software.[31,32] Only isolates scored at each locus were considered for CCA. Analysis was performed with both mono-infected isolates and reconstructed multilocus genotype data. The significance of the canonical axes was tested with a Monte Carlo permutation test.[32] This also allowed estimation of the 95% confidence intervals of the centroid of each population.

Linkage disequilibrium. Deviations from the hypothesis of random association of alleles at distinct loci was tested by permutation procedure using FSTAT version 2.9.4.[28] for each parasite population. We conducted separate analysis of mono-infected isolates and reconstructed multilocus genotypes data. In the case of significant linkage disequilibrium, analyses were performed once again after removing repeated multilocus genotypes from the data set. Associations between all pairs of loci were also estimated by a R^2 coefficient akin to a squared coefficient of correlation[29] and was computed using FSTAT. The sequential Bonferroni procedure was applied to consider the multiple testing enhanced type I error.[33,34]

RESULTS

The number of infected blood samples in which *P. falciparum* populations were detected by PCR genotyping was 42, 37, 43 and 118 in the cities of Djibouti, Dakar, Niamey and the rural area of Zouan-Hounien, respectively (Table 2).

Multiplicity of infections. The proportions of isolates that were multi-allelic and the mean multiplicities of infection estimated with each microsatellite locus and with the three panels are shown in Table 2. The proportions of multi-infected isolates did not differ significantly between cities, but did differ between the cities and the rural area of Zouan-Hounien

TABLE 1
Primer sequences and amplification conditions of 22 microsatellite loci from the *Plasmodium falciparum* genome*

Loci	Panel	GenBank Accession no.	Size, basepairs	Chromosome	Primer sequences	Ta, °C
C3M27	B	G37769	154	3	ATGATCATATTTGGTTAGATC HEX—TTTGGTTAACAAATTTCCTAC	58
C4M79	A and B	G42726	220	3	TTTATATCAAGAATGACAACC HEX—TAGCAACAATAAACAATATGG	55
C3M35	B	G37953	218	4	GGAAATATATATCATACTTGG 6-FAM—TTTTTGGTGTCGGTTATTTTT	55
C4M69	A and B	G37956	362	4	GAAATGGAGATAAACTATTAC TET—AATTACACAACAGATGTGAA	57
SSP	B	G37773	188	4	AATACAGATGAAGGAGAC 6-FAM—TTGCAACGAACAGTCATC	59
Pf2802	A and B	G37818	136	5	GTATAAAAGGAAATACCTA TET—CAGACTATCTTAAGGGAA	54
3E7	B	G37785	188	7	AAGAATGAAAGTATTTTTAGC TET—CCCCTTCAAAAAGGAAATAACAC	59
7A11	A and B	G38831	92	7	ATGTGTAAGGAGATAGTATA 6-FAM—CAACTTTCTCTTTTTAAATATTAC	56
PE14F	B	G38846	126	7	CTGTGGATAATGATATTC 6-FAM—GTCCATTGAAAAGATAGG	54
C9M57	B	G44479	202	9	TGCTTTTTATGTATGCGTAAA TET—TTCTTCTTTCTTTTCAAGTTC	59
MMSA	B	G37834	131	9	TGTAGGAGTAAAATATGT HEX—AATATCACTATTCCTGTA	49
Pf9735	B	G37791	114	11	TATATCATCGGGATGCTA TET—AGGTAATAAGAGTGTTAA	55
Pf2689	A and B	G37854	86	12	TATGCACACACGTTTCTA 6-FAM—CTCCAAGGCATTCACGTA	57
TRAP	A and B	G37858	134	13	CATAATAGTAGCAAGAGA HEX—GATTATATATAGCGATTTAC	49
CAL	B	G37870	256	14	CATAATAGTAGCAAGAGA TET—GATTATATATAGCGATTTAC	49
RRR1	B	G37865	137	14	GTTGTTATAGCTAATGAG TET—ATTATGAACAATTCAGAC	55
TA40	C	G388581	243	10	TET—GAAATTGGCACCACCACA AAGGGATTGCTGCAAGGT	54
TAA42	C	G38832	200	5	6-FAM—TAGAAACAGGAATGATACG GTATTATTACTACTACTAAAG	52
TAA81	C	G38836	118	5	HEX—TGGACAAATGGGAAAGGATA TTTCACACAACACAGGATT	56
TAA87	C	G38838	95	6	6-FAM—AATGGCAACACCATTCAAC ACATGTTCATATTACTCAC	54
Pfg377	C	G37851	95	12	GATCTCAACGGAAATTAT TET—TTATCCCTACGATTAACA	48
2490	C	G37790	80	10	TTCTAAATAGATCCAAAG HEX—TAGAATTATTGAATGCAC	46

