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Genetic polymorphism of *msp2* in *Plasmodium falciparum* isolates among asymptomatic malaria infections from two ecological settings in Cameroon

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Abstract

Background The high genetic diversity of *Plasmodium falciparum* parasites is one of the challenges for malaria control and elimination in endemic areas. A better knowledge of parasite genotypes circulating in different disease endemic areas could help to optimize local malaria interventions. This study aimed at determining *P. falciparum* genetic diversity from isolates collected in forest (Mfou) and humid savanna (Tibati) eco-epidemiological settings in Cameroon.

Methods Dried blood spots collected from asymptomatic individuals in 2018 and 2019 were used to determine the *Plasmodium* infection status and distinguish the *Plasmodium* spp. by real-time PCR. Allelic polymorphism of the *msp2* gene was assessed in the *P. falciparum* positive samples by nested PCR followed by capillary electrophoresis for revelation of the fragment allelic size. Multiplicity of infection (MOI) was defined as the number of coinfecting genotypes within an infection. General linear mixed models were fitted to evaluate the impact of study site, participant age, gender and bed net ownership on genetic diversity.

Results Malaria prevalence among the asymptomatic individuals reached 59.2% (876/1480) in Mfou and 63.4% (808/1274) in Tibati. A total of 36 and 42 different *msp2* alleles were detected in Mfou and Tibati, respectively. No genetic differentiation was observed between the two study sites. The *msp2* IC/3D7 family was the most polymorphic and the most prevalent in both areas. Overall, more than 60% of the isolates had multiclonal infections. The frequency of multiclonal infections and MOI was higher in Mfou (68.9%, MOI = 2.08) than in Tibati (57.29%, MOI = 1.80). In Mfou, a negative correlation was found between MOI and age. Similarly, a gender effect was observed in Mfou, with males having higher MOI than females.

Conclusion This study reported high malaria prevalence and a high allelic diversity in the *msp2* gene among asymptomatic carriers from two epidemiological settings of Cameroon. Despite results reflects high transmission intensity

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in both areas, analysis indicated distinct epidemiological patterns in Mfou and Tibati. These findings will provide valuable baseline information to monitor the impact of malaria control measures implemented in these areas.

Keywords Genetic diversity, *Plasmodium falciparum*, Merozoite surface protein 2, Multiclonal infections, Heterozygosity

Background

Malaria remains a major public health problem despite global efforts to fight against the disease. In malaria endemic countries, control interventions include preventive measures such as insecticide-treated bed nets (ITNs), intermittent preventive treatment in infants and pregnant women (IPTi and IPTp), and, in areas where malaria is seasonal, seasonal malaria chemoprevention (SMC). Additionally, case management through early diagnosis and treatment plays a crucial role and is fundamental for accurate surveillance. Recently, the introduction of malaria vaccines into routine childhood immunization programs has provided a new intervention tool. The latest World Health Organization (WHO) report pointed to 263 million cases, 11 million more cases compared to 2022, and 597 000 deaths worldwide in 2023, with 94% of cases occurring in the WHO African region and almost 99% of malaria cases due to *Plasmodium falciparum* [1].

Cameroon is among the highest burden countries, with a malaria prevalence of about 30% and almost 4 000 malaria deaths reported each year [1]. According to the National Malaria Control Programme (NMCP) report, approximately 28% of hospital morbidity and 18.3% of deaths were due to malaria in 2019 [2]. The burden of the disease disproportionately affects children under 5 years of age and pregnant women, who account for over 70% of malaria cases [2]. Malaria endemicity is further influences by geographical and ecological variations across the country, which affect malaria vector distribution and transmission intensity [3–6]. In particular, the East, Central and South regions, with a perennial transmission, and Adamawa, where transmission is seasonal, are the most affected, classified as hyperendemic [7]. Four human *Plasmodium* species have been documented in Cameroon, including *P. falciparum, Plasmodium ovale, Plasmodium malariae* and *Plasmodium vivax* [3, 8]. However, *P falciparum* remains the most dominant species, and this species is responsible for the most severe clinical forms of the disease and malaria associated deaths in children [3, 9, 10].

So far, in Cameroon, malaria control strategies were mainly based on vector control tools through the massive deployment of long-lasting insecticidal nets (LLINs), the IPTi and IPTp and the prompt treatment of clinical malaria cases with artemisinin-based combination therapy (ACT) [3, 11]. Although these malaria control measures have been intensified, Cameroon has experienced a resurgence of malaria cases over the last past years [2]. Even if this can be due in part to increased resistance to insecticides and drugs [11, 12], other factors such as global warming, population growth, urbanization or impairment of control programmes during the Covid-19 pandemic may have lead to the current situation [13, 14]. The RTS,S/AS01 malaria vaccine has been introduced in Cameroon in January 2024 [15]. The vaccine is intended to complement the existing tools for malaria prevention and control [15] but no data on its effective implementation and impact in Cameroon have been published so far.

The high genetic diversity of *P. falciparum* has been identified as a key mechanism enabling the parasite to evade host immune defenses [16, 17]. Additionally, the extensive polymorphism in *Plasmodium* proteins, particularly in merozoite surface proteins, has hindered the efficacy of anti-malarial drugs and the development of effective malaria vaccines [18, 19]. Molecular surveillance will be crucial to evaluate changes in the parasite populations following vaccine deployment and identify genotypes that could potentially evade vaccine-induced protection [20, 21]. Monitoring the genetic diversity and transmission dynamics of parasites will also be essential to evaluate the impact of control measures and environmental factors on the genetic structure of the parasite populations [22–24]. Both the genetic diversity of *P. falciparum* and the number of co-infecting genotypes correlate with malaria infection intensity [22, 25]. In agreement with the high intensity of malaria transmission in Cameroon, *P. falciparum* presents a large genetic polymorphism [26, 27] and up to 15 genotypes have been recorded within a single infection [27]. As multiclonal infections are carrying mixture of anti-malarial drug resistance mutations, they may impact the spread of drug resistance and malaria epidemiology [28, 29].

Polymorphic molecular markers are widely used to distinguish parasite genotypes within multiclonal infections or to assess the impact of malaria control interventions. The most commonly used molecular markers for genotyping are the genes encoding for the merozoite surface proteins 1 and 2 (MSP1 and MSP2) and the glutamate-rich protein (GLURP) of *P. falciparum* [30–34]. The *msp2* gene located on chromosome 2 codes for a glycoprotein expressed on the surface of merozoites and consists in

five blocks of which the central block, block 3, is the most polymorphic. Variations in length and sequence in the *msp2* block 3 allow to distinguish two main allelic families, IC/3D7 and FC27, and polymorphisms in the block 3 are largely used to describe the genetic diversity of *P. falciparum* populations in malaria endemic areas [31, 35].

