Genetic Diversity of *Plasmodium falciparum* Merozoite Surface Protein-1 Block 2 in Sites of Contrasting Altitudes and Malaria Endemicities in the Mount Cameroon Region

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Abstract. The present study analyzed the relationship between the genetic diversity of *Plasmodium falciparum* and parasitologic/entomologic indices in the Mount Cameroon region by using merozoite surface protein 1 as a genetic marker. Blood samples were collected from asymptomatic children from three altitude zones (high, intermediate, and low). Parasitologic and entomologic indices were determined by microscopy and landing catch mosquito collection/ circumsporozoite protein–enzyme-linked immunosorbent assay, respectively. A total of 142 randomly selected *P. falciparum*-positive blood samples were genotyped by using a nested polymerase chain reaction–based technique. K-1 polymerase chain reaction products were also sequenced. As opposed to high altitude, the highest malaria prevalence (70.65%) and entomologic inoculation rate (2.43 infective/bites/night) were recorded at a low altitude site. Seven (18.91%), 22 (36.66%), and 19 (42.22%) samples from high, intermediate, and low altitudes, respectively, contained multiclonal infections. A new K-1 polymorphism was identified. This study shows a positive non-linear association between low/intermediate altitude (high malaria transmission) and an increase in *P. falciparum* merozoite surface protein 1 block 2 polymorphisms.

INTRODUCTION

Knowledge of the molecular epidemiology of parasites in an area is essential to understand the transmission of a disease such as malaria. Malaria is the most serious tropical parasitic disease and one of the major threats to humans worldwide. It causes 1–3 million deaths and more than 300 million clinical cases annually with more than 90% occurring in sub-Saharan Africa.[1–3] Most of the cases are caused by *Plasmodium falciparum*, and children and pregnant women are the most affected group.[3,4]

Malaria control may be achieved by three complementary methods: chemotherapy, vaccination, and vector control. Nevertheless, the appearance and spread of resistance to anti-malarial drugs and insecticides have become a primary concern for human health care.[5–7] Although the need for a safe and effective malaria vaccine is more urgent than ever, antigenic and strain diversities of the parasite remain a major obstacle, which highlight mandatory appreciation of the diversity of the local parasite population before development of any immunologic interventions.[8] This appreciation would be a step forward generating current and comprehensive information on the diversity in the genes that encode malaria vaccine candidates of *P. falciparum* and its implications on the epidemiology of malaria, immunity, and development of control measures.[9] *Plasmodium falciparum* is genetically diverse at all levels of endemicity, which is not surprising because genetic diversity has been shown to be a function of transmission in a given area.[10–12] The inherent variability of the *P. falciparum* is particularly prevalent in merozoite surface antigens or proteins (MSPs).[13] These proteins provides multi-ple effective evasion and drug resistance mechanisms for the parasite. They also represent a major challenge for development of an effective malaria vaccine.

Merozoite surface protein 1 is the most commonly used genetic marker for the determination of the genetic diversity of the malaria parasite.[14–16] Twenty-four major versions of the MSP-1 gene have been identified,[17,18] and its coding sequence may be divided into 17 blocks among which 7 are variable blocks interspersed with conserved and semi-conserved regions. In some variable blocks, the variation is dimorphic and sequences may be grouped into one of the two allelic families (K-1 and MAD20). Block 2 represents an exception to dimorphism; it is the most polymorphic part of the gene having a third allelic family or variant RO33.[19] Genetic diversity at the MSP-1 locus may be generated by exchanging blocks of sequences during sexual (meiotic) recombination and by putative strand-slippage events during asexual (mitotic) replication of the parasites, which lead to rearrangements of block 2 tripetide repeats.[17,20] High meiotic recombination rates within MSP-1 have been estimated for parasites in areas of intense malaria transmission in Africa, where most human infections consist of mixture of genetically distinct allelic variants.[21] However, it should be noted that meiotic recombination is rare between allelic types, although it occurs in block 2 between MAD20 and RO33 and creates a fourth allele family known as MR.[22] The effect of altitude and estimated rainfall on indices of malaria infection/transmission has been described in a study carried out in Tanzania in which *P. falciparum* prevalence was negatively associated with altitude. However, the relationship varied according to ecological setting, climate, vector species, topography, and host and parasite genetics.[23]

Malaria prevalence in the Mount Cameroon region is high (≥ 90%).[24,25] It has previously been shown that malaria transmission is heterogeneous, and the highest transmission rate has been recorded at lower altitudes.[26] The malaria transmission

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pattern in the Mount Cameroon region is greatly influenced by altitude, climatic, and bio-ecological variations. Serious environmental alterations that have taken place in the region that have been caused by rapid growth in populations, roads, and houses and agro-industrial activities of the Cameroon Development Cooperation have led to ecological changes, which together with other factors, such as rainfall, temperature, and humidity, affect the structure of the vector population and thus transmission of infection and probably the genetic diversity of the parasites circulating in the area.[10] Although previous entomologic and parasitologic studies in this region have shown the influence of these changes on the heterogeneity of the malaria transmission pattern observed, none had been conducted to determine whether this variability necessarily translates into variation in the genetic diversity of *P. falciparum* in the region.

The purpose of this study was to investigate genetic variations in the *P. falciparum* MSP-1 block 2 in samples collected from asymptomatic school children in six localities in the Mount Cameroon region, including two regions at high altitudes where transmission is low, two regions at the intermediate altitudes where transmission has a mixed pattern, and two other regions at low altitudes where transmission is high. This genetic altitude-based characterization of *P. falciparum* infections in the Mount Cameroon area will provide new essential data on the parasite population diversity and implications for the epidemiology of malaria and development of appropriate control measures.

MATERIALS AND METHODS

Study design. The present study aimed to correlate genetic variation in the MSP-1 block 2 of *P. falciparum* with malaria endemicity in six locations of contrasting altitudes in the Mount Cameroon region. Geographic parameters of the study area such as temperature and area of residence of participants, and demographic data such as age and sex of participants were recorded during the sampling period. Climatic parameters were obtained from the Cameroon Development Cooperation weather station. Blood samples were collected during March–July 2006, which is the mild rainy season during which transmission of malaria is high in the region. Malaria prevalence, parasite density, and *Anopheles* biting and entomologic inoculation rates (EIRs) were determined.

