Am. J. Trop. Med. Hyg., 94(6), 2016, pp. 1259–1265
doi:10.4269/ajtmh.15-0832

Analyzing Deoxyribose Nucleic Acid from Malaria Rapid Diagnostic Tests to Study *Plasmodium falciparum* Genetic Diversity in Mali

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Abstract. We evaluated the use of positive malaria rapid diagnostic tests (mRDTs) to determine genetic diversity of *Plasmodium falciparum* in Mali. Genetic diversity was assessed via multiple loci variable number of tandem repeats analysis (MLVA). We performed DNA extraction from 104 positive and 30 negative used mRDTs that had been stored at ambient temperature for up to 14 months. Extracted DNA was analyzed via quantitative polymerase chain reaction (qPCR), and MLVA genotyping was then assessed on positive qPCR samples. Eighty-three of the positive mRDTs (83/104, 79.8%) and none of the negative mRDTs were confirmed *P. falciparum* positive via qPCR. We achieved complete genotyping of 90.4% (75/83) of the qPCR-positive samples. Genotyping revealed high genetic diversity among *P. falciparum* populations in Mali and an absence of population clustering. We show that mRDTs are useful to monitor *P. falciparum* genetic diversity and thereby can provide essential data to guide malaria control programs.

Genetic diversity analysis of *Plasmodium falciparum* in humans and parasite population dynamics are useful to monitor malaria control and elimination strategies. Indeed, several multiple loci variable number of tandem repeats analysis (MLVA) studies using microsatellite markers have shown that *P. falciparum* population genetics correlated with malaria transmission intensity in Africa and provided epidemiologically relevant information.[1–5] Assessing malaria parasite genetic diversity over time can also help to evaluate malaria control programs. For instance, a *P. falciparum* microsatellite-based survey in Djibouti has revealed a significant decline in genetic diversity between 1998 and 2009, which was compatible with pre-elimination goals.[5]

Plasmodium falciparum genetic diversity has been well described in humans throughout the world[1–5]; however, this type of study is difficult to manage in the field. Indeed, collection, transportation, and storage of large amounts of blood samples remain difficult in remote areas with a tropical climate. Sampling of dried blood spots on filter papers partially circumvents these technical difficulties, thereby enabling detection and genotyping of *P. falciparum* from archived specimens.[1,4,5] Nevertheless, patient consent is required as long as specific blood sampling is required for the study.

Malaria rapid diagnostic tests (mRDTs) detect the presence of circulating *P. falciparum*-specific antigens, such as histidine-rich protein 2 (PfHRP2) and lactate dehydrogenase. They are highly recommended by the World Health Organization for systematic malaria diagnosis before artemisinin-based combination therapy of uncomplicated malaria cases.[6] As mRDTs are already used for case management, they are widely available in malaria-endemic countries and therefore represent a potential source of *P. falciparum* DNA for large population studies. Furthermore, patient consent is not required for such investigations, as mRDTs are passively collected during patient care and would normally been discarded once interpreted. Previous studies have shown that used mRDT nitrocellulose strips harbor *P. falciparum* DNA,[7–10] and thereby enable the detection of antimalarial drug-resistant genes via the single nucleotide polymorphism genotyping.[8] This study aimed to assess whether used mRDTs stored at room temperature provide sufficient quality DNA to conduct MLVA genotyping of *P. falciparum* in Mali.

Between October 2013 and January 2015, we randomly selected a total of 134 used mRDTs (104 PfHRP2 positive and 30 PfHRP2 negative) from four sites in Mali (Figure 1, Table 1). Blood samples were collected via finger prick (5–10 μL) and absorbed onto mRDTs (SD BIOLINE Malaria Ag P.f® and P.f/Pan®, Standard Diagnostics, Kyonggi, Republic of Korea) by Malaria Research and Training Center (MRTC) clinicians during the systematic testing of symptomatic febrile patients. Used mRDT samples were stored and transported at ambient temperature to the MRTC Central Laboratory in Bamako, Mali. The samples were then randomly selected and air transported to Marseilles, France, where *P. falciparum* DNA was extracted from January 2015 to August 2015.

