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Authors
Abderrazak, Souha Ben
Oury, Bruno
Lal, Altaf A
et al.

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Plasmodium falciparum: Population Genetic Analysis by Multilocus Enzyme Electrophoresis and Other Molecular Markers

Souha Ben Abderrazak,[1] Bruno Oury, Altaf A. Lal,* Marie-France Bosseno,[2] Pierre Force-Barge, Jean-Pierre Dujardin,[2] Thierry Fandeur,† Jean-Francois Molez,‡ Finn Kjellberg,§ Francisco J. Ayala,¶ and Michel Tibayrenc[3]

*Centre d'Etudes sur le Polymorphisme des Microorganismes (CEPM), UMR CNRS/IRD 9926, IRD, BP 5045, 34032 Montpellier Cedex 01, France; *Molecular Vaccine Section, Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Mail Stop F-12, 4770 Buford Highway NE, Chamblee, Georgia 30341-3724, U.S.A.; †Institut Pasteur de la Guyane Française, 97301 Cayenne Cedex, French Guiana; ‡Laboratoire de Paludologie, Centre IRD, BP 1386, Dakar, Senegal; §CEFE-CNRS, BP 5051, 34033 Montpellier Cedex, France; and ¶Department of Ecology and Evolutionary Biology, University of California, Irvine, California 92697-2525, U.S.A.*

Abderrazak, S. B., Oury, B, Lal, A. A., Bosseno, M.-F., Force-Barge, P., Dujardin, J.-P., Fandeur, T., Molez, J.-F., Kjellberg, F., Ayala, F. J., and Tibayrenc, M. 1999. *Plasmodium falciparum:* Population genetic analysis by multilocus enzyme electrophoresis and other molecular markers. *Experimental Parasitology* **92,** 232–238. The population structure of *Plasmodium falciparum*, the agent of malignant malaria, is uncertain. We have analyzed multilocus enzyme electrophoresis (MLEE) polymorphisms at 7–12 gene loci in each of four populations (two populations in Burkina Faso, one in Sudan, one in Congo), plus one "cosmopolitan" sample consisting of parasite cultures from 15 distant localities in four different continents. We have also performed random amplified polymorphic DNA analysis (RAPD) and restriction fragment length polymorphism (RFLP) and characterized gene variation at four antigen genes in the Congo population. All genetic assays show abundant genetic variability in all populations analyzed. With the isoenzyme assays, strong linkage disequilibrium is apparent in at least two local populations, the Congo population and one population from Burkina Faso, as well as in the cosmopolitan sample, and less definitely in the other Burkina Faso population. However, no linkage disequilibrium is detected in the Congo population with the molecular assays. We failed to detect any nonrandom association between the different kinds of genetic markers; that is, MLEE with RAPD or RFLP, RAPD with RFLP, and so on. Although isoenzyme data show statistical departures from panmictic expectations, these results suggest that in the areas under survey, *P. falciparum* populations do not undergo predominant clonal evolution and show no clear-cut subdivisions, unlike *Trypanosoma cruzi*, *Leishmania* sp., and other major parasitic species. We discuss the epidemiological and taxonomical significance of these results.

Index Descriptors and Abbreviations: strain typing; epidemiological tracking; clonal evolution; self-fertilization; population structure; discrete typing unit.

INTRODUCTION

Little is known about the population structure of *Plasmodium falciparum*, the agent of malignant malaria, although

[1]Present address: Institut Pasteur de Tunis, Laboratoire d'Epidémiologie et d'Ecologie Parasitaire, 13 place Pasteur, BP 74, Tunis-Belvedere, 1002 Tunis, Tunisia.

[2]Present address: UMR CNRS/IRD 9926, Universidad Nacional Autonoma de Mexico, Instituto de Investigaciones Biomedicas, Apartado Postal 70-228, Ciudad Universitaria, Mexico 04510, DF, Mexico.

[3]To whom reprint requests should be addressed. Fax: 33-4-67-416299. E-mail: michel.tibayrenc@cepm.mpl.ird.fr.