A census was used for selection of participants. Patients eligible for this study were asymptomatic school children of both sexes 4–16 years of age. This particular age group was chosen for their relative settled way of life, thus reducing bias that can be caused by movements of populations and acquisition of different strains of parasite from other areas. Government schools were chosen because they were found in all localities, and most parents send their children to these schools because fees are moderate. Objectives and schedules of the study were explained to the parents or guardians of the eligible children, and those who agreed to allow their children to participate signed an informed consent form. Eligible children were enrolled in the study. This study was approved by the ethical review board of Kumba station and the Cameroonian Ministry of Public Health.

Study area. The study was conducted in six localities that had contrasting altitudes, different malaria transmission profiles, and contrasting climatic/environmental features: (Bonakanda = 1,197 meters above sea level) and Likoko-Membea (800 meters above sea level) were considered high-altitude areas, Meanja (300 meters above sea level) and Mutengene (220 meters above sea level) were considered intermediate-altitude areas, and Debundscha (50 meters above sea level) and Tiko (10 meters above sea level) were considered low-altitude areas. Mount Cameroon in Buea, Cameroon, is the highest mountain in western Africa and is an active volcano that rises from the Atlantic Ocean at the Gulf of Guinea and has an altitude of 4,100 meters above sea level. The mountain is formed of a continuous pile of terraces from the base to the summit. At an altitude of 50 meters from the coast is a sedimentary plain that extends from Tiko to Debundscha. From Mutengene, the altitude of the terrain gradually increases to an altitude of 800–1,200 meters in Buea (Bonakanda).

Hydrologically, there are 20 streams in the study area that empty into the Atlantic Ocean. Two of these streams, Ombe and Onge, flow southeast and northwest, respectively. In this forested area of southern Cameroon, the equatorial climate is modified by the influence of the ocean and the mountain. At higher altitudes, the temperatures are lower than in other areas of the southern part of the country: mean values of the minimum temperatures are 20°C in December and 18°C in August, and mean values of the maximum temperature are 35°C in August and 30°C in March. Rainfall was also an important factor in this study. Debundscha, located at the western flanks of Mount Cameroon, has up to 11,000 mm of rainfall, making this locality the second wettest place in the world. The Mount Cameroon region has a long rainy season that starts in March and ends in November, and maximum rainfall occurs in August and September. The dry season starts in November and ends in February.

Approximately 1.3 million persons live near Mount Cameroon in the towns of Tiko, Limbe, Mutengene, Buea, and Muyuka. The population is composed of indigenous Bakweri, Creoles (from Liberia and Sierra Leone), and Nigerians and immigrants from other parts of Cameroon, especially the North West Province. Additional details on the features of villages studied in this work are shown in Table 1.

TABLE 1
Climatic, environmental, and parasitologic characteristics of the study sites in the Mount Cameroon area

	Geographic coordinates	Site	Altitude (meters above sea level)	Mean temperature (°C)	Relative humidity (%)	Rainfall (mm)	Malaria prevalence (%)	Geometric mean parasite density/µL
High altitude	4°21′N, 9°27′E	Bonakanda	1,197	19.5	86.4	2,400	12.33	522
	4°14′N, 9°10′E	Likoko Membea	800	22.5	88.8	2,654	16.82	496
Intermediate altitude	4°07′N, 9°21′E	Meanja	300	27.5	61.1	2,475	62.26	839
	4°05′N, 9°18′E	Mutengene	220	27.5	83.1	1,854	46.80	700
Low altitude	4°10′N, 9°00′E	Debundscha	50	27	89.6	10,000	42.66	650
	4°07′N, 9°36′E	Tiko	10	27.9	83.1	4,524	70.65	656

Collection of blood samples and parasitologic examinations. Using sterile needles and syringes, we collected 2 mL of venous blood from each participant into tubes containing EDTA according to routine clinical practice. Some blood was also collected onto grease-free slides for the preparation of thin and thick blood films and immediately spread to prevent clotting. In the laboratory, serum samples were separated from the blood cells by centrifugation at 2,000 rpm for 5 minutes, transferred into sterile Eppendorf (Hamburg, Germany) tubes, labeled accordingly, and stored at –20°C for future use. Blood cells were also stored at –20°C for DNA extraction. Thick and thin blood films were prepared and stained with 5% Giemsa according to the method described by Cheesbrough.[27] Giemsa-stained films were examined by using the oil immersion objective (×100) of a light microscope for detection and identification of malaria parasites according to procedures of the World Health Organization.[28] Slides were considered positive when asexual forms and/or gametocytes of *P. falciparum* were present in the blood film and negative after observing approximately 100 high-powered microscopic fields without seeing any parasites. For each positive slide, parasites were counted against 200 leukocytes and expressed as parasites per microliter of blood, assuming a leukocyte count of 8,000 leukocytes/μL of blood.[27]

***Anopheles* mosquito collection, human biting rates, and EIRs.** Mosquitoes were collected by the same work force at all stations (localities) during the entire study by standardized landing-catch collections. Mosquito collectors were given prophylactic treatment for malaria. All mosquitoes collected were morphologically identified by using the key of the Afro-Tropical Region and procedures for identification of *Anopheles*.[29–31] Heads and thoraces of female *Anopheles* mosquitoes were obtained and tested for circumsporozoite proteins of *P. falciparum* by enzyme-linked immunosorbent assay as described.[32] Human-biting rates (also known as aggressiveness of the species) (HBRs) were calculated directly from landing-catch collections as the average number of *Anopheles* bites experienced by a collector during an entire night of collection. The sporozoite rate was expressed as the proportion of *Anopheles* positive for sporozoites by an enzyme-linked immunosorbent assay for circumsporozoite protein of the total number tested. The EIR, expressed as the number of infective bites per person per night of collection, was calculated by multiplying the HBR by the sporozoite rate.[33]

Genomic DNA isolation from blood samples. A total of 142 *P. falciparum*-positive blood samples were selected for genomic DNA extraction by using the QIAamp DNA blood midi Kit (QIAGEN, Crawley, United Kingdom) according to the manufacturer's instructions. In brief, 300 μL of whole blood from each sample was lysed and loaded onto a DNA affinity spin column. Genomic DNA bound to the membrane was purified by using two wash buffers to remove impurities. Genomic DNA was then was eluted with 100 μL of distilled water and stored at –20°C until use. The concentration and purity of isolated DNA samples were measured by using a spectrophotometer.