* Panel A (C4M79, Pf2689, TRAP, Pf2802, 7A11, and C4M69); panel B (C4M79, Pf2689, TRAP, Pf2802, 7A11, C4M69, PE14F, 3E7, C3M27, CAL, C3M35, RRR1, SSP, C9M57, MMSA, and Pf9735); panel C (TA40, TA42, TA81, TA87, Pfg377, and 2490). Size in basepairs from 3D7 clone. Primer sequences are 5′→ 3′ with fluorescent label (HEX, TET, 6-FAM) and annealing temperature (Ta, °C). Polymerase chain reaction (PCR) conditions for panels A and B; the PCR (15 μL) contained 2 μL of template, 1.5 μL of 10× buffer, 0.1 units *Taq* polymerase (Eurogentec, Seraing, Belgium), 1.5 mM $MgCl_2$, and 0.3 μM of each primer. Thermocycling was performed in a Biometra (Goettingen, Germany) 96-well T3 thermocycler with an initial denaturation at 94°C for 2 minutes; 30 cycles at 94°C for 20 seconds, the locus-specific annealing temperature for 30 seconds, 72°C for 30 seconds, and a final elongation step at 72°C for 30 seconds. Programs were ended at 60°C for 30 minutes. PCR conditions for panel C, volume reaction = 20 μL, 2 μL of template, 2.5 μL of 10× buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, primers 0.075 μM, and 0.5 units *Taq* polymerase (Eurogentec). Themocycling conditions were an initial denaturation at 94°C for 2 minutes; 30 cycles at 94°C for 20 seconds, 45°C for 10 seconds, 40°C for 10 seconds, and 60°C for 30 seconds, and a final elongation step at 72°C for 15 minutes.

($P < 0.003$, by Fisher's exact test). The same pattern was observed with the mean multiplicities of infection. The estimated percentage of multi-infected isolates and the mean multiplicity of infection were higher with panel B (16 loci) than with panel A (6 loci).

Genetic diversity. The number of distinct alleles observed per locus, the unbiased expected heterozygosity (He) estimated per locus and with each panel of loci, are shown in Table 3. The mean He estimated with loci in panel A differed significantly between Djibouti and Niamey ($P < 0.025$) and between Djibouti and Zouan-Hounien ($P < 0.005$). It was similar in Dakar, Niamey, and the rural area of Zouan-Hounien. The mean He did not differ according to the panel of loci used in each site.

Genetic differentiations between geographic sites. Table 4 shows a pairwise comparison between sites of the differentiation coefficients (Fst) estimated with mono-infected isolates and reconstructed multilocus genotypes. Figure 1 shows the results obtained with panel B loci. The centroid of each population is surrounded by its 95% confidence interval. Results of CCA were consistent with Fst estimates. A Monte Carlo test permuting genotypes among the populations (i.e., Dakar, Niamey, and Djibouti) showed that the axes of the CCA could explain differentiation between populations ($P = 0.001$ for 1,000 permutations). The analysis showed that 95% of the genetic diversity observed for the Djibouti population was much smaller than for the two others sites (Figure 1).

TABLE 2
Multiplicity of *Plasmodium falciparum* populations from Djibouti, Dakar, Niamey, and Zouan Hounien*