So far, very limited information on *P. falciparum* genetic diversity in different eco-epidemiological settings in Cameroon exists [26, 27, 36–38]. This study aimed to characterize the genetic diversity of the *msp2* gene in *P. falciparum* field isolates collected from asymptomatic individuals in two distinct eco-epidemiological settings in Cameroon, forested and humid savannah areas. By analysing the *msp2* polymorphism, this research seeks to enhance understanding of parasite antigenic diversity in areas with different transmission patterns, which will provide critical insights for the implementation of malaria control strategies and surveillance in the region.

Methods

Ethical statement

The research protocol was reviewed and approved by the Cameroon National Ethics Committee for Research on Human Health under agreements 2018/05/1011/CE/CNERSH/SP and 2019/05/1161/CE/CNERSH/SP. Volunteers were recruited through community information meetings held in collaboration with local authorities. During these community sensitizations, the study objectives, procedures, and participants'right to withdraw were clearly explained, with opportunities for questions. All participants provided signed informed consent**;** for minors, signed authorization was obtained from parents or legal guardians. Volunteers were monitored throughout the study and those who were found parasite-positive received a free treatment with artesunate-amodiaquine (ASAQ) according to national guidelines.

Study site description

The study was conducted between June and July 2018 in Mfou (3°43′17.0"N; 11°38′39.0"E) and July–August 2019 in Tibati (6°27′57.0"N; 12°37′29.0"E), two localities in Cameroon with different eco-epidemiological settings (Fig. 1).

Mfou is a semi-urban area located in the Central region of Cameroon. It is characterized by a dense vegetation and an equatorial climate with two dry seasons (November–February and June–July) and two rainy seasons (August–October and March–May). Malaria transmission is perennial with a higher prevalence during the rainy seasons. The hydrographic network comprises

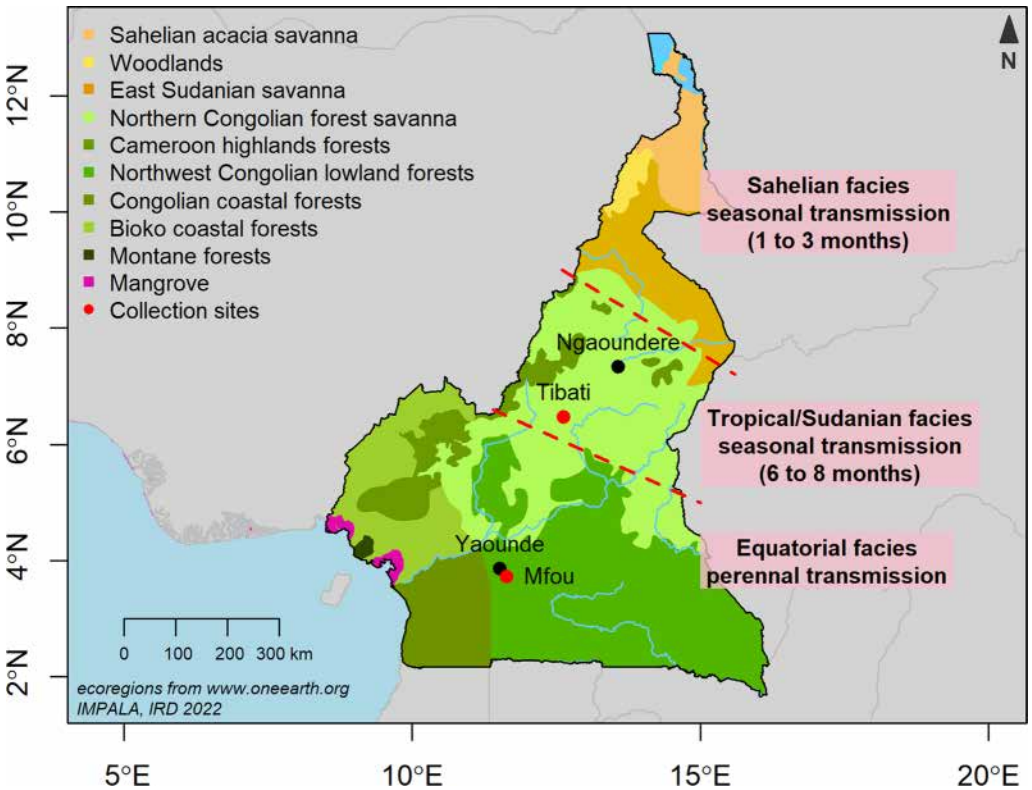

Fig. 1 Map of the study sites in Cameroon. The figure displays the ecoregions and the two study sites

several rivers and the most important are Nsoh, Meben, Olo'o, Etoa, Osomvele and Mefou rivers. The Mfou district covers a surface area of 3338 km^2 and has an estimated population of 42,000. Economic activities in the region are mainly agriculture and fishing. Mfou is a great health district with 12 health facilities available. Previous studies in the Mfou district indicated a malaria prevalence around 70% among asymptomatic individuals [39, 40], and the 2022 Cameroon Malaria Indicator Survey reports an overall 49% prevalence among children less than 5 years of age in the Centre region [7]. The 2019 annual report of the NMCP indicates an incidence of 120 cases per 1000 habitants and a mortality rate of 9 deaths per 100,000 population [2]. Annual transmission was estimated at 129 infective bite/person/year and each person receives about 20 mosquito bites per night [41].

Tibati, located in the Adamawa region, is characterized by a humid savannah climate, with one rainy season lasting over 6 months, from March to October. Malaria transmission is seasonal in the locality. Tibati is crossed with several lakes, ponds, agricultural sites and swamps favorable to the reproduction of the malaria vectors. Tibati is around 8000 km^2, with an estimated population of 108,502. Human activities are mainly agriculture and fishing in the site. Tibati district also has 12 health facilities. According to the 2019 annual report of the NMCP, malaria prevalence in the Adamawa region was 34%, with an estimated incidence of 141 cases per 1000 habitants and a mortality rate of 36 deaths per 100,000 population [2]. However, local heterogeneity exists, as a 61% prevalence was documented in Tibati among asymptomatic individuals [9]. Human biting rate has been estimated at 30 bites/person/night, with an average entomological inoculation rate (EIR) of 77 infective bite/person/year [42].

In both areas, *P. falciparum* is responsible for more than 90% of malaria infections, and *Anopheles gambiae* and *Anopheles funestus* are the main malaria vectors [3].

Study population

A random survey was carried out in each site and study participants were recruited among asymptomatic individuals in the communities. A total of 23 villages were screened, 14 in Mfou and 9 in Tibati. Volunteers who met inclusion criteria: aged at least one year, no fever (axillary temperature $\leq$37.5 °C) within the previous 48 h, no ongoing antimalarial treatment, and who signed an informed consent form were enrolled in the study. Signs and symptoms of severe malaria, use of an anti-malarial within the last two weeks, pregnancy were exclusion criteria. A questionnaire was used to collect demographic and clinical information from all volunteers.