Amplification of MSP-1 allelic variants by polymerase chain reaction. Amplification of MSP-1 block 2 was performed by using two rounds of nested polymerase chain reaction (PCR) according to the protocol described by Snounou and others.[14] All oligonucleotide primers were obtained from Eurogentec (Seraing, Belgium). The polymorphic repetitive regions (block 2 of MSP-1) were amplified in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA) with the following cycling parameters: denaturation at 95°C for 5 minutes; followed by 35 cycles (first round) or 45 cycles (second round) at 95°C for 1 minute, 61°C (first round) or 58°C (second round) for 1 minute, and 72°C for 2 minutes; and final extension at 72°C for 5 minutes.

In the first-round PCR, sequences spanning blocks 1–5 of the MSP-1 gene were amplified with the outer sense primer M1-OF (5′-CTAGAAGCTTTAGAAGATGCAGTATTG-3′) and the outer antisense primer M1-OR (5′-CTTAAATAG-TATTCTAATTCAAGTGGATCA-3′) and 1 μL of purified genomic DNA as template. Reactions were carried out in a final volume of 25 μL containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM MgCl$_2$, 0.25 mM of each of the four dNTPs, 0.4 μM of each specific primer, and 0.04 units/μL of *Taq* polymerase.

Three second-round nested reactions were subsequently performed to determine the presence of allelic variants from the MAD20, Kl, and RO33 types of the MSP-l block 2 by using each of the following pairs of nested specific primers: M1-MF 5′-AAATGAAGGAACAAGTGGAACAGCTGT-TAC-3′ and M1-MR 5′-ATCTGAAGGATTTGTACGTCT-TGAATTACC-3′; M1-KF 5′-AAATGAAGAAGAAATTA-CTACAAAAGGTGC-3′ and M1-KR 5′-GCTTGCATCAG-CTGGAGGGCTTGCACCAGA-3′; M1-RF 5′-TAAAG-GATGGAGCAAATACTCAAGTTGTTG-3′ and M1-RR 5′-CATCTGAAGGATTTGCAGCACCTGGAGATC-3, respectively. In all cases, 1 μL of a 1:70 dilution of amplification product from the first-round PCR was used in the three nested reactions. Positive controls (DNA from a parasite containing a specific MSP-1 block 2 allelic variant) and a negative control (without DNA) were included in each amplification.

The PCR products were subjected to electrophoresis on 2% agarose gels containing 0.1 mg/mL of ethidium bromide and visualized under UV light by using the Doc-print photographic system (Fisher Scientific Bioblock, Illkirch, France). Electrophoretic analysis of K-1-type PCR products showed bands of different sizes. To investigate the nature of the DNA fragments obtained by K-1 PCR-typing, these fragments were purified and cloned into the pGEM®-T Easy vector (Promega, Madison, WI) according to the manufacturer's recommendations and sequenced by GATC Biotech (Konstanz, Germany) by using the automated Sanger method (dideoxy-mediated chain termination).[34] Homology searches in databases were performed using the basic local alignment search tool (BLAST) network service (National Center for Biotechnology Information, Bethesda, MD).

Data analysis. Data was analyzed by using SPSS version 15 (SPSS Inc., Chicago, IL), and all tests were performed at a 5% level of significance. Different allelic variant frequencies were estimated by calculating the percentage of fragments assigned to one allelic variant type among the overall number of fragments detected for MSP-1 block 2. Gene types were defined according to the method of Da-Silveira and others[18] as the unique combination of allelic types in variable blocks. The complexity of infection (mean number of lines or fragments) per sample was calculated for MSP-1 block 2 as the average number of distinct fragments (K-1, MAD20, and RO33) per PCR-positive sample.[14] Parasite density was expressed as the

geometric mean parasite density (GMPD) of *P. falciparum* per microliter of blood.

Significant changes in malaria prevalences and frequencies of allelic variants (K-1, MAD 20, and RO33) between localities and altitude zones were compared by using the chi-square test. The Kruskal-Wallis test was used to check for differences in the mean number of genotypes per sample between the three altitude zones. This test was used to account for the small sample size at high altitude and also because samples were not normally distributed. In addition, a non-parametric multiple comparison was performed by using the Dunn test to check for differences in the mean number of genotypes per sample between high and low altitudes, high and intermediate altitudes, and low and intermediate altitudes. This test was chosen because it is appropriate when the Kruskal-Wallis test fails to reject the null hypothesis and because it accounts for disparities in sample sizes. A Spearman correlation rank test was used to check the association between altitude and complexity of infection, malaria prevalence, GMPD, mosquito biting rate, and EIR.

RESULTS

Parasitologic indices. Blood samples were collected from 876 children. The overall prevalence of malaria parasites observed in the study population was 45.32% (397 of 876). The highest (70.65%) prevalence rate of malaria was recorded in Tiko (low altitude site), and the lowest (12.33%) was recorded in Bonakanda (high altitude site). An unexpected higher prevalence (62.26%) was obtained in Meanja (intermediate zone) than in Debundscha (42.66%), which is a low altitude site (Table 1). There was a significant difference in malaria prevalence between sites at different altitudes ($P = 0.01$), but the correlation between malaria prevalence and altitude was not significant ($r = –0.771$, $P = 0.072$). Globally, there was a trend of decreasing malaria prevalence with altitude (Figure 1). The GMPD of asexual stages of *Plasmodium* species was highly heterogeneous among different localities and intermediate zones had the highest value. A GMPD value of 839.42 parasites/μL of blood (range = 40–29,520 parasites/μL of blood) was recorded in Meanja (intermediate altitude site), and the lowest GMPD of 496.14 parasites/μL of blood (range = 120–4,200 parasites/μL of blood) was recorded at high altitude sites (Table 1 and Figure 2). No significant
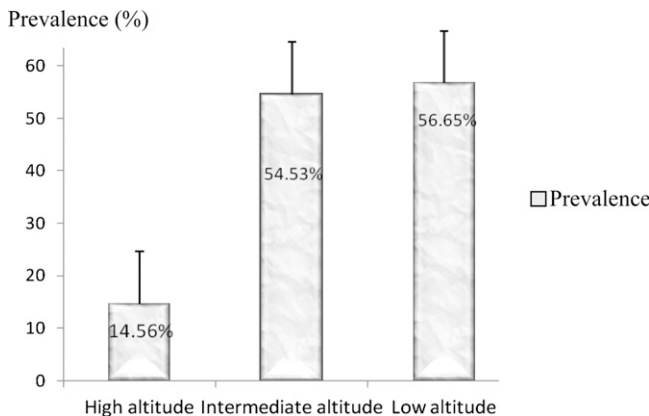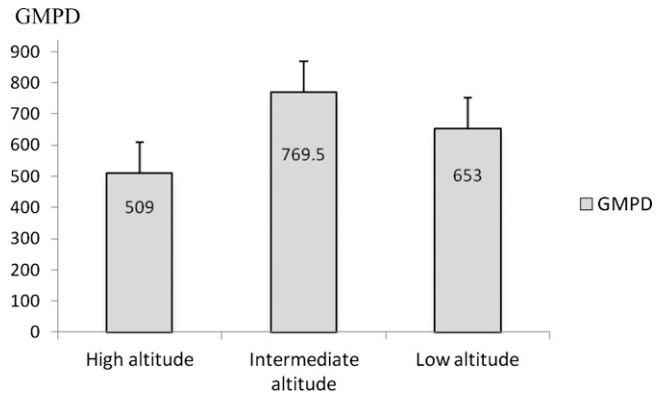