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it is often assumed that it approximates a "potentially panmictic" model (Walliker 1985). We have earlier proposed that published data suggest nonrandom association between gene loci in certain natural populations of this parasite (Tibayrenc, Kjellberg, and Ayala 1990; Tibayrenc *et al.* 1991). This proposition has been controversial (Dye, Davis, and Line 1990; Walliker *et al.*, and Luzzatte 1990; Tibayrenc, Kjellberg, and Ayala 1991) and evidence has been presented against the occurrence of linkage disequilibrium in *P. falciparum* (Babiker *et al.* 1991; Conway and McBride 1991). Babiker *et al.* (1994) have analyzed the products of meiosis of *P. falciparum* zygotes in the mosquito vector by the polymerase chain reaction and found a high rate of cross-fertilization in a Tanzanian population of *P. falciparum*. Nevertheless, the issue of this parasite's population structure is not settled. Indeed: (i) although PCR analysis of meiosis products provides valuable information about the basic biology of the parasite, it is poorly informative about what happens 'downstream' from the mosquito, i.e., about the population structure of the parasite in humans, which is the more relevant question for epidemiologists (Tibayrenc 1994, 1995); (ii) in some areas (Papua New Guinea), contrary to the observations in Tanzania, high rates of self-fertilization in *P. falciparum* zygotes have been observed (Paul *et al.* 1995); (iii) the 'strain model' proposed to account for antigenic variation in *P. falciparum* (Gupta and Day 1994; Gupta *et al.* 1994) is hardly compatible (Tibayrenc 1994; Tibayrenc and Lal 1996) with a model of random genetic recombination (Walliker 1985); (iv) few data are available about *P. falciparum* population structure in human populations; and (v) recent results dealing with antigenic diversity suggest clonality in *Plasmodium falciparum* (Rich, Hudson, and Ayala 1997).

The matter is epidemiologically and taxonomically relevant. If *P. falciparum* natural populations are panmictic, this parasite's genotypes must be considered as ephemeral individual variants that are hence unsuitable markers for epidemiological tracking purposes. Moreover, if genetic recombination is frequent, there will be no tendency for the species *P. falciparum* to get structured into stable, separately evolving subdivisions ('Discrete Typing Units' or DTUs; Tibayrenc 1998).

We report here the results of a population genetic analysis of five different *P. falciparum* populations, characterized by multilocus enzyme electrophoresis (MLEE; five populations) and three additional kinds of genetic markers (only one population). These results were obtained in order to explore the population structure of *P. falciparum*.

MATERIALS AND METHODS

Parasite stocks. We have studied five samples of *P. falciparum* (Table 1). Four are local samples, Bobo 1 and Bobo 2 (Burkina Faso), Sudan, and Congo. The fifth is a "cosmopolitan" sample consisting of 29 stocks from a total of 15 locations in different continents; namely Africa (total: 22 stocks; Burkina Faso: 1 stock; Cameroon: 5; Gambia: 4; Ghana: 1, Guinea: 1; Kenya: 1; Mali: 1; Uganda: 1; Zambia: 1; undetermined: 5), Asia (total: 4 stocks; China: 1; Thailand: 3), Latin America (Brazil and Honduras: 1 stock each), and Papua New Guinea (1 stock). Bobo 1 and Bobo 2 are samples taken 1 year apart from infected placentas obtained in the maternity of Bobo Dioulasso (Burkina Faso). The Sudan and Congo samples were collected within 2 years previous to our electrophoresis analysis. These and the cosmopolitan stocks were cultured following established procedures (Trager and Jensen 1976).

All populations were characterized by MLEE, whereas only the Congo population was analyzed by additional molecular markers (see below).

Isoenzyme electrophoresis. Following previously described methods for cellulose acetate isoenzyme electrophoresis (Ben Abderrazak *et al.* 1993), we have studied 12 gene loci coding for enzymes. Seven loci were analyzed in all populations, namely: glucose phosphate isomerase (GPI, E.C. 5.3.1.9); glutamate dehydrogenase (GDH, E.C.1.4.1.2); hexokinase (HK, E.C.2.7.1.1); lactate dehydrogenase (LDH; E.C.1.1.1.27); leucine amino peptidase (LAP, EC 3.4.11); peptidase 1, substrate L-leucyl-leucine-leucine (PEP1, EC 3.4.11); and peptidase 2, substrate L-leucyl-Lalanine (PEP2, EC 3.4.11). Two loci, namely glutathione reductase (GSR, EC 1.6.4.2) and 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), were studied in all populations except Bobo 2; and 3 loci, namely adenosine deaminase (ADA, E.C.3.5.4.4), isocitrate dehydrogenase (IDH, EC 1.1.1.42), and nucleoside hydrolase, substrate inosine (NH-i, EC 3.2.2.), were studied in the Sudan, Congo, and cosmopolitan samples.