The sample size was calculated using the formula $N = Z^2\ P\ (1-P)/d^2$, where N is the sample size, Z the standard normal variate (1.96 at 95% confidence interval), P the expected prevalence and d the margin of error (5%) [43]. The expected malaria prevalence, P, was estimated according to data provided by the NMCP in its 2019 report, Mfou (47%) and Tibati (32%) [2]. This provided an estimated sample size of 383 individuals in Mfou and 334 in Tibati.

Sample collection

Finger-prick blood samples were collected for malaria diagnosis upon microscopical examination of Giemsa-stained thick blood smears. Blood films were air-dried, stained with 10% Giemsa for 20 min, and examined under a light microscope (Leica DM750; Leica Microsystems GmbH, Wetzlar, Germany) at 100 × magnification for the detection of asexual stages. Thick blood smears were screened across 200 fields under oil immersion. Simultaneously, blood samples were spotted on a Whatman® grade17 filter paper (Whatman® Grade 17 Cellulose Chromatography Paper, GE Healthcare, Chicago, USA) and air-dried. Dried blood spots (DBS) were conserved individually in paper bags containing silica gel and stored at −20 °C until molecular analyses. DBS samples from Mfou were processed 2 months after collection and those from Tibati 8 months after collection.

DNA extraction and molecular identification of *Plasmodium* species

Genomic DNAs from dried blood spots were extracted using the chelex-100 method as previously described [44]. After extraction, DNA concentration and purity were estimated for each sample using the Nano Drop spectrophotometer (Thermo Scientific NanoDrop™ 2000) and stored at −20 °C until molecular analyses. *Plasmodium* spp were detected and identified by real-time PCR according to Mangold et al. [45]. The PCR assay differentiates the four plasmodial species (*P. falciparum, P. vivax, P. ovale* and *P. malariae*) based on melting curve analysis, using species-specific melting temperature (Tm) and amplicon sizes. PCR amplification and analysis were performed on a Light Cycler® 96 instrument (Roche Molecular Systems, Indianapolis, USA) with SYBR Green fluorescence detection.

Genotyping of *Plasmodium falciparum* parasites

Only samples identified as *P. falciparum*-positive by real-time PCR were considered for *msp2* genotyping analysis. The repetitive polymorphic regions in the block 3 of the *msp2* gene were amplified by nested PCR as previously described [33]. Amplified products from the secondary PCR were submitted to capillary electrophoresis to reveal

the different alleles of the two allelic families (FC27 and IC/3D7). PCR reactions were processed with minor modifications; the initial denaturation was set at 94 °C instead of 95 °C and the final extension lasted 5 min at 72 °C instead of 2 min. Briefly, the primary reaction was carried out in a final volume of 15 µL containing 1× PCR buffer, 2 mM $MgCl_2$, 125 µM dNTP, 0.02 units/µl of EurogentecTaq® DNA polymerase, 250 nM of each primer (Eurogentec, Belgium) and 2 µl of DNA template. The cycle conditions were as follows: an initial denaturation at 94 °C for 2 min followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 2 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. For the secondary PCR, a 15 µl PCR mixture was used containing: 2 µl of the primary reaction product as template, 1× PCR buffer, 1 mM $MgCl_2$, 125 µM dNTP, 0.02 units/µl of EurogentecTaq® DNA polymerase and either 125 nM of each primer for the FC27 family or 300 nM for the IC/3D7 family. Secondary PCRs for the two different allelic families were performed in separate reactions. The cycle conditions were as follows: an initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The 5′-end of the forward primers was tailed with a 7-bp tail to avoid genotyping errors, according to Liljander et al. [46]. The reverse primers were labeled with different fluorophores at the 5′-end: FC27 with 6-FAM™ (blue) and IC/3D7 with VIC® (green) (Additional file 1: Table S1).

Allele-specific positive (DNA from 3D7 and HB3 cultures) and negative (sterile water) controls were included in each set of reactions. The nested *msp2* PCR products were separated by gel electrophoresis on a 2% agarose gel containing ethidium bromide and the gel was observed on a transilluminator under UV light. Positive samples were subsequently prepared for capillary electrophoresis analysis.

Fragment allelic analysis by capillary electrophoresis

The fragment analysis was carried out on a 3500xL DNA sequencer (Applied Biosystems), using POP-7™ polymer (Applied Biosystems). 1 µl product of each nested PCR of the *msp2* allelic type (FC27 and IC/3D7) were pooled and diluted 1:50 in sterile water to achieve peaks < 8000 rfu. 1 µl of the pooled product was added to 13.9 µl of Hi-Di formamide and 0.1 µl size standard (GeneScan™ 1200 LIZ®, Applied Biosystems) per well on 96-well plates. The separation was run at 19.5 kV for 25 min. Fragment sizes were determined by comparison to the GeneScan™ 1200 LIZ size standard and analyzed using GeneMapper® Software v4.0 (Applied Biosystems). A cut-off value of 300 relative fluorescent units (rfu) was applied for allele calling. Binsets were predefined for each allele family based on fragment sizes of controls and previously characterized samples to enable automatic scoring. To ensure accuracy, electropherograms were manually inspected to distinguish artifact and stutter peaks, that could exceed 300 rfu, and fragments that felt outside the binset ranges. Alleles were then identified by both fragment size and fluorescent dye, FC27 with 6-FAM™ (blue) and IC/3D7 with VIC® (green). Because of the 7-bp tail on the forward primers, the estimated sizes include an extra 8 bp segment (7 bp tail + A) [46].

Data analysis

Multiplicity of infection (MOI) was defined as the number of distinct parasite alleles present in a given infection. The frequency of FC27 and IC/3D7 allelic families was calculated as the proportion of the given allele out of the total of alleles detected.

Statistical analyses were performed using R version 4.4.2 [47]. Generalized Linear Mixed Models (GLMMs) were fitted using the glmmTMB package [48] to assess the effects of study site (categorical, two levels: Mfou and Tibati), gender (categorical, two levels: male and female), age (numeric), and bed net ownership (categorical, two levels: yes or no), along with their interactions, on three outcomes: (i) malaria prevalence, (ii) multiclonality, both modeled using a binomial distribution, and (iii) MOI, modelled using a zero-truncated Poisson distribution, as MOI values are strictly positive integers and cannot equal zero. The effect of site, gender, age and interactions on bed net owenership was analysed using a binomial GLMM. In each model, village was included as a random effect to account for clustering (i.e. 14 villages in Mfou and 9 in Tibati). The statistical significance of fixed effects was evaluated using the Type II Wald chi-square test, implemented via the Anova function in the car package [49]. Diversity indexes were calculated to characterize the allelic diversity for each allelic family in the two study sites using the Vegan package [50]. Expected heterozygosity was calculated using the formula $He = [n/(n-1)][1 - \sum p_i^2]$, where n is the number of genotyped samples and p_i the frequency of the *i* allele at a given locus [51]. Differentiation measures between study sites and allele distribution were calculated using the SpadeR package [52]. The level of statistical significance was set at $P \leq 0.05$.