The mRDT nitrocellulose strips (cut into five identical pieces) were incubated for 48 hours at ambient temperature in 800 μL of lysis buffer (bioMérieux, Marcy l'Etoile, France). The extracted DNA was eluted in 100 μL of elution buffer using a NucliSENS EasyMAG instrument (bioMérieux).[11] Screening for *P. falciparum* was performed via quantitative polymerase chain reaction (qPCR) using a LightCycler 480 PCR system (Roche Diagnostics, Meylan, France) with specific primers targeting the *18S rRNA* gene.[12] Each experimental run included both a negative (no template) and a positive (*P. falciparum 18S rRNA* plasmid) control. Standard curves were generated with serial 10-fold dilutions of the plasmid to allow for species-specific quantification of parasite density (number of parasites/μL of blood). We assumed that each genome of *P. falciparum* has five copies of the *18S rRNA* gene (as observed in the 3D7 genome).[13] The qPCR-positive samples were genotyped using eight polymorphic microsatellite markers specific for *P. falciparum* (Poly α, TA109, TA1, TA81, TA42, ARA2, PfPK2, and Pfg377).[1] Microsatellite

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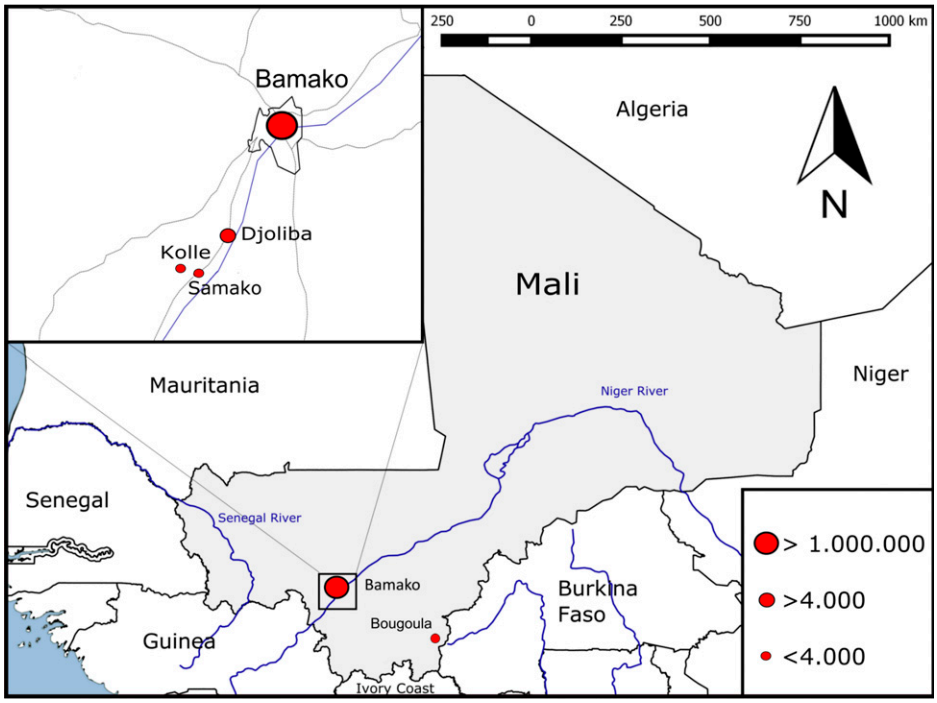

FIGURE 1. Map of the four study sites in Mali. The size of the red dots indicates the relative approximate number of inhabitants of Bamako, Bougoula, Djoliba, Samako, and Kolle. Samako and Kolle were pooled as a single site because of geographic proximity.