DNA preparation. DNA was prepared from culture parasites (2–5% parasitemia) as follows. The parasite erythrocyte pellet obtained after centrifugation of the culture was resuspended in 0.15% saponin in phosphate-buffered saline (PBS) and then incubated at room temperature for 15 min. The lysed erythrocyte suspension was then transferred to a microcentrifuge tube and spun for 10 min at 8000*g*. The hemoglobin supernatant and red cell ghosts were removed. The parasite pellet was then resuspended in cold PBS and the centrifugation step was repeated. After removing the supernatant and residual ghosts, the parasite pellet was lysed by the addition of 400 μl of lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 50 mM EDTA, pH 8.0, 1.0% SDS) supplemented with 100 μg/ml of proteinase K. The extraction was carried out by incubation at 42°C for up to 2 h. The DNA was extracted twice with an equal volume of phenol–chloroform–isoamyl alcohol (25/24/1) and was precipitated with ethanol in the presence of 0.1% NaCl (5.0 M). RNA was then digested with RNase (Boehringer Mannheim) (100 μg/ml) for 1 h at 37°C, followed by phenol–chloroform–isoamyl alcohol (25/24/1) extraction. The genomic DNA was precipitated in the presence of salt and ethanol and reconstituted in 50 μl of TE buffer (0.01 M, pH 8.0).

Random amplified polymorphic DNA (RAPD). RAPD analysis was performed according to Williams *et al.* (1990) with slight modifications. RAPD reactions were performed on the genomic DNAs using each of

20 10-mer primers (kit A; obtained from Operon Technologies, Alameda, CA, U.S.A.). The following six primers were used in this study: OPA 02 (5′-TGCCGAGCTG-3′), OPA 07 (5′-GAAACGGGTG-3′), OPA 08 (5′-GTGACGTAGG-3′), OPA 09 (5′-GGGTAACGCC-3′), OPA 10 (5′-GTGATCGCAG-3′), and OPA 18 (5′-AGGTGACCGT-3′). Each reaction was carried out in 15 μl of reaction mixture containing 1× reaction buffer II (100 mM Tris–HCl, pH 8.3; 500 mM KCl, Perkin–Elmer–Cetus, Norwalk, CT, U.S.A.), 1.0 mM $MgCl_2$, 0.2 μm primer, 0.2 mM each dNTP, 2.5 UI of *Taq* DNA polymerase (Gibco BRL, Grand Island, NY, U.S.A.). This reaction mixture was added to 10 μl of template DNA. Two DNA concentrations were used for each sample: 12 and 4 ng per reaction. The RAPD PCR analysis for each primer was repeated at least once. Negative controls for each primer contained all of the above components except for 10 μl of distilled water in place of *P. falciparum* DNA. Thermocycling was performed with a GeneAmp PCR system 9600 thermocycler (Perkin–Elmer–Cetus), using the following thermal profile: 94°C for 5 min, then 45 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min, followed by a final 72°C extension for 7 min. Amplification products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining.

Polymerase chain reaction (PCR) amplification of antigen gene. We used published sequences of genes that have conserved 5′ and 3′ ends but contain a region with blocks of repeated sequences which vary in size and DNA sequence from strain to strain (Kemp *et al.* 1987). We selected highly conserved regions and chose primers that spanned the repeats in four surface antigens: ring-infected erythrocyte surface antigen (RESA, Favalaro *et al.* 1986), the precursor of the major merozoite surface antigen-1 (MSA-1; Machay *et al.* 1985), the major surface antigen-2 (MSA-2; Fenton *et al.* 1991; Smythe *et al.* 1991), and circumsporozoite surface protein (CSP; Dame *et al.* 1984).