Panel	Loci	Djibouti n = 42			Dakar n = 37			Niamey n = 43			Zouan Hounien n = 118		
		n	% Multi-infection	Mean multiplicity	n	% Multi-infection	Mean multiplicity	n	% Multi-infection	Mean multiplicity	n	% Multi-infection	Mean multiplicity
A and B	C4M79	42	0	1.0	37	13.5	1.1	43	20.9	1.2	76	48.7	1.7
	Pf2689	42	11.9	1.1	37	13.8	1.2	43	23.3	1.3	100	56	1.8
	TRAP	42	4.8	1.1	37	3.7	1.1	43	27.9	1.3	106	58.5	2.0
	Pf2802	41	12.2	1.1	37	11.4	1.2	43	31	1.3	95	51.6	1.9
	7A11	42	2.4	1.0	37	16.2	1.1	42	23.8	1.3	109	63.3	2.2
	C4M69	42	14.3	1.1	37	15.2	1.2	42	35.7	1.4	91	64.8	2.2
B	PE14F	40	2.5	1.0	29	13.5	1.2	43	23.3	1.3	NA	NA	NA
	3E7	38	0	1.0	27	8.3	1.0	41	22	1.3	NA	NA	NA
	C3M27	41	0	1.0	35	18.9	1.1	40	17.5	1.2	NA	NA	NA
	CAL	39	10.3	1.1	33	8.1	1.2	42	40.5	1.5	NA	NA	NA
	C3M35	42	2.4	1.1	36	11.1	1.1	43	18.6	1.3	NA	NA	NA
	RRR1	40	12.5	1.1	36	10.8	1.1	40	27.5	1.3	NA	NA	NA
	SSP	42	2.4	1.0	37	5.4	1.1	39	15.4	1.2	NA	NA	NA
	C9M57	42	0	1.0	37	2.7	1.1	35	2.9	1.0	NA	NA	NA
	MMSA	40	0	1.0	37	18.9	1.0	38	13.2	1.1	NA	NA	NA
	Pf9735	40	0	1.0	32	6.3	1.1	43	34.9	1.3	NA	NA	NA
C	TA40	33	24.2	1.2	25	20	1.2	NA	NA	NA	NA	NA	NA
	TAA42	37	16.2	1.2	31	6.5	1.1	NA	NA	NA	NA	NA	NA
	TAA81	37	8.1	1.1	32	31.3	1.3	NA	NA	NA	NA	NA	NA
	TAA87	38	5.3	1.1	37	18.9	1.2	NA	NA	NA	NA	NA	NA
	Pfg377	40	2.5	1.0	33	12.1	1.1	NA	NA	NA	NA	NA	NA
	2490	39	0	1.0	32	15.6	1.2	NA	NA	NA	NA	NA	NA
	Panel A	42	26.2	1.3	37	37.8	1.4	43	48.8	1.6	115	75.4	2.7
	Panel B	42	35.7	1.4	37	40.5	1.5	43	62.8	1.8	NA	NA	NA
	Panel C	41	33.3	1.3	37	35.1	1.3	NA	NA	NA	NA	NA	NA

* n = number of isolates with detected *P. falciparum* population; % Multi-infection = percentage of isolates that exhibit more than one population; Mean multiplicity = mean number of parasite populations per isolate. The 22 microsatellite loci are grouped together in panels A, B, and C. NA = not analyzed. Multi-infections and mean multiplicity from each panel with the locus that exhibits the highest number of alleles are shown in the bottom three rows.

Linkage disequilibrium. Complete genotyping of the six loci of panel A identified 69 distinct multilocus genotypes among 86 mono-infected isolates from all sites. Using the genotypes of the mono-infected isolates at the six loci of panel A, we observed that the Djibouti *P. falciparum* population had three significantly associated pairs of loci ($P < 0.0009$) among 15 possible pairs. All associated loci were located on different chromosomes. Among the 14 distinct multilocus

TABLE 3
Genetic diversity of *Plasmodiium falciparum* populations from Djibouti, Dakar, Niamey, and Zouan Hounien*

Panel	Loci	Djibouti			Dakar			Niamey			Zouan Hounien		
		n	A	He	n	A	He	n	A	He	n	A	He
A and B	C4M79	42	4	0.18	37	13	0.81	43	15	0.85	76	25	0.84
	Pf2689	42	3	0.44	37	7	0.60	43	4	0.54	100	10	0.56
	TRAP	42	8	0.68	37	14	0.83	43	14	0.81	106	18	0.78
	Pf2802	42	8	0.70	37	7	0.69	43	9	0.72	95	17	0.76
	7A11	42	6	0.47	37	6	0.64	42	9	0.84	109	13	0.81
	C4M69	42	7	0.71	37	7	0.84	42	10	0.82	91	10	0.85
B	PE14F	40	4	0.39	29	8	0.81	43	9	0.81	NA	NA	NA
	3 E7	38	4	0.20	27	10	0.53	41	14	0.88	NA	NA	NA
	C3M27	41	7	0.48	35	15	0.92	40	19	0.94	NA	NA	NA
	CAL	39	6	0.68	33	22	0.95	42	36	0.98	NA	NA	NA
	C3M35	42	10	0.73	36	17	0.91	43	18	0.94	NA	NA	NA
	RRR1	40	6	0.61	36	14	0.93	40	21	0.92	NA	NA	NA
	SSP	42	8	0.75	37	17	0.92	39	16	0.92	NA	NA	NA
	C9M57	42	4	0.31	37	4	0.26	35	6	0.34	NA	NA	NA
	MMSA	40	5	0.51	37	5	0.66	38	5	0.54	NA	NA	NA
	Pf9735	40	6	0.69	32	10	0.85	43	11	0.83	NA	NA	NA
C	TA 40	33	6	0.71	25	15	0.95	NA	NA	NA	NA	NA	NA
	TA42	37	5	0.42	31	5	0.44	NA	NA	NA	NA	NA	NA
	TA 81	37	3	0.59	32	10	0.84	NA	NA	NA	NA	NA	NA
	TA87	38	5	0.43	37	9	0.80	NA	NA	NA	NA	NA	NA
	Pfg377	40	4	0.21	33	7	0.61	NA	NA	NA	NA	NA	NA
	2490	39	4	0.16	32	5	0.44	NA	NA	NA	NA	NA	NA
		Mean A	Mean HE	SD	Mean A	Mean He	SD	Mean A	Mean HE	SD	Mean A	Mean He	SD
Panel A		6	0.53	0.21	9	0.73	0.11	10.2	0.76	0.12	15.5	0.76	0.11
Panel B		6	0.53	0.19	11	0.76	0.19	13.5	0.80	0.18	NA	NA	NA
Panel C		4.5	0.41	0.21	8.5	0.67	0.22	NA	NA	NA	NA	NA	NA