amplification was performed applying a semi-nested PCR strategy using fluorescent end-labeled primers.[1] PCR products were analyzed using an ABI 3130XL capillary sequencer (Applied Biosystems, Foster City, CA). To differentiate allele peaks from stutter peak artifacts, we scored multiple alleles per locus if minor peaks were > 33% of the height of the major peak, corresponding to the predominant allele.[1] We also discarded peaks with a fluorescence intensity < 100 units. Genetic diversity metrics were assessed using Arlequin v3.5 (Excoffier & Lischer 2010) software based on complete genotypes. For a haploid organism, genetic diversity was defined as a measure of the probability to randomly draw a pair of different alleles from an allelic pool. Potential values ranged from 0 (no diversity, 100% similarity between alleles) to one (maximal diversity, 100% of the alleles are different). Because of the frequent occurrence of multiclonal infections, we only considered major peaks when determining genotypes for genetic diversity and haplotype analysis.[1–5] To analyze the relationship between *P. falciparum* haplotypes, we computed a dendrogram using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (BioNumerics v7.1 Software, Applied Maths, Ghent, Belgium).

Eighty-three of the positive mRDTs (83/104, 79.8%) and none of the negative mRDTs were confirmed *P. falciparum*-positive via qPCR (Table 1). Among the 83 qPCR-positive samples, 75 (90.4%) were successfully genotyped for all test loci (Table 1). The eight remaining samples displayed one ($N = 3$), two ($N = 2$), three ($N = 1$), and four ($N = 2$) negative loci of the eight loci tested (see Table 3 for complete microsatellite data). Mean PCR cycle threshold (C_t) values were 31.94 ± 3.15 for the 75 samples that were completely genotyped (estimated parasitemia ranging from 11 to 512,000

TABLE 1

Epidemiological and sampling characteristics as well as the qPCR and MLVA results of the 104 mRDT-positive samples collected from four sites in Mali

Study sites	Djoliba	Kolle (K) Samako (S)	Bamako	Bougoula	Total
Sampling period	October 2013 to January 2014	November 2014 (K) December 2014 (K) January 2015 (K) (S)	December 2014 January 2015	January 2015	
Endemicity*†	Hyperendemic	Hyperendemic	Hypoendemic	Hyperendemic	
Patients sampled, no.	34	27	28	15	104
Positive PCR for *Plasmodium falciparum*, *n* (%)	29 (85.3)	24 (88.9)	20 (71.4)	10 (66.7)	83 (79.8)
Complete genotype ($N = 8$ loci), *n* (%)	28 (96.5)	21 (87.5)	18 (90.0)	8 (80.0)	75 (90.4)
Genetic diversity (mean ± SD)	0.76 ± 0.18	0.75 ± 0.16	0.78 ± 0.17	0.69 ± 0.36	0.75 ± 0.22

MLVA = multiple loci variable number of tandem repeats analysis; mRDTs = malaria Rapid Diagnostic Tests; qPCR = quantitative polymerase chain reaction; SD = standard deviation.

*Data from various Malian official reports and studies reviewed in a published thesis in French. (Doumbo O, 1992. Epidémiologie du paludisme au Mali, étude de la chloroquinorésistance, essai de stratégie de contrôle basée sur l'utilisation de rideaux imprégnés de permethrine associée au traitement systématique des accès fébriles. Thèse de Doctorat. Sciences Biologiques, Montpellier II, France.)

†Malaria endemicity levels based on *P. falciparum* prevalence among children aged 2–10 years ($PfPR_{2-10}$), according to the World Health Organization classification: hypoendemic, 0–10%; hyperendemic, 50–75%.