GMPD

FIGURE 2. Geometric mean parasite density (GMPD) at different altitude zones in the Mount Cameroon area. Each bar represents the mean ± SD GMPD at a given altitude zone.

correlation was found between altitude and GMPD ($r = –0.42$, $P = 0.397$).

Entomologic indices. Four *Anopheles* species were found to prevail in the region (*An. gambiae*, *An. funestus*, *An. hancocki*, and *An. nilli*). Globally, *An. gambiae* was found to be the most aggressive species with a high HBR (32.99 bites/person/night) in Tiko (Table 2). The highest total HBR (38.84 bites/person/night) was recorded in Mutengene, the only site in which *An. funestus* appeared to be a major vector with an HBR of 25.19 bites/person/night. In general, the four *Anopheles* species were found to be more aggressive at intermediate and low altitudes. *Anopheles funestus* and *hancocki* were more aggressive at intermediate zone and had cumulative HBRs of 35.90 bites/person/night and 5.82 bites/person/night, respectively. *Anopheles gambiae* was aggressive at low and intermediate altitudes (Table 2). No significant association was found between HBR and altitude ($r = –0.714$, $P = 0.111$).

Infected *An. funestus* and *An. hancocki* were found only at high and intermediate altitudes. Infected *An. gambiae* was found at all altitude zones, and the highest sporozoite rate (7.36%) was recorded in Tiko (low altitude). As opposed to *An. gambiae*, *An. nilli* was found only in Mutengene and had a sporozoite rate of 7.14% (Table 3). *Anopheles gambiae* was the most aggressive vector and the most active vector in malaria transmission in the Mount Cameroon region (overall EIR = 3.93 infective bites/person/night). This vector had EIRs in all altitude zones, and the highest EIR (2.43 infective bites/person/night) was recorded at a low altitude (Tiko). In contrast, the three other *Anopheles* species had EIRs only at high and intermediate altitudes. The highest EIRs of 1.38 infective bites/person/night and 0.14 infective bites/person/night for *An. funestus* and *An. nilli*, respectively, were obtained at

Prevalence (%)

FIGURE 1. Malaria prevalence at different altitude zones in the Mount Cameroon area. Error bars indicate mean ± SD.

TABLE 2

Human-biting rates in relation to *Anopheles* species and locality, Mount Cameroon area

| Locality | Human biting rate (bites/person/night) | | | | |
	An. funestus	*An. gambiae*	*An. hancocki*	*An. nilli*	Total
Bonakanda	0	0	0	0	0
Likoko Membea	4.38	1.38	4.92	0	10.68
Meanja	10.71	19.98	0.90	0	31.59
Mutengene	25.19	4.73	4.92	1.92	36.76
Debundscha	0	27.20	0	0	27.20
Tiko	2.54	32.99	0	0.67	36.20

TABLE 3

Sporozoite rates in relation to *Anopheles* species and locality, Mount Cameroon area

Locality	Sporozoite rate (%) of *Anopheles* species				
	An. funestus	*An. gambiae*	*An. hancocki*	*An. nilli*	Average
Bonakanda	0	0	0	0	0
Likoko Membea	3.96	3.04	5.19	0	3.05
Meanja	3.24	3.47	1.39	0	2.03
Mutengene	5.50	5.68	4.46	7.14	5.70
Debundscha	0	1.85	0	0	0.46
Tiko	0	7.36	0	0	1.84
Average (%)	2.12	3.56	1.84	1.19	

intermediate altitudes. The highest EIR for *An. hancocki* (0.25 infective bites/person/night) was recorded at a high altitude (Table 4). In all localities, *An. hancocki* and *An. nilli* were found to be minor vectors. Overall, the EIRs were higher at low and intermediate altitudes than at high altitudes. The intermediate zone appeared to have the highest EIR. The same pattern was observed with GMPD (Figures 2 and 3). However, for malaria prevalence, an unexpected lower EIR was also obtained in Debundscha (50 meters above sea level). A significant positive correlation was also found between malaria prevalence and EIR (r = 0.943, *P* = 0.005). As opposed to HBR, there was a significant negative correlation between EIR and altitude (r = –0.829, *P* = 0.04).

Sampling and *P. falciparum* status in the Mount Cameroon region. A total of 142 *P. falciparum*-positive blood samples were randomly selected for genotyping: 30 from Meanja, Mutengene, and Tiko respectively, and 19, 18, and 15 from Likoko Membea, Bonakanda, and Debundscha, respectively. Thus, we analyzed 37 samples from high altitude sites, 60 samples from intermediate altitude sites, and 45 samples from low altitude sites. Because of the discrepancy in sample size, all proportions were calculated with respect to the total number of sample tested at each altitude zone to make them comparable. Of the 142 samples genotyped, 93 (65%) were positive for MSP-1 block 2. Of the 93 positive samples, 18 (19.35%), 43 (46.24%), and 32 (34.41%) were from high, intermediate, and low altitudes sites, respectively.