* n = number of typed isolateds; A = number of alleles; He = biased expected heterozygosity1271 for haploid organisms are shown for each microsatellite loci. NA = not analyzed.

TABLE 4
Genetic differentiation of *Plasmodium falciparum* populations between Africans sites*

		Djibouti		Dakar		Niamey	
	Sample size	Fst	95% CI	Fst	95% CI	Fst	95% CI
Mono-infected isolates							
Djibouti	PA n = 52						
	PB n = 44						
	PC n = 54						
Dakar	PA n = 35	0.109†	0.025–0.181				
	PB n = 33	0.173†	0.112–0.236				
	PC n = 36	0.194†	0.096–0.246				
Niamey	PA n = 34	0.197†	0.102–0.280	0.042‡	–0.008–0.094		
	PB n = 26	0.232†	0.154–0.325	0.020‡	–0.003–0.049		
Zouan-Hounien	PA n = 41	0.243†	0.155–0.325	0.107†	0.005–0.237	0.122†	0.015–0.252
Reconstructed Multilocus Genotypes							
Djibouti	PA n = 30						
	PB n = 22						
	PC n = 28						
Dakar	PA n = 23	0.128†	0.053–0.220				
	PB n = 20	0.154†	0.109–0.193				
	PC n = 24	0.182†	0.102–0.262				
Niamey	PA n = 21	0.195†	0.104–0.278	0.057†	0.003–0.121		
	PB n = 16	0.202†	0.151–0.267	0.042†	0.013–0.076		
Zouah-Hounien	PA n = 27	0.162†	0.069–0.292	0.044†	0.008–0.096	0.031†	–0.005–0.086

Fst-statistic (Weir & Cockernams) computed with Fstat version 2.9.4.
* Results were obtained from mono-infected isolates and from reconstructed data (genotypes inferred from multi-infected isolates displayed at only one locus) are shown in the upper and lower parts of the table, respectively. For each pairwise comparison between sites, sample size, Fst and its 95% confidence interval (CI) are shown according to the three microsatellite panels: PA = panel A (C4M79, Pf2689, TRAP, Pf2802, 7A11, and C4M69); PB = panel B (C4M79, PE14F, 3EF, C3M27, Pf2689, CAL, TRAP, C3M35, Pf2802, 7A11, RRR1, SSP, C9M57, MMSA, C4M69, and Pf9735); PC = panel C (TA 40, TA 42, TA 81, TA 87, Pfg377, and 2490). Confidence intervals were estimated after 1,000 bootstrap simulations over loci. The departure of Fst from zero was tested after 10,000 boostrap simulations and using a Bonferroni corrected *P* value.
† Statistically significant.
‡ Not significant.