TABLE 2
Quantification of samples via qPCR cycle threshold and estimated parasitemia based on the number of positive loci

	Complete genotypes (8 positive loci; $N = 75$)	Incomplete genotypes (< 8 positive loci; $N = 8$)
PCR cycle threshold (mean ± SD)	31.94 ± 3.15	37.52 ± 1.92
Estimated parasitemia* (parasites/μL blood) (mean ± SD)	43,067 ± 82,558	749 ± 1,280
Range of estimated values of parasitemia* (parasites/μL blood)	11–512,000	0.01–3,620

qPCR = quantitative polymerase chain reaction; SD = standard deviation.
*Estimation of parasite density: ([copy number of the gene in 1 μL of DNA] × [100/25]). We assumed that each genome of *Plasmodium falciparum* has five copies of the *18S rRNA* gene,[13] that 5 μL of blood was spotted onto malaria rapid diagnostic tests, and that extracted DNA was eluted into 100 μL.

parasites/μL) versus 37.52 ± 1.92 for the eight incompletely genotyped samples (estimated parasitemia ranging from 0.01 to 3,620 parasites/μL) (Table 2).

Genetic diversity was high (mean value of 0.75) and did not differ between the urban site of Bamako and the rural sites of Samako/Kolle (which were pooled together because of geographical proximity) and Djoliba (Table 1). The dendrogram based on the analysis of the 75 complete haplotypes (Figure 2) demonstrates that each haplotype was unique, which is consistent with the high level of isolate genetic diversity. The absence of major haplotype clustering between and within study sites indicates a lack of local clonal expansion of *P. falciparum*.

Our findings show that mRDTs are a very accessible, convenient, and efficient tool to detect and genotype *P. falciparum* from patient blood samples. As mRDTs are already widely used for malaria case management, such samples can be collected and stored in large-scale in malaria-endemic countries. Systematic collection of used mRDTs can be considered for genetic diversity studies without specific population sampling procedures or patient consent requirements. Malaria RDTs are also suitable for field conditions, as they can be transported and stored at ambient temperature, even for extended periods. Indeed, after a 14-month storage period at ambient temperature, DNA quality and quantity were sufficient to perform complete genotyping of 90.4% of the qPCR-positive samples. In addition, mRDTs can be transported in simple packages; this feature is critical as it is increasingly difficult to ship biological samples because of the expansion of international biological risk.

Of 104 mRDT-positive samples, 21 tested negative via qPCR. This discrepancy may be due to false-positive mRDT results associated with the persistence of PfHRP2 antigenemia following malaria treatment, as previously observed.[10,14] Malaria RDT-positive samples that yielded negative qPCR results could also be interpreted as false-negative qPCR results because of the presence of PCR inhibitors or DNA extraction failure. Insufficient parasitic material may also yield a false-negative result, as the blood volume spotted onto mRDTs is low (5–10 μL) compared with the blood volume usually spotted on filter papers (50 μL). However, previous studies have reported the absence of PCR inhibitors in samples obtained from mRDTs.[7,9] Furthermore, the estimated qPCR sensitivity from mRDT samples is much higher than that of mRDT antigen capture and field microscopy (both estimated to 100 parasites/μL).[15] Indeed, the presence of a single parasite in a 5–10 μL blood sample spotted onto an mRDT should be sufficient to yield a positive qPCR result.[9]

Eight of 83 samples (9.6%) were not successfully genotyped for all test loci despite a positive qPCR result. This outcome may be due to low parasite density, as indicated by the higher C_t values (mean C_t of 37) compared with the successfully genotyped samples (mean C_t of 32). This result may also be due to unsuccessful primer recognition because of high polymorphism or local DNA alterations of the target sequence. Nevertheless, 90.4% of samples were completely genotyped for all eight test loci, which provided sufficient data to examine *P. falciparum* genetic diversity.

Our study demonstrates high genetic diversity among *P. falciparum* populations in Mali (mean genetic diversity = 0.75), which indicates a high intensity of malaria transmission in the country. These results are compatible with local malaria epidemiology, as Mali has yet to reach the malaria pre-elimination stage. Microsatellite studies performed in other malaria-endemic countries in Africa have also reported high genetic diversity of *P. falciparum*, ranging from 0.72 to 0.8.[1–4]

These techniques of DNA extraction and genotyping from used mRDTs have already been transferred to the MRTC in Mali and will serve to monitor genetic diversity of *P. falciparum* in the context of malaria control. This approach will also facilitate the monitoring of drug resistance via genotyping of resistance genes. Using mRDTs, future large-scale *P. falciparum* genetic studies would be relatively cost-effective and easy to carry out, as they circumvent specific blood sampling, storage, and transportation. This technique thus represents a breakthrough in the capacity to bolster field malaria epidemiological studies.