Results

Characteristics of malaria infections

Malaria prevalence was not significantly different between the two studied areas, 59.2% (876/1480) in Mfou vs 63.4% (808/1274) in Tibati, (LRT $X^2_1 = 1.5$, $P = 0.214$; Additional file 1: Table S2). *Plasmodium falciparum* was the predominant *Plasmodium* species in

both sites, present in > 95% of total infections, 95.8% (839/876) in Mfou and 98% (791/808) in Tibati. *Plasmodium malariae* infections were present only in Mfou, in 4.2% (37/876) samples and 54% (20/37) were mixed *P. falciparum/P. malariae* infections. In contrast, *P. ovale* was only identified in Tibati, in 2% (17/791) samples and 29.4% (5/17) were mixed *P. falciparum/P. ovale* infections.

ITN ownership was higher in Mfou as compared to Tibati (58.4% vs 37.5%, LRT $X^2_1 = 9.7$, $P = 0.002$) (Table 1). Females were owning an ITN more frequently than males, 51.7% (737/1425) vs 45.6% (601/1317) (LRT $X^2_1 = 8.4$, $P = 0.004$). Participants who reported owning an ITN were less likely to be infected, 58.4% (782/1338) vs 63.9% (900/1414) for those that had no ITN (LRT $X^2_1 = 6.4$, $P = 0.01$) (Additional file 1: Fig. S1 A, Tables S2 and S3), albeit the ITN effect on malaria prevalence was only significant in Mfou (LRT $X^2_1 = 4.1$, $P = 0.04$). In particular, owning an ITN was associated with a 20% reduction in the odds of malaria infection (OR = 0.801, 95% CI [0.680–0.943]). Males had an overall higher prevalence of malaria, 64% (843/1317) vs 58.6% (835/1425) for females (LRT $X^2_1 = 4.4$, $P = 0.035$) (Additional file 1: Fig. S1B). However, the association was only significant in Mfou (63.7% for males vs 54.8% for females; LRT $X^2_1 = 6.4$, $P = 0.012$) (Additional file 1: Fig. S1B). Prevalence significantly decreased with age (LRT $X^2_1 = 154$, $P < 0.001$) (Additional file 1: Table S3), with a more pronounced decline in Mfou compared to Tibati (significant age-by-site interaction: LRT $X^2_1 = 10.4$, $P = 0.001$) (Additional file 1: Fig. S1 C and Table S3).

Table 1 Characteristics of the study participants

	Both sites	Mfou	Tibati
Own an ITN, n (%)			
Yes	1338 (48.6)	865 (58.4)	473 (37.5)
No	1414 (51.4)	615 (42.6)	799 (62.8)
Sex, n (%)			
Female	1425 (52.0)	755 (51.1)	670 (53.1)
Male	1317 (48.0)	725 (48.9)	592 (46.9)
Age			
Mean (± SD)	13.9 (± 14.1)	13.4 (± 13.4)	14.6 (± 14.7)
Range	1–97	1–87	1–95

Frequency of the allelic families of *Plasmodium falciparum msp2* gene

A total of 611 and 377 isolates from Mfou and Tibati, respectively, were successfully genotyped for *msp2* (Table 2). The genotyping success rate was lower for samples from Tibati (377/791; 48%) compared to those from Mfou (611/839; 73%), and this might be due to differences in storage conditions. Specifically, DBS specimens from Tibati experienced prolonged transport to Yaoundé and were kept at ambient temperature in silica gel for several days before final preservation at −20 °C in the laboratory, which could have compromised DNA quality. In Mfou, the IC/3D7 and FC27 allelic families were detected in 527/611 (86.3%) and 454/611 (74.3%) of the samples, respectively. Among the samples from Tibati, 312 (82.8%) and 256 (67.9%) were carrying the IC/3D7 and FC27 type alleles, respectively. In both areas, the IC/3D7 type alleles were the most frequent ones (Mfou, $X^2 = 27$, $P < 0.001$ and Tibati, $X^2 = 22$, $P < 0.001$). The frequency of FC27 type alleles was significantly higher in Mfou as compared to Tibati (74.3% vs 67.9%; $X^2 = 4.4$, $P = 0.036$). The isolates that carried both IC/3D7-FC27 allelic families were found more frequently in Mfou 60.6% (370/611) than in Tibati 50.7% (191/377) ($X^2 = 8.9$, $P = 0.003$).

Allelic diversity of *Plasmodium falciparum msp2* gene

The different *msp*2 alleles were classified according to size (in base pairs) and family type for each study site and diversity indices were computed for each allelic family (Fig. 2, Table 3). A total of 36 and 42 allelic variants of *msp*2 were detected in Mfou and Tibati, respectively. In the FC27 family, only 6 and 7 alleles were found in Mfou and Tibati, respectively, 5 being shared in both sites (Fig. 2A and C). 30 different IC/3D7 allelic fragments were identified in Mfou, 35 in Tibati, and 26 alleles were shared between the two sites (Fig. 2B and D). The allelic diversity was higher in the IC/3D7 family than in the FC27 family in both sites, as revealed by the Shannon values (Table 3). The Pielou's evenness indices indicate a balanced distribution of alleles in the IC/3D7 family

Table 2 Frequency of the *msp2* allelic families IC/3D7 and FC27 in the two studied sites

msp2 families	Both sites N = 988	Mfou N = 611	Tibati N = 377	P-value
IC/3D7, n (%)	839 (84.92)	527 (86.25)	312 (82.76)	0.162
FC27, n (%)	710 (71.86)	454 (74.30)	256 (67.90)	0.036
IC/3D7–FC27, n (%)	561 (56.78)	370 (60.56)	191 (50.66)	0.003

The frequency of alleles for each family was compared between Mfou and Tibati using a Chi square test