The primers used for the RESA gene were 5′ primer AL 594 (5′-GATCAAGGAGGAGAGAACC-3′) and 3′ primer AL 595 (5′-CAGCATTAACACCAACACC-3′). For the MAS-1 gene, the following primers were used: 5′ primer AL 596 (5′-GAAGATGCAGTATTGACAGG-3′) and 3′ primer AL 597 (5′-GAGTTCTTTAATAGT-GAACAAG-3′). For the amplification of the MSA-2 gene, the following primers were used: 5′ primer AL 598 (5′-GAGTATAAGGAGAAGTATGG-3′) and 3′ primer AL 599 (5′-CCTGTACCTTTATTCTCTGG-3′). The sequence of the primers used to amplify the CSP gene were 5′ primer AL 600 (5′-ATAGTAGATCACTTGGAGA-3′) and 3′ primer AL 601 (5′-GCATATTGTGACCTTGTCCA-3′).

A total of 1 to 5 μg of DNA was amplified in a final volume of 100 μl in the presence of 200 μM (each) deoxynucleoside triphosphate, 1 μM (each) primer, and 2.5 U of *Taq* DNA polymerase (Gibco BRL) in the buffer supplied by Gibco BRL. Thermocycling was performed with a GeneAmp PCR system 9600 thermocycler (Perkin–Elmer–Cetus), using the following thermal profile: initial denaturation for 2 min at 94°C, 20 s at 55°C, and 20 s at 72°C (Wooden 1992). PCR products were detected by electrophoresis of 20 μl of each reaction on 2% agarose gels. Gels were made and run in 1× TBE buffer (Gibco BRL). The DNA was stained with ethidium bromide.

Restriction fragment length polymorphism (RFLP). *P. falciparum* DNA was digested with *Hinf* I (Gibco BRL), fractioned by agarose gel electrophoresis, and blotted on GeneScren Plus membrane (Biotechnology Systems). Probe pC4H32 was radiolabeled by the Random Primers DNA Labeling System (Gibco BRL). DNA isolation, endonuclease restriction, agarose gel electrophoresis, and filter hybridizations were performed with standard methods (Sambrook, Fritsch, and Maniatis 1989).

The pC4.H32 insert contains a 0.5-kb imperfectly repeated sequence found in subtelomeric regions of multiple chromosomes. Restriction site variations both within and outside of the 0.5-kb repeat contribute to the fingerprint polymorphisms.

Data analysis. We have earlier developed methods suitable for testing deviations from random mating in populations of parasitic protozoa (Tibayrenc, Kjellberg, and Ayala 1990; Tibayrenc *et al.* 1991). The segregation tests used in earlier studies are not applicable to *P. falciparum*, because the parasite forms we have sampled (i.e., asexual forms) are haploid. However, the recombination tests previously proposed, namely d1, d2, e, and f (Tibayrenc, Kjellberg, and Ayala 1990) are usable whatever the ploidy level of the organism under study. These recombination tests are based on the null hypothesis of random genetic exchange and appraise different consequences of linkage disequilibrium between loci.

Test d1 relies upon a combinatorial analysis and gives the probability of sampling the most frequent genotype as many as or more times than actually observed in a population. Test d2 measures the probability of observing as many as or more individuals of any genotype than actually observed of the most common genotype. Test e gives the probability of observing as few as or fewer different genotypes than found in the sample. Test f estimates the probability of observing a linkage disequilibrium as high or higher than actually found. The d2, e, and f tests are based on Monte Carlo simulations, with 10^3 iterations in the present study.

RESULTS AND DISCUSSION

Overall genetic diversity. All isoenzyme loci are polymorphic, with 75–100% loci polymorphic in any given population (Table 1). Three electrophoretically distinguishable alleles occur at each of four loci; two alleles at the other eight loci. The heterozygosity, or genetic diversity ($1 - \Sigma x_i^2$, where x_i is the allele frequency) per locus has an average of 0.353 for the total data set, with a range from 0.142 to 0.516 in a given population.

Table 1 gives, for each population, the number of different multilocus genotypes observed, which is 42 for the 114 stocks. The "diversity index" is the ratio of the number of different genotypes to the number of stocks, calculated for the seven loci assayed in every population. The diversity index for pooled data is 0.37, which is lower than for any local population (0.56–0.71; 0.52 for the cosmopolitan set), indicating that the local samples are genetically heterogeneous (Table 1).