Received November 18, 2015. Accepted for publication February 15, 2016.

Published online March 21, 2016.

Acknowledgments: We would like to express our gratitude to the study population and volunteers as well as the MRTC field and laboratory staff. We would also like to acknowledge APPLIED MATHS for use of the BioNumerics software platform.

Financial support: Sample collection in Mali was supported by EDTCP1-WANETAM and WANECAM grants. Sample genotyping was supported by the Parasitology and Mycology Laboratory, La Timone Hospital, Marseilles, France.

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TABLE 3
Microsatellite data of the 83 genotyped samples

Site	Sample	Poly α				TA109				PfPK2			ARA2			Pfg377			TA42			TA81			TA1			
Kolle	T10	166	163	157		183				168	171		76	82		104	107		192			125	128		168	180			
Kolle	T11	157				171				180			67			104			207			143			180				
Kolle	T12	160				168				165			76			107			207			125			174				
Kolle	T13	157	136			180				180			64			104			192			137			180				
Kolle	T15	157				192				168			73			104			192			131			180	168			
Kolle	T16	157				183				165			70			104			192			131			171				
Kolle	T17	154				183	195			180			67			104			192			131	137		168	189			
Kolle	T18	169				183	171			165	183		64	70		104			183			131			183	180			
Kolle	T19	148	166			183				171	168	174	82	70	67	107			207	192		125	131		189	174	183		
Kolle	T129	163				180				177			67			107			192			134			171				
Kolle	T130	163				180				168						107			192			125			165				
Kolle	T131	163				213				177			82			107			192			131			174				
Samako	T20					168				180	171					107			192			128							
Samako	T21	163				168	183			174	168		70	67		107			192			131	125		180				
Samako	T22	163	157			180				174			82			107			207			128			174				
Samako	T23	169	172	157		168	180			171			70	64		104	107		192			134	143		168	177	189		
Samako	T24					180	198			174						104			192			128			189	186			
Samako	T25	151	160			183				174	171		67	79		104			192			128	131		165	168			
Samako	T26	157				168				174	171		64	79		107			192			128	125		183	180			
Samako	T28	154	157			186	201			177	174		73			104			192			137			168	171			
Samako	T124	157	142	166	175	168	183	180	198	168	171		70	64	67	82	107			192	207		125	131		165	171	189	198	
Samako	T125	109				168	182			168	174	180	82	73		104	113		192			131	137		171	189			
Samako	T126	157				168				177			70			107			192			131			180				
Samako	T127	157	172			189	180	168		165	195		73	64		107			207	192	183	125			183	165			
Djoliba	T29	178				180				177			73			107			192			131			177				
Djoliba	T30	169				168				174			79			104			207			131			165				
Djoliba	T31	154				186				189			73			107			192			128			174				
Djoliba	T32	169	154			171				186	168		76			107			192			137	125		180				
Djoliba	T33	160	157			168	180			171	165		76			104	107		192			134	128		177	165			
Djoliba	T34	163				183				192			94			107			207			131			174				
Djoliba	T35	172	184			168	174			171	195		70			107			192	189		128			174				
Djoliba	T36	157				180	183			171	165		70			104			207	222		131	137		171	183			
Djoliba	T37	163				204				171			85			107			207			122			174				
Djoliba	T38	163	184			195	183	171		174	177		73			107			192			128			171				
Djoliba	T39	115	160			159				168	171		70			104			192			125	170		174				
Djoliba	T40	154	166			153				171			76	73		113			207			131			171				
Djoliba	T41	178				183				168			73			107			192			125	128		174				
Djoliba	T42	154				207	171			168			94	64	73	107			192			131			183	171			
Djoliba	T43	169	160			180				180	165		79			107			207			125			171				
Djoliba	T44	175				171				165			79			104			192			125			183				
Djoliba	T45	184				189				174			70			101			192			125			174				
Djoliba	T46	157				183							73						207			140			174				
Djoliba	T47	148				180				171			73			110			192			125			168				
Djoliba	T48	160				171				165	171		79	70		101	107		195			143	134		174				
Djoliba	T50	157	160	163		183	171	186	204	168	171	174	76	67	70	104	101	107	192			131	128	134	165	174	168	177	
Djoliba	T51	166				171				174			70			107			192			140			165				
Djoliba	T52	157				183	168			183	177	171	76	67		107			192			125			168				
Djoliba	T54	154				180				198	162		76			104			192	207		128			171				
Djoliba	T55	160	154			168				168	177		76			107	104		192			125	128	134	171	168			
Djoliba	T56	169				162				186	177		70			107			192	207		128			189	177			