***Plasmodium falciparum* MSP-1 block 2 allelic variant frequencies in sites of contrasting altitudes.** Among the 93 *P. falciparum* MSP-1 positive cases, 32 were positive for the allelic variant K-1. Six (16.21%) of 37 were from high altitude sites, 11 (18.33%) of 60 from intermediate sites, and 15 (33.33%) of 45 from low altitude sites. There was a significant difference in the frequency distribution of this allelic variant between study sites (*P* = 0.021). Sixty-nine of 93 samples were positive for the allelic variant MAD20. Nine (24.32%),

TABLE 4

Entomologic inoculation rates in relation to *Anopheles* species and locality, Mount Cameroon area

Locality	Entomologic inoculation rate (infective bites/person/night)				
	An. funestus	*An. gambiae*	*An. hancocki*	*An. nilli*	Total
Bonakanda	–	–	–	–	–
Likoko Membea	0.17	0.04	0.25	–	0.46
Meanja	0.35	0.69	0.01	0	1.05
Mutengene	1.38	0.27	0.23	0.14	2.02
Debundscha	0	0.50	0	0	0.50
Tiko	0	2.43	0	0	2.43
Total	1.9	3.93	0.49	0.14	

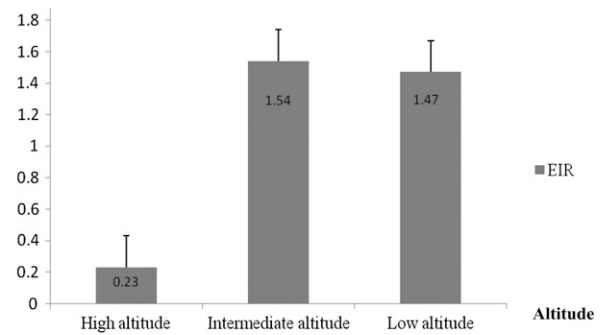FIGURE 3. Entomologic inoculation rate (EIR) at different altitude zones in the Mount Cameroon area. Each bar represents the mean ± SD EIR at a given altitude zone.

36 (60%), and 24 (54.33%) samples were from sites at high, intermediate, and low altitudes, respectively. There was also a significant difference in the frequency distribution of the allelic variant MAD20 between study sites (*P* < 0.001). Fifty samples were positive for the allelic variant RO33. Ten (27.02%), 24 (40%), and 16 (35.55%) were observed at high, intermediate, and low altitudes, respectively. The difference in the frequency distribution of the allelic variant RO33 between the study sites was not significant (*P* = 0.282).

Merozoite surface protein 1 block 2 type genetic diversity and altitude. Allelic families of MSP-1 block 2 were diverse and classified according to the size of their PCR-amplified fragments. The allelic K-1 type yielded only one fragment per sample in all the localities except Debundscha (low altitude) and Mutengene and Meanja (intermediate zone) where some samples produced ≤ 2 fragments. There was a high level of polymorphism between samples that yielded one fragment (Figure 4A). Fragment sizes were within the expected range (80–459 basepairs). DNA sequencing of these K-1 PCR-products identified five DNA sequences. Database searches with the BLAST Network Service confirmed that those sequences were from the malaria parasite *P. falciparum*. Deduced sequence homologies are presented in Supplementary Table 1. Our sequences shared high similarity (98–100%) with K-1 sequences registered in GenBank. The fifth sequence of 250 basepairs did not match any described K-1 polymorphism. The corresponding amino acid sequence (5210B) had an atypically long region starting with SAQ and terminating with SGPSGT amino acids and consisted of 13 SGT tripeptide repeats (Figure 5). The amino acid sequence 5210B was deposited in GenBank under accession no. JF968467 as a new *P. falciparum* MSP-1 block 2 K-1 polymorphism identified in the Mount Cameroon malaria-endemic region.

The allelic variant MAD20 yielded ≤ 4 four bands. The major finding here was that samples with four and three bands were mostly recorded in Debundscha, Tiko, and Mutengene (low and intermediate altitude sites) (Figure 4B). Only a limited number of samples were found to have ≤ 4 bands in Bonakanda (high altitude/low transmission). The MAD20 fragment sizes were also within the expected range (80–350 basepairs).

The allelic RO33 type was the least polymorphic. Only one sample in Debundscha resulted in amplification of ≤ 2 (Figure 4C). Most fragments were between 156 and 165 basepairs.

A **K-1 polymorphism between different samples from Tiko (low altitude)**
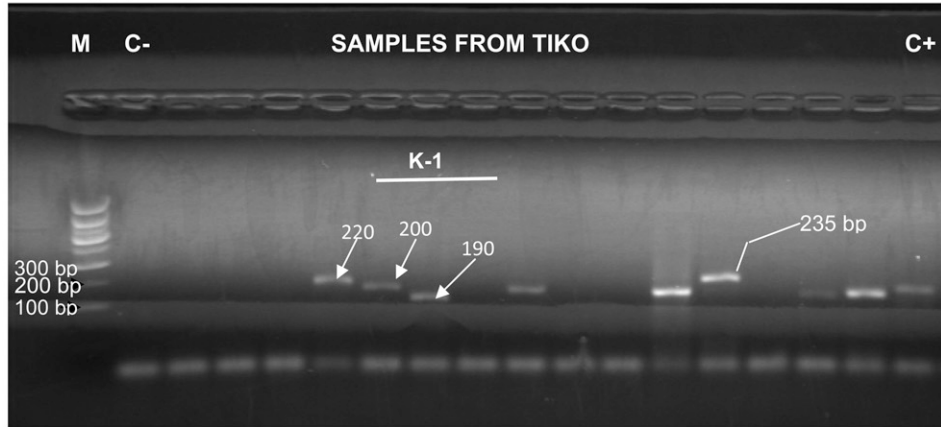
B **MAD20 polymorphism between different samples from Mutengene (intermediate altitude)**
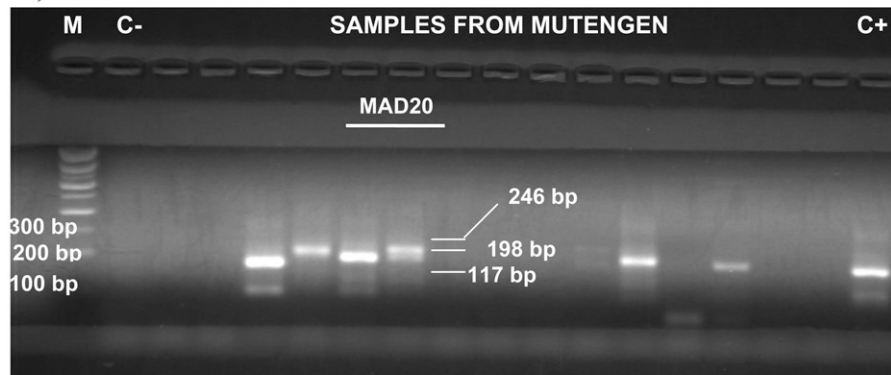
C **Lack of RO33 polymorphism between different samples from Debundscha (low altitude)**

FIGURE 4. Merozoite surface protein 1 (MSP-1) block polymorphism in the Mount Cameroon area. M = molecular marker; C– = negative control; C+ = positive control. Other lanes contain the second-round polymerase chain reaction products of different samples amplified with **a**, K-1, **b**, MAD20, and **c**, RO33 specific primers. Bp = basepairs.