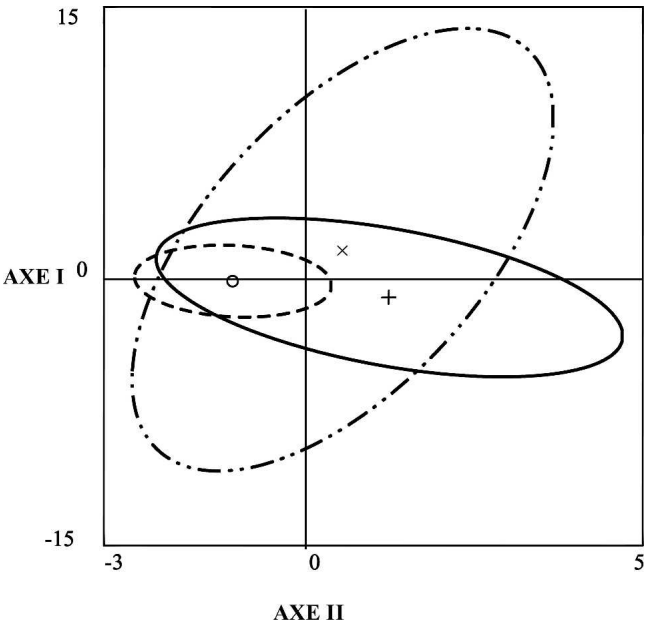

FIGURE 1. Results of canonical correspondence analysis of mono-infected isolates of each population at 16 loci (C4M79, Pf2689, TRAP, Pf2802, 7A11, C4M69, PE14F, 3E7, C3M27, CAL, C3M35, RRR1, SSP, C9M57, MMSA, and Pf9735). Centroids of populations are surrounded by 95% confidence intervals (○ and dashed oval = Djibouti; + and solid oval = Niamey; × and dotted and dashed oval = Dakar).

genotypes identified in 29 mono-infected isolates from Djibouti, five multiple repeated multilocus genotypes were observed. When we analyzed the reconstructed data set with panel A, nine pairs of loci among 15 possible pairs were associated ($P < 0.0009$).

Using the genotypes of the mono-infected isolates at the 16 loci of panel B and the 6 loci of panel C, we showed that the Djibouti *P. falciparum* population had 9 (58 with reconstructed data) and 0 (1 with reconstructed data) associated pairs of loci ($P < 0.0009$), among 120 and 15 possible pairs, respectively. These associations between loci do not remain significant when we counted only the repeated genotype once to perform linkage disequilibrium analysis. The *P. falciparum* populations of Dakar, Niamey, and the rural area of Zouan-Hounien did not exhibit linkage disequilibrium.

DISCUSSION

Our findings provide evidence for support structured *P. falciparum* populations in Africa, and suggest that malaria epidemiology in urban areas depends on local transmission, geographic isolation, and parasite flow between the city and the surrounding rural areas. The proportion of multi-infected isolates and the mean multiplicity of infection in urban dwellers were low and slightly increased in Djibouti, Dakar, and Niamey. This is consistent with the low level of malaria transmission and endemicity in these cities. Genetic diversity did not differ in Dakar, Niamey, and the rural area of Zouan-Hounien and was similar to that previously observed in Africa (Uganda, Congo, and Zimbabwe; He range = 0.76–0.80).[8] In the rural site, the higher percentage of multi-infected isolates and higher mean multiplicity of infections were consistent with the level of transmission, which was100-fold higher than that in the three cities.

In Djibouti, the genetic diversity of *P. falciparum* populations was low. The expected heterozygosity was similar to that observed in Asia (Shoklo, Thailand; He = 0.51) and higher than that observed in South America (Colombia, Bolivia and

Brazil; He range = 0.30–0.40).[8] This could result from isolation and low levels of transmission. Allele fixation due to genetic drift limits genetic diversity. The low *P. falciparum* prevalence rate reported in Djibouti and the rest of the country suggests that the parasite population is small and drift may have a greater effect because the parasite population size is smaller. Low levels of malaria transmission near Djibouti may also limit acquiring genetic diversity from surrounding areas.

Genetic diversity of *P. falciparum* populations sampled in the cities did not depend solely on the low level of transmission in urban conditions. Because African cities are communication nodes and drain human populations from surrounding areas, genetic diversity observed in the cities could reflect genetic diversity of *P. falciparum* populations of surrounding areas. Malaria transmission and endemicity are higher in the savannah around Dakar and Niamey than in the sub-desert area of Djibouti, whereas malaria transmission, endemicity, and complexity of infections are similar in these three cities. Thus, flow of parasite populations between urban and rural areas might be important and explain the differences in genetic diversity observed between Dakar and Niamey, and Djibouti. This implies that the easy access to anti-malarial drugs in the cities and the resulting drug pressure could have an impact on the selection of drug resistance not only in the urban areas but also in the surrounding rural areas. Conversely, the flow of parasite populations from the rural to the urban areas could dilute drug-resistance traits.