In the case of the Congo population, additional markers (RAPD, RFLP, and antigen genes) show a similarly high level of genetic variability (see Table 1).

Population structure. Results of the tests for nonrandom association between loci are given in Table 1. For isoenzyme data, there are, in the whole sample, 17 instances when a

TABLE 1
Genetic Variation and Population Genetic Tests for Five *Plasmodium falciparum* Populations

Population	Sample size	Gene loci		Multilocus genotypes observed	Incidence of most common genotype		Linkage disequilibrium tests			
		N	% Polymorphic		Observed	Expected	d1	d2	e	f
Bobo 1 MLEE	17	9	78	13	3	0.25	0.002	NS	NS	$<10^{-3}$
Bobo 2 MLEE	18	7	86	10	5	1.63	0.02	NS	NS	NS
Sudan MLEE	19	12	75	16	2	0.40	0.06	NS	NS	NS
Congo MLEE	31	12	100	25	2	0.005	10^{-5}	NS	NS	$<10^{-3}$
Congo RAPD	31	6	100	31	—	—	NS	NS	NS	NS
Congo RFLP	30	1	100	—	2	ND	ND	ND	ND	ND
Congo Ag	15	4	100	14	2	1.14	NS	NS	NS	NS
Cosmopolitan MLEE	29	12	100	19	4	0.53	0.002	0.006	$<10^{-3}$	$<10^{-3}$
Total	114	7	100	42	15	8.54	0.023	0.03	$<10^{-3}$	$<10^{-3}$

Note. N, number of genetic loci tested; ND, not done; NS, nonsignificant; MLEE, multilocus enzyme electrophoresis; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; Ag, antigene genes.

particular parasite isolate exhibits two electromorphs at one or more loci. Since the parasite forms sampled are haploid, we interpret these two-allele instances as mixtures and have removed them for calculating the statistics for nonrandom association, given that the multilocus associations are ambiguous whenever two alleles are observed at two or more loci. We have repeated the statistical tests for nonrandom associations using the allelic frequencies obtained when the mixed-culture data are included; the results are not materially different from those shown.

For the d1 test, the observed and expected (assuming random association between loci) frequencies of the most common genotype are shown in Table 1, in the two columns preceding d1. The test is statistically significant for the Bobo 1, Bobo 2, and Congo populations and also for the cosmopolitan sample and the total data set. All other tests are statistically significant for the cosmopolitan sample and the combined total; the f test is significant also for the Bobo 1 and the Congo populations.

In summary, when isoenzyme data are considered, there is evidence of significant linkage disequilibrium in two (Bobo 1 and Congo) of the four local populations studied; and d1 indicates nonrandom multilocus associations also in Bobo 2.

Other molecular assays were performed, but only in the Congo population. All tests for linkage disequilibrium based on these assays are negative (see Table 1). No statistically significant correlation is found, either between different pairs of markers, that is, between isoenzymes and RAPD, or between RAPD and RFLP, or any other pair combination. Correlation between independent sets of genetic markers is strong evidence for linkage disequilibrium and is the basis for the 'g' test (Tibayrenc, Kjellberg, and Ayala 1990; Tibayrenc 1995). In the present case, lack of correlation between different genetic markers is illustrated in Fig. 1, which shows for the Congo sample that the unweighted pair-group method with arithmetic averages (UPGMA) dendrograms (Sneath and Sokal 1973) based on isoenzymes are not congruent with those based on RAPD. A totally different picture is obtained with *Trypanosoma cruzi*, the agent of Chagas' disease, in which dendrograms based on different sorts of markers are congruent with one another (Tibayrenc *et al.* 1993).

In summary, several cases of significant linkage disequilibrium have been found in different populations of this sample for the isoenzyme data. But there is no evidence of linkage disequilibrium in the Congo population, on the basis of the other molecular data, whether the different sets of markers are considered separately or in combination.