Multiplicity (complexity) of infection. Forty-eight (54.02%) samples among which 7 (18.91%) of 37. 22 (36.66%) of 60, and 19 (42.22%) of 45 samples from high, intermediate and low altitude sites, respectively, contained multiclonal infections. There was a trend of increasing proportion of multiple-type infections with a decrease in altitude. The overall estimated mean ± SD number of clones per sample was 2.08 ± 1.34. There was also a trend of increasing mean ± SD number of clones per sample with a decrease in altitude. A total of 1.58 ± 0.8, 2.1 ± 1.24, and 2.32 ± 1.47 clones per sample were recorded in high, intermediate, and low altitude areas, respectively Up to six genotypes were found per sample at low (high malaria transmission) at intermediate altitudes; at high altitude (low malaria transmission), most samples had 2–3 genotypes (Figure 6). The Kruskal-Wallis test showed no significant differences between median numbers of clones per altitude zone (H = 0.632, P = 0.729). Similar to the Kruskal-Wallis test, the Dunn test did not detect any significant differences in multiplicity of infection between high and low altitudes (Q = 0.795, P > 0.05), high and intermediate altitudes
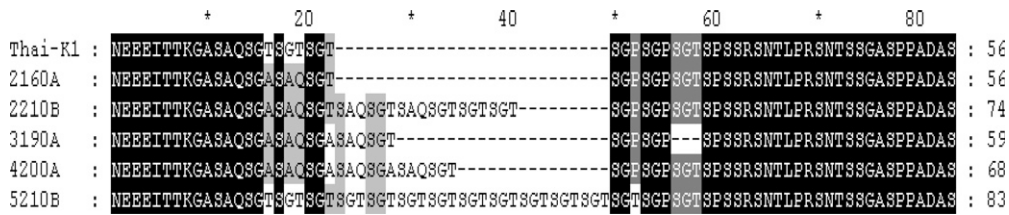
FIGURE 5.   Deduced amino acid sequences of five alleles for merozoite surface protein 2(MSP-2) block 2 K-1 type from *Plasmodium falciparum* isolates identified in the Mount Cameroon area. The protein alignment, which also includes the *P. falciparum* Thai-K1 isolate reference sequence (GenBank accession no. CAA27070), was constructed by using the MUSCLE algorithm (http://www.ebi.ac.uk/Tools/muscle/) and is shown by using the GeneDoc program (http://www.nrbsc.org/gfx/genedoc/index.html). Regions of consensus at the amino acid level are shaded and dashes represent gaps.

(Q = 0.123, *P* > 0.05), and low and intermediate altitudes (Q = 0.981, *P* > 0.05).

At high altitude, of the 18 positive samples obtained, single-type infections accounted for 61.12% and samples containing RO33 were most prevalent. The proportion of mixed-type infections was 38.88% and samples containing MAD 20 and RO33 prevailed. However, no samples were found with the three allelic variants (Figures 7 and 8). At intermediate altitude, of 43 samples amplified, 51.15% had mixed-type infections. Single-type infections with MAD 20 (27.77%) and mixed-type infections with MAD 20/RO33 (27.9%) were more common. No mixed-type infections with K-1/RO33 were observed at this level (Figure 7). At low altitude, of the 32 samples that were positive (amplified), 54.54% had mixed-type infections. Single and mixed-type infections were similarly represented at low altitude except that single-type infections with K-1 were less frequent. No mixed-type infections with K-1/RO33 were seen at this altitude (Figure 7).

The Spearman correlation rank test showed no significant association between altitude and complexity of infection (r = –0.6, *P* = 0.208) or between EIR and multiplicity of infection (r = 0.486, *P* = 0.329). However, just as higher EIRs were recorded at low and intermediate zones, infections appeared to be more complex in these zones.

DISCUSSION

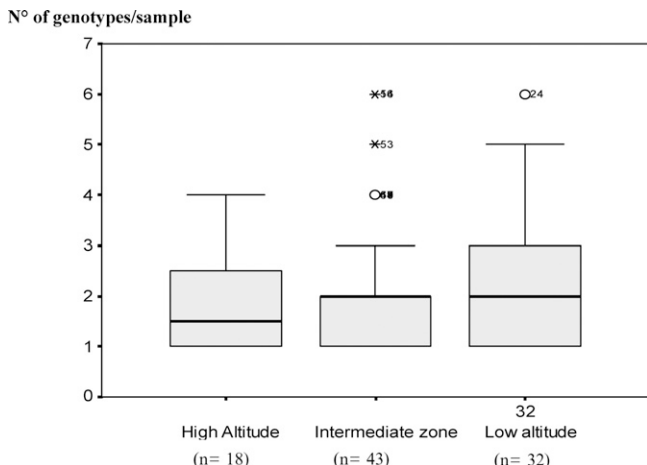This is the first report on the genetic diversity of *P. falciparum* field isolates from sites of contrasting altitudes or sites of contrasting malaria transmission profiles in the Mount Cameroon area in the South-West region of Cameroon. It is of great importance to understand the molecular characteristics of the parasite population because they can provide information for vaccine development.

This study demonstrated that the altitude of an area has an influence on prevalence and intensity of malaria infection. There was a decrease in the prevalence of malaria infection with an increase in altitude, and the difference at various altitudes was significant. A correlation test also showed a negative association between prevalence and altitude, although not significant. This finding is consistent with that of another study conducted in Tanzania, in which prevalence of malaria parasites decreased with an increase in altitude.[23] There was also a strong negative association between altitude and EIR. This finding indicates that malaria transmission decreases with altitude and can also explain malaria prevalence rates obtained in this study. However, the unexpected high malaria prevalence observed in Meanja (intermediate altitude) could be explained by a dam that created an ecosystem appropriate for *Anopheles* mosquito vector development. Conversely, low malaria prevalence recorded in Debundscha (low altitude) could be attributed to rainfall in the locality. Excessive rainfall probably decreases vector activity hence low malaria transmission and prevalence. This finding is confirmed by the low EIR in the locality.