N number of isolates analysed

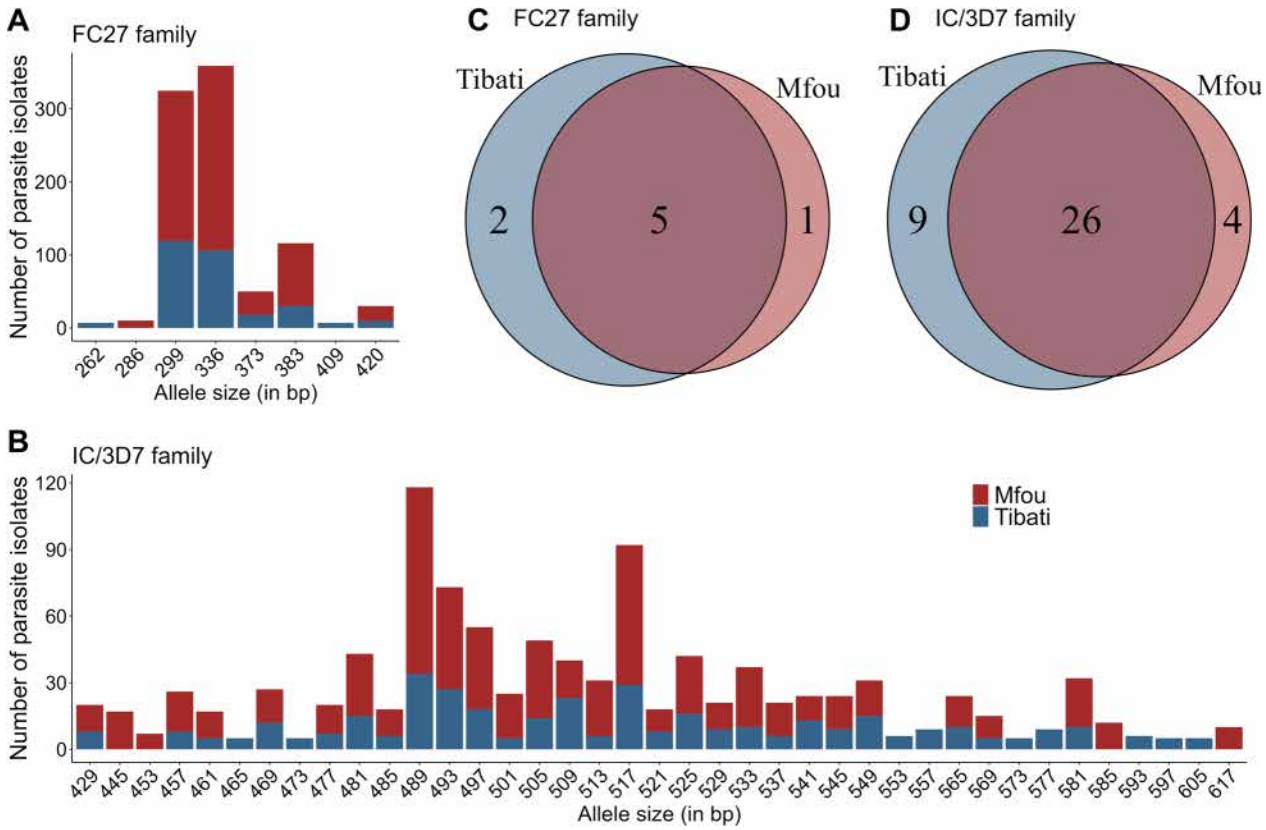

Fig. 2 Diversity and distribution of the *msp2* alleles in Mfou and Tibati. **A** FC27 allele family distribution. **B** IC/3D7 allele distribution. **C** and **D** Schematic representation of distinct and shared alleles in the FC27 and IC/3D7 families

Table 3 Diversity indices and differentiation between Mfou and Tibati for both IC/3D7 and FC27 allelic families

Allelic family	**IC/3D7**		**FC27**	
Study site	**Mfou**	**Tibati**	**Mfou**	**Tibati**
Shannon	3.153	3.344	1.344	1.347
Pielou's evenness	0.937	0.949	0.749	0.733
Cumulated alleles	661	382	607	297
Allelic richness	30	35	6	7
Shared alleles	26		5	
Heterozygosity *He* (SD)	0.94 (0.0021)	0.96 (0.0018)	0.69 (0.0074)	0.81 (0.0112)
Gst differentiation (SD)	0.0014 (0.0007)		0.0038 (0.0026)	

(0.94 in Mfou and 0.95 in Tibati), while the smaller values in the FC27 family (0.75 in Mfou and 0.73 in Tibati) reflects the dominance of certain alleles. And indeed the 299 bp and 336 bp alleles of the FC27 family were the most prevalent in both areas, with proportions over 30% (Additional file 1: Fig. S2). Expected heterozygosity was high at both IC/3D7 and FC7 loci (Table 3), reflecting high genetic variability. Pairwise comparison of the allelic composition between the study areas did not detect genetic differentiation for both allelic families (Gst $=0.0014 \pm 0.0007$ for IC/3D7 and Gst $=0.0038 \pm 0.0026$ for FC27; Table 3), which is suggestive of high gene flow between the study sites.

Diversity indices were calculated using the VEGAN package (version 2.5–7) [28] and the SpadeR package [29]. SD. estimates were obtained by 100 bootstrap replications. *He* was computed according to Nei [51].

Multiplicity of *P. falciparum* infections

MOI was defined as the cumulated number of alleles identified at IC/3D7 and FC27 within each infection and multiclonal infections as samples with MOI > 1.

Overall, 64.47% (637/988) of *P. falciparum* isolates harbored two or more clones. The prevalence of multiclonal infections was higher among the isolates from Mfou as compared to those from Tibati, 68.9% (421/611) in Mfou and 57.3% (216/377) in Tibati (LRT $X^2_1 = 9.2$, $P = 0.002$) (Fig. 3A, Table 4, Additional file 1: Table S4). Owning an ITN did not significantly influenced the prevalence of multiclonal infections (LRT $X^2_1 = 0.63$, $P = 0.43$) (Fig. 3A, Table 4, Additional file 1: Table S4).

Males more frequently harboured multiclonal infections (69.9%, 355/510 for males vs 58.8%, 280/476 for females; LRT $X^2_1 = 11$, $P < 0.001$), although the difference was significant only in Mfou (75.2%, 240/319 for males vs 62%, 181/292 for females; LRT $X^2_1 = 11$, $P < 0.001$) (Fig. 3B, Table 4, Additional file 1: Table 4). Overall, prevalence of multiclonal infections decreased with age (LRT $X^2_1 = 4.7$, $P = 0.03$) (Additional file 1: Table S4). However, this association was significant only in Mfou (significant age-by-site interaction: LRT $X^2_1 = 8.7$, $P = 0.003$) (Fig. 3C, Additional file 1: Table S4).

The overall mean MOI was 1.97 (± 0.94). Notably, the mean MOI value was significantly higher in Mfou compared to Tibati (2.08 ± 0.97 vs 1.80 ± 0.85; LRT $X^2_1 = 8.4$, $P = 0.004$) (Fig. 4A, Table 4, Additional file 1: Table S5).