(continued)

TABLE 3
Continued

Site	Sample	Poly α			TA109			PfPK2			ARA2			Pfg377		TA42			TA81			TA1		
Djoliba	T57	157			168			171			73			107		192			116	128		165		
Djoliba	T58	160	178		177	189		174			70	64		107		192	207		125			168	174	
Djoliba	T59	142			183	189		168			76	67		107		192			125			177	183	
Bougoula	T133	160			183			186			76			107		192			116			171		
Bougoula	T134	157			201														128	125		132		
Bougoula	T135	151	154		180	183		192			85	73		107		192			125			171		
Bougoula	T137	181			171			171			82			107	113	192	204	207	128			168		
Bougoula	T138	166	154		168	183		168	180		76	70	82	107	92	192			125			171		
Bougoula	T139	157	166		177	168		168			76	70		107		192			128			165	174	
Bougoula	T140	151			171	168	180	171			70			107		192	207		134	131		177	180	174
Bougoula	T141	160	157		165	192		168	165		82			107		192			131	134	128	174		
Bougoula	T144	151			189			174			70			104		192			122			183		
Bougoula	T146				168			186			64			107		192			128	134		171		
Bamako	T1	169			177			174			82			104		192			134	128		171		
Bamako	T3	154	178		189			174			70			104		207			128			201		
Bamako	T6	166			165			168			70	82		104		192			131			186		
Bamako	T7	139			180			165			70			110		207			128			168		
Bamako	T9	154			189			168			67			98		192			134	128		174		
Bamako	T155				180			171			76			107		192			140			171		
Bamako	T156	157			171			168			70	73		107		192			134			186		
Bamako	T157	157			168			171			79			104		192			131			165		
Bamako	T159	169			168			174			76			107		207			131			180		
Bamako	T160	139			171			165			70	73		110		207			125	131		174	165	
Bamako	T162	172			183	186		171	162		64	73		101	107	192			125	134		177		
Bamako	T163	172			183			171			76			104		192			128			177		
Bamako	T171	178			168			165			70			110	95	192			128			174		
Bamako	T172	157			171	180		174			67	73		104	110	192			131			168	180	
Bamako	T174	166			183			171			70			107		192			128			186		
Bamako	T176	163			183			171			76			104	89	192			134			192		
Bamako	T177	154			183			177											131					
Bamako	T178	154	157		168			177			73			107	92	192			125			180		
Bamako	T179	160			180			168			79			107	92	192			128			177		
Bamako	T180	157	169	148	180	168	183	177	171	180	73	76	85	107		192			125			171	174	

The allele length (in base pairs) for each *Plasmodium falciparum* tested loci (Poly α, TA109, TA1, TA81, TA42, ARA2, PfPK2, and Pfg377) is provided. The empty cells of the table correspond to negative loci.