It is therefore apparent that in the Congo population of *P. falciparum* there is no evidence of predominant clonal evolution, nor of clear-cut subdivisions ('discrete typing units' or DTUs; Tibayrenc 1998), such as are observed in other parasitic species, like *Trypanosoma cruzi* or *Leishmania* (Tibayrenc, Kjellberg, and Ayala 1990; Tibayrenc 1995). Indeed, the evidence of linkage disequilibrium obtained in the present study of *P. falciparum* is far weaker than that for these other parasites. This result is not likely to be due to lack of resolution of the tests, owing to low levels of genetic variability that would lead to statistical type II errors (Tibayrenc 1995). All the samples included in the present study show notable levels of genetic diversity (Table 1).

In the UPGMA dendrogram elaborated for the Congo

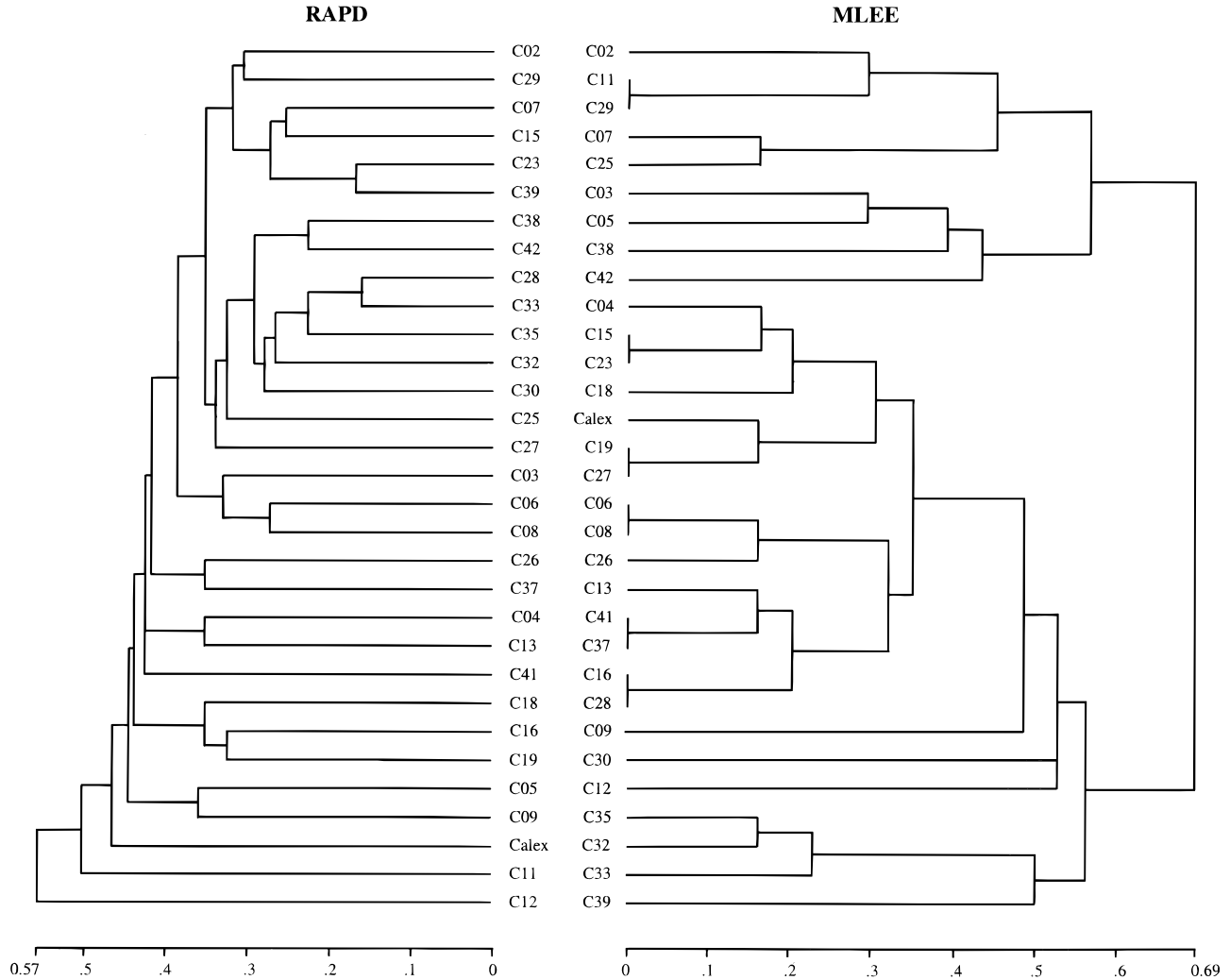

FIG. 1. Two UPGMA dendrograms (Sneath and Sokal 1973) showing the genetic relatedness revealed by MLEE (right) and RAPD (left) analyses for 31 stocks of *Plasmodium falciparum* from the Congo. Lack of agreement between the two dendrograms is taken as an indication that linkage disequilibrium is not strong within this sample.