Table 4 Multiclonality and multiplicity of infection (MOI) in *P. falciparum* infections from Mfou and Tibati

Study site	Both sites	Mfou	Tibati
Multiclonal infection, n (%)	637/988 (64.5)	421/611 (68.9)	216/377 (57.3)
Own an ITN			
Yes	296/463 (63.9)	226/333 (67.9)	70/130 (53.8)
No	340/524 (64.9)	195/278 (70.1)	145/246 (58.9)
Sex			
Females	280/476 (58.8)	181/292 (62.0)	99/184 (53.8)
Males	355/510 (69.6)	240/319 (75.2)	115/191 (60.2)
MOI, mean (± sd)	1.97 (± 0.94)	2.08 (± 0.97)	1.80 (± 0.85)
Own an ITN			
Yes	1.98 (± 0.97)	2.05 (± 0.98)	1.79 (± 0.79)
No	1.96 (± 0.90)	2.11 (± 0.97)	1.82 (± 0.94)
Sex			
Females	1.88 (± 0.93)	1.94 (± 0.95)	1.78 (± 0.88)
Males	2.05 (± 0.94)	2.20 (± 0.99)	1.81 (± 0.81)

N Number of parasite isolates for each site

The number of *msp*2 alleles per isolate (MOI) varied from 1 to 6 in Mfou and 1 to 5 in Tibati. Owning a bed net did not influence MOI (LRT $X^2_1 = 0.14$, $P = 0.71$) (Fig. 4A). Overall, males had higher MOI than females (LRT $X^2_1 = 6.13$, $P = 0.013$) (Table 4, Additional file 1: Table S5) but this was especially true for Mfou (2.2 ± 0.99 in males vs

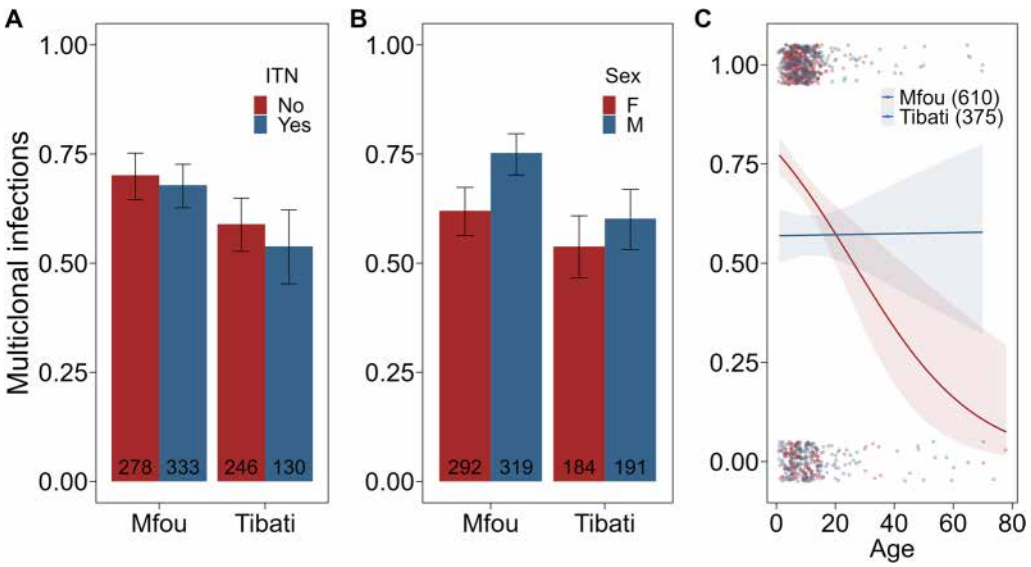

Fig. 3 Effects of ITN (insecticide-treated net), sex, and age on the prevalence of multiclonal infections (proportion of individuals harboring more than one *P. falciparum* clone) in Mfou and Tibati. **A** Prevalence of multiclonal infections by ITN in Mfou and Tibati. **B** Prevalence of multiclonal infections by sex in Mfou and Tibati. *F* Females, *M* Males. In **A** and **B**, error bars represent the 95% CI, and sample sizes are displayed at the base of the bars. **C** Relationship between age and the prevalence of multiclonal infections at the Mfou and Tibati sites. The regression line represents the fitted logistic model. The shaded region indicates the 95% CI for the model. Sample sizes for each site are indicated within bars for **A** and **B** and within the legend for **C**

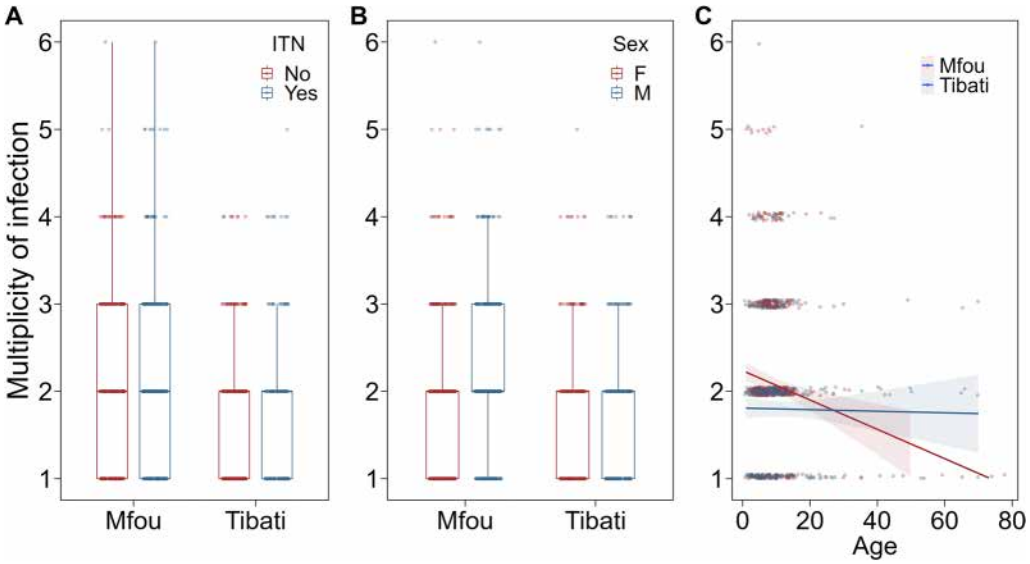

Fig. 4 Effects of ITN, sex, and age on the MOI (number of *msp2* alleles) in Mfou and Tibati. **A** MOI by ITN (insecticide-treated net) ownership in Mfou and Tibati. Yes: own an ITN, No: no ITN. **B** MOI by sex in Mfou and Tibati. *F* females, *M* Males. **C** Relationship between age and the prevalence of multiclonal infections at the Mfou and Tibati sites. The regression line represents the fitted truncated Poisson model. The shaded region indicates the 95% CI for the model. Sample sizes for each site are indicated in parentheses

1.94 ± 0.95, LRT $X^2_1 = 7.4$, P = 0.007) (Fig. 4B, Table 4). Finally, there was a significant age-by-site interaction (LRT $X^2_1 = 5.1$, P = 0.024) (Additional file 1: Table S5) with MOI decreasing with age in Mfou but not in Tibati (Fig. 4C).