In general, it can be suggested from the EIR and sporozoite rates of different *Anopheles* species found in this study that *An. gambiae* plays a major role in malaria transmission at low

FIGURE 6.   Multiplicity of infection at different altitude zones in the Mount Cameroon area. Error bars indicate mean ± SD.
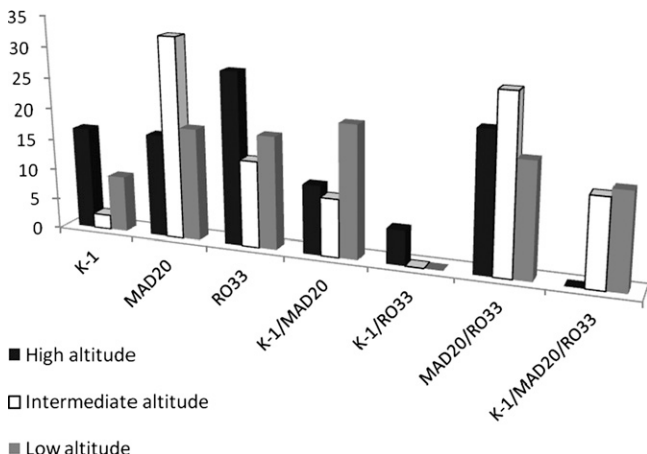

FIGURE 7.   Comparison of single- and mixed-type infections at different altitude zones in the Mount Cameroon area. Percentages were calculated with respect to total positive samples at each altitude zone.
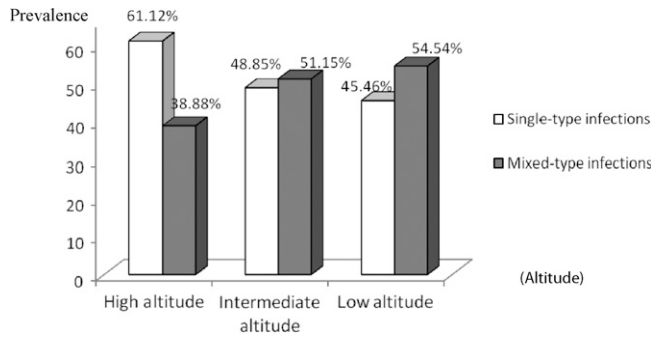
FIGURE 8. Proportions of single and mixed-type infections at high attitude, intermediate altitude, and low altitude in the Mount Cameroon area. Proportions were calculated with respect to total positive samples at each altitude zone.

altitude. At intermediate and high altitudes, *An. funestus* and to a lesser extent *An. hancocki* and *An. nilli* are also found, but they appear to be minor vectors. The EIR reflected the HBR; low and intermediate altitudes had higher values than high altitudes. This finding could be explained by the fact that the mosquito population decreases with altitude.[26] The mosquito population is reduced at high altitude because of the steepness of the slope and absence of streams or stagnant pools of water that normally play a major role in creation of breeding sites. This zone is also characterized by low temperature and low humidity, which are not favorable for mosquito vector development. The EIR at different altitude zones (Figure 3) and the GMPD (Figure 2) also showed the same pattern; the intermediate zone had relative higher values. This finding might imply an association between malaria infection intensity in the host and that in the vector. Despite the relative higher EIR value obtained in the intermediate zone, a significant negative correlation was found between EIR and altitude. This finding corroborates results obtained in Tanzania[23] because in our study, a significant positive correlation was found between EIR and prevalence.

The study also demonstrates that genetic diversity of *P. falciparum* may be a function of transmission (the relationship is non-linear). This result is similar to those of studies conducted in Thailand.[10,14] In general, the *P. falciparum* MSP gene was similarly represented in all sites included in this study. Some samples failed to amplify, which might be caused by low recovery of *P. falciparum* DNA during DNA extraction. Furthermore, because the MSP gene seems to be polymorphic, single-nucleotide polymorphisms might be present

in regions for which primers were designed and this may hinder proper primer annealing.

The three allelic variants K-1, MAD20, and RO33 were observed in all six localities. However, allelic variant K-1 was highly distributed in low altitude sites compared with intermediate and high altitude sites, but MAD20 and RO33 allelic variants were common at an intermediate altitude than at a low altitude (Table 5). This low distribution of K-1 at a high altitude could be accounted for by the low number of samples used, but using the sample size at each altitude zone in calculating the different proportions did not result in a different distribution pattern. If one considers that K-1 was rarely observed in all the localities in the Mount Cameroon region, it could be suggested that certain *P. falciparum* strains had single nucleotide polymorphisms in DNA regions to which primers were designed, which interfered with proper annealing for this particular allelic type. If not true, it might suggest a relative negative natural selection in the region because overall distribution of K-1 in the region was low. However, this observation does not corroborate most findings in areas of holoendemic, mesoendemic, and hyperendemic malaria, in which the allelic variant K-1 was predominant.[8,15,35]

Conversely, The MAD20 allelic variant frequency was high in all sites and the same trend was observed for allelic variant combinations containing this allelic variant. This result is consistent with those of other studies. Snounou and others[14] also found a high prevalence of the allelic variant MAD20 in comparison with K-1 and RO33 allelic variants. Other studies conducted in Colombia, southern Vietnam, the Brazilian Amazon, and some countries in Africa such as the Gambia, Nigeria, and Gabon reported the predominance of MAD20 and RO33 allelic variants.[36–40] These findings were attributed to a positive natural selection for these two allelic variants in *P. falciparum*. However, these results cannot explain the result obtained in this study with certainty because the high frequency of MAD 20 we observed is with respect to the frequency of K-1, which may be caused by poor annealing as explained. Another peculiarity to our study is that RO33 allelic variant, which has been shown in many studies to be the least predominant, was the second most prevalent allelic variant type. Nevertheless, this finding corroborates findings of other studies.[36–40] The high frequency of the RO33 allelic variant in the region may be associated with reduced risk of clinical malaria.[41] Most patients who have this parasite allelic variant had high parasitemias ($\leq$ 29,520 parasites/μL). However, children did not show any clinical manifestations of malaria.