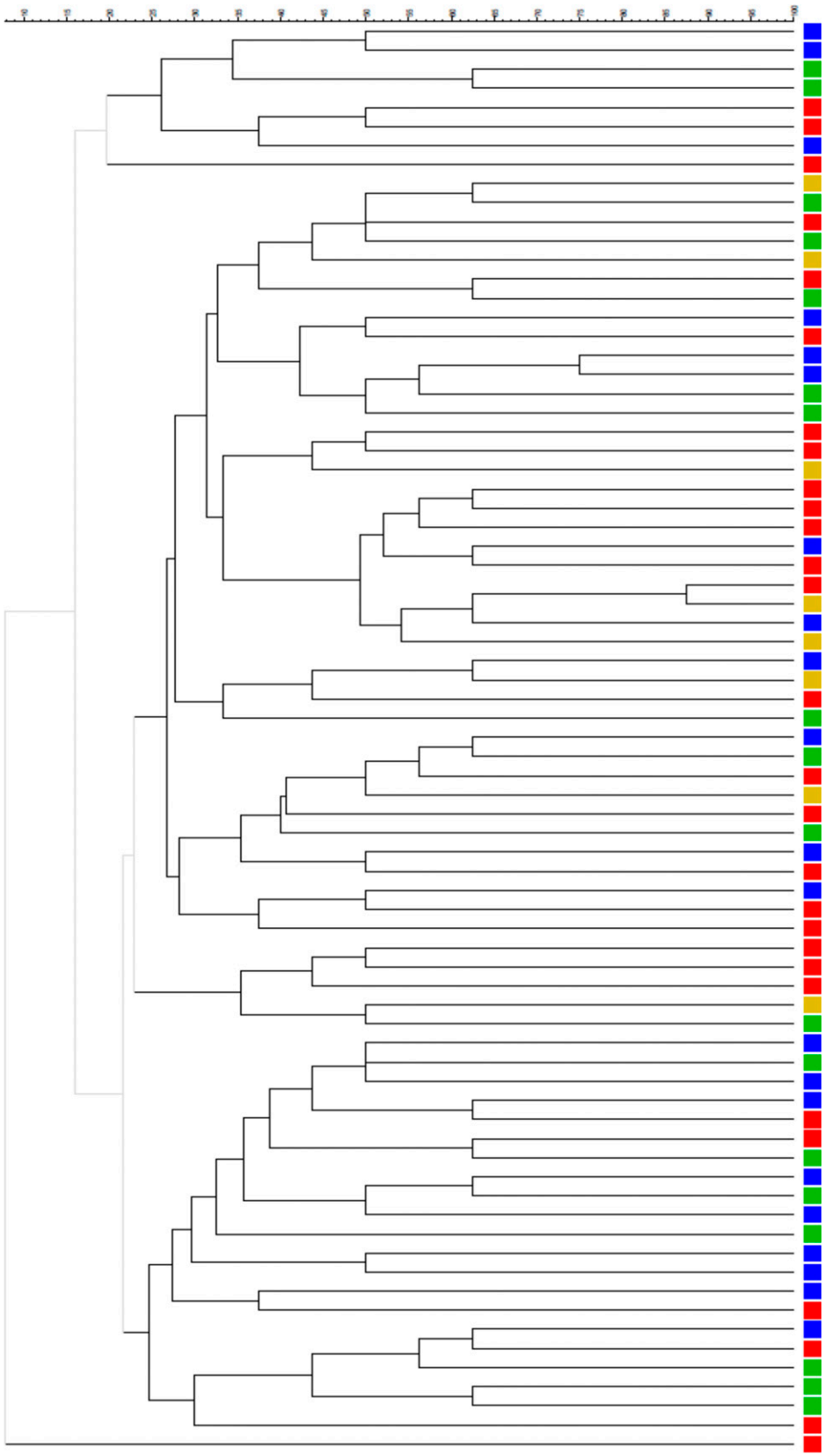

FIGURE 2. UPGMA dendrogram displaying the relationship between the *Plasmodium falciparum* haplotypes (N = 75). The isolates are colored according to geographic location in the dendrogram. Red: Djoliba; blue: Samako/Kolle; green: Bamako; and gold: Bougoula.

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