Discussion

Knowledge of the genetic diversity of malaria parasite populations in different endemic settings is essential to monitoring the effectiveness of malaria control interventions. Here, genetic diversity of *P. falciparum* was assessed by genotyping the highly polymorphic *msp2* gene among asymptomatic infections from two ecological settings with different variation of malaria transmission, perennial transmission in Mfou and seasonal transmission in Tibati.

The prevalence of malaria infections among the asymptomatic participants in the studied areas was 61.1% and the vast majority, > 95%, were due to *P. falciparum*, in line with previous works in the same localities [9, 39]. *Plasmodium ovale* was only found in Tibati in this study. However, its circulation has previously been reported in Mfou, at low level, and its presence may have been missed due to sample size and its low prevalence [39]. The absence of *P. malariae* in Tibati is more surprising, as it was reported with a prevalence of 23% among asymptomatic individuals screened in 2017 during a same period of malaria transmission (June – July, rainy season) [9]. This is likely linked to a different exposure of the sampled populations between the two studies as the prevalence of *Plasmodium*, including *P. malariae*, is influenced by several factors, including rainfall patterns, temperature, and human behaviour [13, 53].

Prevalence of malaria infections was higher among younger participants, reflecting the typical pattern observed in regions with high endemicity [54–56]. In such areas, acquired immunity in largely exposed adults allows to cure the infection while in younger children, who have had fewer malaria infections, immunity is still insufficient to control parasitaemia [57]. A sharper decline in malaria prevalence with age was observed in Mfou and it will be important to monitor the entomological inoculation rate (EIR) in both areas to determine whether varying levels of exposure to infectious mosquito bites could explain this age-by-site interaction. Indeed, previous studies have evidenced that heterogeneous EIR are major factors determining the prevalence of infection [58, 59]. Nevertheless, these findings point the importance to target school-aged children in malaria-control programmes, as school-aged children represent the major contributors to the infectious reservoir [60, 61]. *Plasmodium* infections were more prevalent in males, although the gender difference was only significant in Mfou. A male bias in malaria prevalence has often been reported and ascribed to behavioural differences by which males would be more exposed to mosquito bites [56, 62–65]. The higher malaria prevalence in males could also be due to biological sex-based differences as it has been reported that females cleared asymptomatic infections faster than males [62].

ITN ownership differed between the two studied areas, a greater proportion of participants from Mfou owning an ITN. Multiple factors influence bed net ownership and use in endemic areas, including region of residence and education level [66–69]. Because of the close proximity of Mfou to the capital city, Yaoundé, inhabitants from this area may have a better access to community interventions that promote ITN use and a better knowledge of malaria burden. Owning an ITN provided substantial protection against malaria, ITN owners having a 20% reduction in the odds of being infected. However, community ITN coverage impacted the protection conferred against malaria infection and the protective effect of ITN was only significant in Mfou (OR = 0.801, 95% CI [0.680–0.943]). ITNs are more effective with higher ITN coverage [70, 71] and these results indicate that efforts are needed to enhance malaria prevention strategies aiming at increasing ITN ownership and usage.

A total of 611 and 377 isolates were successfully genotyped for *msp2*, from Mfou and Tibati respectively. The IC/3D7 family was more prevalent as compared to the FC27 family, in both sites (86.3% vs 74.3% in Mfou and 82.8% vs 67.9% in Tibati), and over half of the isolates carried both allelic families. These results are consistent with others studies carried out in Africa, including Cameroon [26, 72–74]. Several studies tried to correlate the prevalence of *msp2* allelic families with malaria clinical status or disease severity [75–77]. Here, parasites isolates were collected from asymptomatic carriers and such associations were not examined. This study revealed a high degree of genetic polymorphism in *P. falciparum* field isolates, that corresponds with the high level of malaria transmission in the studied areas. A total of 42 (IC/3D7: 35; FC27: 7) and 36 (IC/3D7: 30; FC27: 6) different alleles in the *msp2* gene were found among the asymptomatic cohorts in both sites, respectively. Genetic diversity here was higher than that reported in previous recent studies carried out in the South-West region of Cameroon, 27 alleles (IC/3D7: 15; FC27: 12) [26] and in neighbouring Central African countries, such as Republic of Congo, 27 alleles (IC/3D7: 13; FC27: 14) [78], Gabon, 27 alleles (IC/3D7: 16; FC27: 11) [74] or Nigeria, 15 alleles (IC/3D7: 8; FC27: 7) [79]. The limited genetic diversity of *P. falciparum* observed in these earlier studies may stem from clinical status, as these studies focused on uncomplicated infections, which typically exhibit lower genetic diversity [27, 72]. Additionally, the genotyping method could influence diversity results. In the present study, the analysis of allelic fragments was carried out by capillary electrophoresis, which has a much higher resolving power than gel electrophoresis [46, 80, 81]. The IC/3D7 allelic family was the most polymorphic in this study, in agreement with previous reports [26, 32, 73, 75, 81]. Conversely, other studies found similar diversity within the IC/3D7 and FC27 allelic families or a predominance of the FC27 alleles over the IC/3D7 ones [72, 78, 82, 83]. These discrepancies could be related to geographical location, transmission intensity, or sampled populations [84]. No genetic differentiation was observed between the two studied areas, which suggests that the same populations of parasites circulate in Mfou and Tibati. While IC/3D7 alleles were unevenly distributed in the studied areas, two alleles in the FC27 family (299 and 336 bp) were overrepresented, reaching frequencies above 40%. Even if a risk of homoplasy cannot be excluded, *msp2* sequence containing tandem repeats, a biased distribution of alleles has already been reported and it was suggested that the alleles may have a role in the acquisition of immunity [33]. Nonetheless, such high allele frequencies may lead to misclassification of drug failure as a new infection in drug efficacy studies, and it will be crucial to use multiple genetic markers to accurately distinguish recurrent infections during follow-up [33, 85].