The Fst coefficients show evidence of population structure among studied samples spread throughout Africa. Estimated Fsts ranged from 0.109 to 0.243 between Djibouti and the other sites. The Fst was 0.122 between the closest sites geographically, i.e., Niamey and the rural area of Zouan-Hounien. These Fsts were stronger than the estimation reported by Anderson and others[8] in Africa (Fst < 0.01). African *P. falciparum* populations are structured, and at a continental scale these populations are obviously isolated among geographic areas, i.e., they are not panmictic. The slight geographic genetic differentiation between Dakar and Niamey may be related to the road network that covers west Africa at this latitude from Senegal to Niger and permits the easy exchange of people and goods. There was not such interconnection between the other pairs of sites. Moreover, the Fst estimated between Zouan-Hounien and the urban sites (range = 0.107–0.243) suggested substantial geographic isolation of this landlocked rural area. Our results imply that genetic flow between sites is not sufficient to homogenize parasite populations. Selection of drug-resistant *P. falciparum* populations could then act mainly at the local level and the spread of drug resistances in neighboring areas could depend on their isolation. It explains why the *P. falciparum* chloroquine resistance took 12 years to cross Africa from east to west.[35]

Djibouti was the only site that showed linkage disequilibrium, which is consistent with the population structure described earlier in this report. Whatever its origin, linkage disequilibrium will remain longer in an isolated population such as seen in Djibouti. Several non-exclusive hypotheses such as selfing or the Wallhund effect can explain such linkage disequilibrium. An epidemic may lead to a high proportion of a few major multilocus genotypes in remote parasite populations, such as in Djibouti.[19] Moreover, we observed a low number of different multilocus genotypes within blood sample from Djibouti. Therefore, we can expect a high proportion of mating between similar strains (during the sexual phase within the vector) and a limited effect of genetic recombination. If we consider several malaria foci in Djibouti and an epidemic that occurs within one of them, this leads to local selfing that could mime clonal expansion. This would be consistent with the disappearance of linkage disequilibrium after removing the repeated genotype.[19] Although we cannot reject statistical type II error when repeated genotypes are removed, a local epidemic may explain most of the linkage disequilibrium[36] observed in this city. It can be hypothesized that malaria transmission in Djibouti occurs usually in the form of localized micro-epidemics, possibly related to the introduction of new *P. falciparum* populations or to a brief increase in vector densities around focused vector breeding sites.[19] We did not reproduce the linkage disequilibrium observed in Dakar in a previous study,[9] possibly because of differences in sampling.

Our results showed fairly structured *P. falciparum* populations at the scale of the African continent that could be related to geographic isolation and insufficient flow of parasites between sites. Furthermore, we pointed out the potential effect of rural suburbs in generating genetic diversity in towns where malaria transmission is usually low.[13] If one considers urban malaria as malaria of tomorrow, control strategies in towns should take into consideration the malaria situation in rural surroundings and parasite flow between rural and urban areas, which could affect genetic diversity and dispersal of selected drug resistance.

Received August 16, 2005. Accepted for publication November 6, 2005.

Acknowledgments: We thank Professor A. Buguet, V. Buguet, Professor J. Lebras, Dr. F. Ariey, and Dr. R. Prescott for helpful comments on the manuscript. We also thank the people of studied sites for their cooperation during the survey. We are also grateful to the team nurses, health workers, and microscopists who made this study possible.

Financial support. This study was undertaken within the framework of the PAL+ program (DYNAPOP) supported by the French Ministry of Research (PAL+). It is also supported by the Impact Malaria Program of Sanofi-Synthélabo, the Délégation Générale pour l'Armement (PEA 010808), and the Société de Pathologie Exotique (research grant 2003). Patrick Durand and François Renaud are supported by the Centre National de la Recherche Scientifique and the Institut de Recherche pour le Développement.

Disclosure: None of the authors has commercial or other associations that might pose a conflict of interest.

Authors' addresses: Hervé Bogreau, Housem Bouchiba, Bruno Pradines, Thierry Fusai, and Christophe Rogier, Institut de Médecine Tropicale du Service de Santé des Armées, Unité de Recherche en Biologie et Epidémiologie Parasitaire, Boulevard Charles Livon, Parc du Pharo, Marseille, France, Telephone: 33-6-85-94-12-30 or 33-4-91-15-01-50, Fax: 33-4-91-15-01-64, E-mails: hervebogreau@yahoo.fr, housem_b@hotmail.com, bruno.pradines@free.fr, thierry.fusai@free.fr, and christophe.rogier@wanadoo.fr. François Renaud and Patrick Durand, Institut de Recherche pour le Développement, Génétique des Maladies Infectieuses, Unité Mixte de Recherche 2724, Institut de Recherche pour le Développement, Centre National de la Recherche Scientifique, 911 Avenue Agropolis, BP 64501, 34394 Montpellier CEDEX 5, France, E-mails: renaud@mpl.ird.fr and durand@mpl.ird.fr. Serge-Brice Assi, Institut Pierre Richet, Bouaké, Côte d'Ivoire, E-mail: assisergi@yahoo.fr. Marie-Claire Henry, Centre Muraz, 01 BP390, Bobo-Dioulasso 01, Burkina-Faso, E-mail: depauw.henry@fasonet.bf. Eric Garnotel, Hôpital d'Instruction des Armées Alphonse Laveran, Service de Biologie Médicale, 13013