TABLE 5
Frequencies of merozoite surface protein 1 allelic variants with respect to locality, Mount Cameroon area

Characteristic		Allelic type, no. (%)		
		K-1	MAD20	RO33
High altitude	Bonakanda (18)	3	8	6
	Likoko Membea (19)	3	1	4
	Total (37)	6 (16.21)	9 (24.32)	10 (27.02)
Intermediate altitude	Meanja (30)	2	19	16
	Mutengene (30)	9	17	8
	Total (60)	11 (18.33)	36 (60)	24 (40)
Low altitude	Debundscha (15)	3	9	8
	Tiko (30)	12	15	8
	Total (45)	15 (33.33)	24 (53.33)	16 (35.55)
Overall total of each allelic type (142)		32 (22.53)	69 (48.59)	50 (35.21)
Chi-square test		$\chi^2 = 7.731$, $P = 0.021$	$\chi^2 = 15.956$, $P < 0.001$	$\chi^2 = 2.529$, $P = 0.282$

Moreover, most samples with multiple polymorphisms within allelic variants (K-1 and MAD20) were obtained from low altitude sites. Even the one sample that had two polymorphisms for the allelic variant RO33 was from Debundsha (low altitude). Genetic diversity of P. *falciparum* populations seems to be positively associated with high parasite transmission in these geographic locations.[8,12,42] In our study, sizes of MSP-1 allelic variants were within ranges obtained by other authors.[43]

DNA sequencing and BLAST analysis confirmed that polymorphisms obtained from PCR typing of MSP-1 allelic variant K-1 were from *P. falciparum*. Among the five K-1 polymorphisms obtained from *P. falciparum* DNA samples from the Mount Cameroon region; four had 98–100% homology with described K-1 polymorphisms. Sequences in GenBank were from *P. falciparum* isolates from several countries in Africa. Therefore, a vaccine based on MSP-1 allelic variant K-1 may give immune protection against malaria in those regions in Africa. However, the fifth K-1 sequence identified as 5210B-T7, which was different from any of the described K-1 sequences, could be a new polymorphism of K-1. This polymorphism could also be the basis of a malaria vaccine. However, for such an assertion to be made, further immunologic characterization of this candidate polymorphism needs to be conducted. In addition, sequencing and immunologic characterization of other allelic variants (MAD20 and R033) should also be conducted to obtain more useful information.

Detection of more than one allelic type for block 2 provided evidence for multiple *P. falciparum* infections of many persons on the basis of block 2 typing. Forty-eight (54%) samples were found to carry multiple allelic variants ranging from two to six distinguishable polymorphisms per infection. The overall mean ± SD number of genotypes per sample was 2.08 ± 1.34, which was similar to that observed in school children in Molyko and Buea (other localities in the Mount Cameroon region).[35] Although there was no significant difference in the median numbers of genotypes/sample, there was a trend of decreasing mean number of *P. falciparum* genotypes or clones per person and of proportions of samples with mixed-type infections with an increase in altitude in the Mount Cameroon region. Persons living in intermediate and low altitude sites had ≤ 6 polymorphisms of *P. falciparum* of different allelic variants, and persons living in high altitude sites had 2–3 polymorphisms. No mixed-type infection harboring the three allelic variants was found at high altitude; this finding might reflect low transmission that prevails at that level. However, given the sample size used, a clear conclusion cannot be made. Moreover, there was a significant negative correlation between altitude and the EIR. This finding is consistent with other studies that demonstrated a decrease in malaria transmission with an increase in altitude,[23,44] which in turn affects the genetic diversity of the parasite population.[20,42]

Genetic diversity is caused by recombination events that occur during sexual reproduction and asexual multiplication in the vector, but these recombination events are rare between allelic types. This finding can justify the high complexity of infection observed at low and intermediate altitudes where malaria transmission also appears to be high. However, the relationship between malaria transmission and multiplicity of infection is not linear. In our study, the complexity of infection was not directly associated with the level of transmission. For instance, the infection was more complex (2.32 polymorphisms/infection) at low altitude, which had an EIR of 1.47 infective bites/person/night than at intermediate altitude (2.1 polymorphisms/person), which had a relatively higher EIR of 1.54 infective bites/person/night. This finding indicates that an increase in transmission intensity does not necessarily translate into a parallel increase in the number of polymorphisms.[10,45] A precise analysis of the influence of transmission intensity on the extent of parasite diversity is complicated by the marked annual variation of the EIR.[46] Moreover, additional factors such as resistance to antimalaria drugs and migration (movement of older persons from one locality to another) have been shown to increase the diversity of parasite population through introduction of foreign polymorphisms.[10,14,45,47] This finding could explain the relatively high complexity found in Bonakanda (1,197 meters above sea level), which in our study was characterized by an EIR close to zero. Mosquito vectors at this altitude are rare because the steepness of the slope, and the absence of streams at this altitude prevent formation of breeding sites. In addition, low temperature ($< 15°C$) and low relative humidity interfere with proper development of the mosquito vector.[26] However with the limited sample size, a clear conclusion cannot be drawn because the relatively high complexity can be accounted for by the limited number of samples tested.

This study has demonstrated that altitude of an area has an influence on the level of endemicity and the genetic diversity of the local malaria parasite. *Anopheles gambiae* appeared to be the major malaria vector at low altitudes. At intermediate and high altitudes, *An. funestus* (which predominates at intermediate zone), *An. hancocki*, and *An. nilli* are also found, but they play minor roles in malaria transmission. Our study showed a positive association between low altitude where malaria transmission is high and MSP-1 block 2 polymorphism and complexity of infection. However, the uneven allelic polymorphisms observed in this study between different sites may be a hindrance for malaria vaccine formulations if not considered in vaccine development studies. More immunologic studies are needed to characterize the new allelic K-1 polymorphism and determine its potential to serve as a suitable vaccine candidate.

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SUPPLEMENTARY TABLE 1

NCBI BLASTanalysis of *P. falciparum* MSP-1 block 2 K-1 type DNA genotyping results from the Mount Cameroon area

Sample	GenBank accession no.	K-1 allele size (bp)	BLASTN highest homology, GenBank description	E value	% Nucleotide identity	% Amino acid identity
2160A		169	M77730.2 *P. falciparum* clone 834B major MSP	9e-75	97	94
2210B	JF968465	223	AF509633.1 *P. falciparum* isolate MSP-1 170	8e-96	95	90
190A		178	AF191061 *P. falciparum* isolate 1/M1 MSP-1	1e-72	94	86
4200A	JF968466	205	AB276007.1 *P. falciparum msp-1* gene for MSP-1	1e-99	100	100
5210B	JF968467	250	EU445566.1 *P. falciparum* type 12 MSP-1	4e-113	96	92

NCBI = National Center for Biotechnology Information; BLAST = basic local alignment search tool; MSP-1 = merozoite surface protein 1; bp = basepairs.