A majority of parasite isolates was carrying multiple genotypes and multiclonal infections were more prevalent in Mfou as compared to Tibati (68.9% vs 57.3%, respectively; LRT $X^2_1 = 9.2$, $P = 0.002$). The difference could reflect geographical heterogeneity and distinct epidemiological pattern between the two areas. Indeed, malaria is seasonal in Tibati, perennial in Mfou and seasonal variations in infection complexity have previously been described [77, 84, 86]. The overall MOI was around 2, 2.1 in Mfou and 1.8 in Tibati, and this corresponds to the MOI range reported in other areas of high malaria transmission using *msp2* genotyping [31, 72, 73, 82, 84]. Higher means of MOI have been associated with a reduced incidence of malaria episodes [30, 87] and this could explain the higher MOI found in Mfou where individuals are exposed to malaria parasites throughout the year and less subject to develop clinical malaria, as compared to Tibati where malaria transmission is seasonal.

In Tibati, age was not correlated with MOI or monoclonal infections while strong effects of age on the number of genotypes were found in Mfou. This site-dependant effect suggests different epidemiological patterns between the two areas and the difference could be due to a different immunological status of the sampled populations. In Mfou, with a perennial transmission, continuous exposure to parasites likely impact on the development and persistence of the anti-parasite immunity. In older individuals, host immune responses contribute to maintain infections at low density and this reduces the likelihood of detecting minority genotypes in adults. Alternatively, in Tibati, human populations are more exposed to infectious mosquito bites during the rainy season regardless of age. Previous

studies indicated that MOI was decreasing with age and linked this association to the acquisition of antimalarial immunity, which aids in clearing more efficiently infections in older individuals [37, 77, 84, 88]. However other studies reported no association between age and MOI [82, 83, 89] and ecological or behavioural factors may lead to distinct exposure to mosquito bites in different areas.

Similarly, the gender effect found in Mfou, where males had higher monoclonal infections and MOI levels as compared to females, may result from different exposure to parasites in this particular area. Although there are indications that sex may influence MOI, the results are not always consistent and depend heavily on the epidemiological context, behavioural factors, and local conditions [26, 77, 90].

This study provides recent data on the genetic diversity of *P. falciparum* in two different areas in Cameroon, however it has several limitations. Samples were collected over a short period at each study site (June–July 2018 in Mfou and July–August 2019 in Tibati), and longitudinal studies would be required to investigate malaria infection dynamics. MOI can vary with the transmission level and seasonal fluctuations in parasite densities may have influenced the genetic diversity in the study sites [90, 91]. Also, a limitation is that parasite densities in the infections were not recorded. Several studies reported a positive association between MOI and parasitaemia, which could be explained by the higher probability of detecting co-infecting genotypes at high parasite densities [30, 89, 92]. Other studies found negative correlation between MOI and parasite density in symptomatic patients and it was suggested that the proliferation of a more virulent genotype of parasites is favored during the onset of a clinical infection [76, 77]. In this study, *msp2* genotyping was performed in samples from asymptomatic individuals, who typically carry low density parasites. The success rate of *msp2* PCR was relatively low in samples from Tibati (377/791, 48%), possibly due to lower parasite densities in this area. However, it is possible that reduced efficiency was caused by DNA degradation. Another limitation of this study is the use of a single genetic marker. The MOI level found in Mfou is lower than that previously found in the same area using different genotyping methods [27, 93] and the MOI values reported here are probably underestimated due to the genotyping method. In addition, *msp2* can elicit immune responses against malaria in a strain-specific manner and this can introduce an immune selection bias [94, 95]. It will be of importance in future studies to use multiple markers and more sensitive methods such as amplicon deep sequencing in genotyping assays to detect minority genotypes in multiclonal infections [27, 80, 96, 97].

Conclusion

This study describes the genetic diversity of *P. falciparum* isolates from asymptomatic individuals in two distinct malaria transmission settings in Cameroon, Mfou and Tibati, and provides data on the genetic polymorphism at the *msp2* gene. Despite Mfou and Tibati have a high malaria transmission profile, a different pattern of genetic diversity was highlighted in this study and this represents valuable information for guiding the decisions of policy-makers to improve anti-malarial strategies in these areas. Even though owning an ITN provides protection against malaria infection, these findings support the need to integrate additional tools into malaria prevention and control strategies. These interventions should be tailored to align with the specific epidemiological patterns of malaria in the considered regions.

Abbreviations

ACT	Artemisinin-based combination therapy
bp	Base pair
DBS	Dried blood spots
EIR	Entomological inoculation rate
glmmTMB	Generalized Linear Mixed Models (GLMMs)
Glurp	Glutamate rich protein
IPTi	Intermittent preventive treatment in infants
IPTp	Intermittent preventive treatment in pregnant women
ITN	Insecticide impregnated bed net
MOI	Multiplicity of infection
NMCP	National Malaria Control Programme
Nested PCR	Nested polymerase chain reaction
Pfmsp1	*Plasmodium falciparum* Merozoite surface protein 1
msp2	Merozoite surface protein 2
qPCR	Quantitative polymerase chain reaction
rfu	Relative fluorescent unit

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05414-6.

Additional file1: Table S1. Sequences of oligonucleotide primers used to genotype *msp2* allelic families of *P. falciparum*. Table S2. Prevalence of *Plasmodium* infections among the study participants. Table S3. Results of the binomial generalized mixed effects modeltesting *Plasmodium* infections. Table S4. Results of the binomial generalized mixed effects modeltesting multiclonality. Table S5. Results of the truncated-Poisson generalized mixed modeltesting MOI. Figure S1. Effects of ITN, sex and age on malaria prevalencein Mfou and Tibati. A, Malaria prevalence by ITN in Mfou and Tibati. B, Malaria prevalence by sex in Mfou and Tibati. F: females, M: Males. In A and B, error bars represent the 95% CI, and sample sizes are displayed at the base of the bars. C, Relationship between age and the proportion of malaria-infected individuals at the Mfou and Tibati sites. The regression line represents the fitted logistic model. The shaded region indicates the 95% CI for the model. Figure S2. Proportion of alleles within the IC/3D7 and FC families in Mfouand Tibati.

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Author contributions
AGB-T, IM, and SEN conceived the experiments. AGB-T, LA, BLF-D, CNM, ANB, and SEN performed the experiments. AGB-T, LA, TL, and IM analyzed the data. LSA, CEE-M, and AB contributed to data acquisition. AGB-T, SEN, and IM wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated during this study are included in the published article and its additional file.

Declarations

Ethics approval and consent to participate
This study was conducted in accordance with the principles of the Declaration of Helsinki. The research protocol was reviewed and approved by the Cameroon National Ethics Committee for Research on Human Health under agreements 2018/05/1011/CE/CNERSH/SP and 2019/05/1161/CE/CNERSH/SP. The CNERSH is officially recognized by the Ministry of Public Health and is not affiliated to any University. Adult participants were enrolled in the study after signing a written informed consent, and a signed authorization was obtained from the legal parent/guardian for minor participants.

Consent for publication
Not applicable.

Competing interest
The authors declare no competing interests.

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