Marseille, France, E-mail: eganotel@mageos.com. Boubacar Wade, Hôpital Principal de Dakar, 1 Avenue Nelson Mandela, BP3006, Dakar, Senegal, E-mail: bwade55@yahoo.com. Eric Adehossi, Département de Médecine Interne B3, BP238, Hôpital National de Niamey, Niamey, Niger, E-mail: eadehossi@yahoo.fr. Philippe Parola, Laboratoire de Parasitologie et Mycologie, Institut National de la Santé et de la Recherche Médicale, Unite 399, Institut Fédératif 48, Faculté de Médecine, Marseille, France, E-mail: philippe.parola@medecine.univ-mrs.fr. Mohammed Ali Kamil, Direction et Laboratoire d'Epidémiologie et d'Hygiène Publique, Djibouti Ville, République de Djibouti, E-mail: drmakamil@intnet.dj. Odile Puijalon, Institut Pasteur Immunologie Moléculaire des Parasites, Centre National de la Recherche Scientifique, Unité de Recherche Associée, 2581, 75015 Paris, France, E-mail: omp@pasteur.fr.

REFERENCES

1. Farooq U, Mahajan RC, 2004. Drug resistance in malaria. *J Vector Borne Dis 41:* 45–53.
2. Smith PG, Milligan PJ, 2005. Malaria vaccine: 3 or 6 months' protection? *Lancet 365:* 472–473.
3. World Health Organization, 2003. Available from http://www.afro.who.int/amd_2003/mainreport.pdf.
4. Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, Rare L, Baisor M, Lorry K, Brown GV, Pye D, Irving DO, Smith TA, Beck HP, Alpers MP, 2002. A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. *J Infect Dis 185:* 820–827.
5. Escalante AA, Cornejo OE, Rojas A, Udhayakumar V, Lal AA, 2004. Assessing the effect of natural selection in malaria parasites. *Trends Parasitol 20:* 388–395.
6. Baum J, Thomas AW, Conway DJ, 2003. Evidence for diversifying selection on erythrocyte-binding antigens of *Plasmodium falciparum* and *P. vivax. Genetics 163:* 1327–1336.
7. Wootton JC, Feng X, Ferdig MT, Cooper RA, Mu J, Baruch DI, Magill AJ, Su XZ, 2002. Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum. Nature 418:* 320–323.
8. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, Whitworth J, Velez ID, Brockman AH, Nosten F, Ferreira MU, Day KP, 2000. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum. Mol Biol Evol 17:* 1467–1482.
9. Leclerc MC, Durand P, de Meeus T, Robert V, Renaud F, 2002. Genetic diversity and population structure of *Plasmodium falciparum* isolates from Dakar, Senegal, investigated from microsatellite and antigen determinant loci. *Microbes Infect 4:* 685–692.
10. Durand P, Michalakis Y, Cestier S, Oury B, Leclerc MC, Tibayrenc M, Renaud F, 2003. Significant linkage disequilibrium and high genetic diversity in a population of *Plasmodium falciparum* from an area (Republic of the Congo) highly endemic for malaria. *Am J Trop Med Hyg 68:* 345–349.
11. Abdel-Muhsin AA, Mackinnon MJ, Awadalla P, Ali E, Suleiman S, Ahmed S, Walliker D, Babiker HA, 2003. Local differentiation in *Plasmodium falciparum* drug resistance genes in Sudan. *Parasitology 126:* 391–400.
12. Keiser J, Utzinger J, Caldas de Castro M, Smith TA, Tanner M, Singer BH, 2004. Urbanization in sub-saharan Africa and implication for malaria control. *Am J Trop Med Hyg 71:* 118–127.
13. Robert V, Macintyre K, Keating J, Trape JF, Duchemin JB, Warren M, Beier JC, 2003. Malaria transmission in urban sub-Saharan Africa. *Am J Trop Med Hyg 68:* 169–176.
14. Trape JF, Pison G, Spiegel A, Enel C, Rogier C, 2002. Combating malaria in Africa. *Trends Parasitol 18:* 224–230.
15. Trape JF, Rogier C, 1996. Combating malaria morbidity and mortality by reducing transmission. *Parasitol Today 12:* 236–240.