population with isoenzyme data (Fig. 1), two clusters are apparent that are defined mainly by three loci: *Gdh*, *Gsr*, and *Ldh*. Three alleles occur at *Ldh*, and two at each of *Gdh* and *Gsr*. The top cluster includes the 8 stocks with genotype *2/2/2* (allele *2* at each locus). The bottom cluster includes all 18 stocks with genotype *1/1/1*, plus 5 stocks representing four additional genotypes (six other genotypes, with an expected joint frequency of 0.264, are not included in the dendrogram). The two complementary genotypes *1/1/1* and *2/2/2* are both present in much greater frequencies than expected (Table 2) and account for most of the genetic disequilibrium in the Congo population. Nevertheless, this clustering pattern is not confirmed by the RAPD dendrogram (Fig. 1).

The fact remains that, when isoenzyme data are considered, there is highly significant linkage disequilibrium in several populations. Several possible explanations that are not exclusive of one another can be explored. First, culture bias selection, which would have eliminated a large portion of the possible genotypes, could play a role. This explanation is not acceptable in the Bobo 1 and Bobo 2 populations, which derive from placenta samples and were not cultured. Second, geographical separation could lead, through genetic drift, to different allelic frequencies among populations and

TABLE 2
Linkage Disequilibrium between the *Ldh*, *Gsr*, and *Gdh* Isoenzyme Loci in the Congo Population

Genotype	Observed	Expected	χ^2	*P*
1/1/1	18	11.69	5.5	<0.02
2/2/2	8	0.47	8.3	<0.01
Four other	5	18.84	25.3	<0.001

Note. Six of the 12 possible genotypes do not occur in the sample. The χ^2 for the genotype *2/2/2* is calculated using 3 as the expected value.

generate some linkage disequilibrium in samples that were derived from different populations that have been inadvertently pooled (Wahlund effect). But this explanation will not work either, because the Bobo and Congo populations have been collected in relatively limited geographical areas (within less than a 20-km-diameter area, an area within which people readily move). Moreover, when a microorganism is found to be panmictic, such as *Neisseria gonorrhoeae*, the evidence of panmixia remains when large geographical areas are considered (see Maynard Smith *et al.* 1993). Third, one possible factor to account for linkage disequilibrium would be natural selection favoring certain multilocus combinations, particularly when the loci are linked on the same chromosome. Once again, however, linkage disequilibrium extending over multiple gene loci, some in different chromosomes, can hardly be accounted for by natural selection, even if this is strong, if panmixia is the case. Therefore, it may very well be the case that some kind of uniparental propagation (Tibayrenc, Kjellberg, and Ayala 1990) obtains in these populations of *P. falciparum*. This might occur as a consequence of high rates of self-fertilization, a situation found in Papua New Guinea populations of *P. falciparum* (Paul *et al.* 1995). In conclusion, it seems that even if the present data are consistent with some level of clonal propagation, this does not seem preponderant in the case of the Congo population of *P. falciparum*, the only one we have surveyed with molecular markers other than isozymes. By extension, we extend this conclusion to the Bobo and Sudan populations, which have been surveyed only with isozymes. Rich, Hudson, and Ayala (1997) have reached, however, a different conclusion based on the analysis of gene sequences involved in antigenic diversity. It seems possible that different populations of *P. falciparum* will show different evolutionary patterns. In low-transmission areas, self-fertilization leading to *de facto* clonal reproduction would be the case, while a closer approximation to panmixia might obtain in high-transmission areas (Ayala 1998). This hypothesis is consistent with unpublished RAPD results obtained with Latin American isolates, which show much stronger indices of linkage disequilibrium than those observed in the present study (L. Urdaneta and M. Tibayrenc, unpublished data).

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