16. Trape JF, Lefebvre-Zante E, Legros F, Druilhe P, Rogier C, Bouganali H, Salem G, 1993. Malaria morbidity among children exposed to low seasonal transmission in Dakar, Senegal and its implications for malaria control in tropical Africa. *Am J Trop Med Hyg 48:* 748–756.
17. Diallo S, Konate L, Ndir O, Dieng T, Dieng Y, Bah IB, Faye O, Gaye O, 2000. Malaria in the central health district of Dakar (Senegal); entomological, parasitological and clinical data. *Sante 10:* 221–229.
18. Julvez J, Mouchet J, Michault A, Fouta A, Hamidine M, 1997. Eco-epidemiology of malaria in Niamey and in the river valley, the Republic of Niger, 1992–1995. *Bull Soc Pathol Exot 90:* 94–100.
19. Rogier C, Pradines B, Bogreau H, Koeck JL, Kamil MA, Mercereau-Puijalon O, 2005. Malaria epidemic and drug resistance, Djibouti. *Emerg Infect Dis 11:* 317–321.
20. Carteron B, Morvan D, Rodhain F, 1978. The question of endemic malaria in Republic of Djibouti. *Med Trop (Mars) 38:* 299–304.
21. Rodier GR, Parra JP, Kamil M, Chakib SO, Cope SE, 1995. Recurrence and emergence of infectious diseases in Djibouti city. *Bull World Health Organ 73:* 755–759.
22. Dugelay F, Adehossi E, Adamou S, Ousmane I, Parzy D, Delmont J, Parola P, 2003. Efficacy of chloroquine in the treatment of uncomplicated, *Plasmodium falciparum* malaria in Niamey, Niger, in 2001. *Ann Trop Med Parasitol 97:* 83–86.
23. Briët OJ, Dossou-Yovo J, Akodo E, Giesen NV, Teuscher TM, 2003. *Anopheles gambiae* density and rice cultivation in savannah zone and forest zone of Côte d'Ivoire. *Trop Med Int Health 8:* 439–448.
24. Nzeyimana I, Henry MC, Dossou-Yovo J, Doannio JM, Diawara L, Carnevale P, 2002. The epidemiology of malaria in the southwestern forests of the Ivory Coast (Tai region). *Bull Soc Pathol Exot 95:* 89–94.
25. Su X, Wellems TE, 1996. Toward a high-resolution *Plasmodium falciparum* linkage map: polymorphic markers from hundreds of simple sequence repeats. *Genomics 33:* 430–444.
26. Anderson TJ, Su XZ, Bockarie M, Lagog M, Day KP, 1999. Twelve microsatellite markers for characterization of *Plasmodium falciparum* from finger-prick blood samples. *Parasitology 119:* 113–125.
27. Nei M, 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics 89:* 583–590.
28. Goudet J, 2003. *Fstat (Version 2.9.4), a Program to Estimate and Test Population Genetics Parameters*. Updated from Goudet [1995]. Available from http://www.unil.ch/izea/softwares/fstat.html
29. Wright S, 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution 19:* 395–420.
30. Weir BS, Cockerham CC, 1984. Estimating F-statistics for the analysis of population structure. *Evolution 38:* 1358–1370.
31. Ter Braack CJF, 1986. Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology 67:* 1167–1179.
32. Ter Braack CJF, 1987. *CANOCO—Fortran Program for Canonical Community Ordination*. Ithaca, NY: Microcomputer Power.
33. Rice WR, 1989. Analyzing tables of statistical tests. *Evolution 43:* 223–225.
34. Holm S, 1979. A simple sequentially rejective multiple test procedure. *Scand J Stat 6:* 65–70.
35. Charmot G, Amat-Roze JM, Rodhain F, Le Bras J, Coulaud JP, 1991. Geographic approach to the epidemiology of chloroquine resistance of *Plasmodium falciparum* in tropical Africa. *Ann Soc Belg Med Trop 71:* 187–197.
36. Smith JM, Smith NH, O'Rourke M, Spratt BG, 1993. How clonal are bacteria? *Proc Natl Acad Sci U S A 90:* 4384–4388.