Genetic Diversity of Plasmodium Falciparum Populations in three Malaria Transmission Settings in Madagascar

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Research

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Abstract

Background

The assessment of the genetic diversity of *Plasmodium falciparum* parasites from various malaria transmission settings could help to define tailored and dedicated local strategies for malaria control and elimination. To date, this information is scarce in Madagascar. To fill this gap, a study aiming at investigating the genetic diversity of *P. falciparum* populations in three epidemiological facies (Equatorial, Tropical and Fringes) in Madagascar was conducted.

Methods

Two hundred sixty-six *P. falciparum* isolates were obtained from patients with uncomplicated malaria enrolled in clinical drug efficacy studies conducted in health centers at Tsaratanana (Equatorial facies), Antanimbary (Tropical facies) and Anjoma Ramartina (Fringes) in 2013 and 2016. Parasite DNA was extracted from blood samples collected prior antimalarial treatment. *Plasmodium* species were identified by nested-PCR targeting *18S rRNA* gene. The genetic profiles of *P. falciparum* parasites were defined by assessing the polymorphic regions of the *msp-1* and *msp-2* genes using allele-specific nested-PCR.

Results

A total of 58 alleles were detected for *msp-1* (18 alleles) and *msp-2* (40 alleles) among *P. falciparum* samples tested. K1 (62.9%, 139/221) and FC27 (69.5%, 114/164) were the most predominant *msp-1* and *msp-2* allelic families, although the proportions of the *msp-1* and *msp-2* alleles varied significantly between sites. Polyclonal infections were more frequent in site located in the Equatorial facies (69.8%) compared to sites in the Tropical facies (60.5%) and Fringes (58.1%). Population genetic measures showed that the genetic diversity was similar between sites and the parasite flow within sites was limited.

Conclusion

This study provides recent information on the genetic diversity of *P. falciparum* populations in three transmission facies in Madagascar and valuable baseline data to further evaluate the impact of the control measures implemented in Madagascar.

Background

P. falciparum malaria still remains one of the major human infectious diseases affecting millions of people in tropical areas, despite the progresses achieved over the last decade, mainly due to the scaling up of key interventions (vector control measures, and improved management of malaria cases) [1]. However, in 2019, 229 million malaria cases leading to 409,000 deaths observed mostly in pregnant women and children under five years old living in sub-Saharan Africa (94%) were recorded by the World Health Organization [1].

In Madagascar (total population 26,969,306 inhabitants in 2019), malaria is a major public health issue, ranked as the fourth cause of morbidity at health center level and the fourth cause of hospital mortality. In 2019, the malaria incidence was estimated to 76.1/1000 inhabitants with a mean number of annual malaria cases and deaths to 2,052,071 (range:1,535,000–2,642,000) and 5,073 cases (range:180-9,580) respectively [1]. This worrying report was mainly due to a significant increase in districts located in high transmission areas, the emergence of several malaria outbreaks in the South and exceptional climatic conditions (cyclones and floods) over the last years [2].

One of the major challenges to the policy makers is that malaria transmission greatly varies across Madagascar, depending on regional variations in rainfall, temperature and altitude. The country is typically divided into five

epidemiological facies, including *(i)* the equatorial facies on the east coast, where malaria transmission is the highest and perennial, *(ii)* the tropical facies on the west coast, with a seasonal transmission spanning around six months (October-April), *(iii)* the sub-desert facies in the south characterized by a dry and hot climate prone to episodic outbreaks and *(iv)* the highland and the fringes facies in the center of the country, where malaria transmission is low and unstable between January and April [3, 4].

Currently, the interventions to control malaria recommended by the WHO are provided free by the Malagasy Malaria Control Programme (MMCP) over the country. These interventions are based on vector control measures (Long-Lasting Insecticide Nets and insectides indoor spraying) and prompt and effective management of the malaria cases detected at health facilities and at community level (*i.e.*, Malaria Rapid Diagnostic Test, RDT and Artemisinin-based Combined Therapies, ACT). No specific and tailored strategies for malaria control are designed and implemented at regional or local levels according to the epidemiological context. Furthermore, the impact of the strategies is assessed only on estimates of the malaria cases recorded by hospital and health center staff and malaria community workers [4].

Genotyping of *P. falciparum* parasites has been shown to be a useful tool to explore the genetic diversity (*i.e.* the complexity and size of the parasite populations) and the multiplicity of infections (MOI; *i.e.* the number of clones per isolate), considered usually correlating well with the transmission intensity. Indeed, in areas of high malaria transmission, parasite genetic diversity and MOI are increased, while in regions where effective malaria control strategies are implemented, the parasite genetic diversity and MOI tend to significantly decrease [5, 6]. To assess the *P. falciparum* genetic diversity and MOI, one of the most widely used techniques is based on the detection of polymorphic genes encoding merozoites surface proteins such as MSP-1 and MSP-2 by PCR [7–10]. For MSP-1 (encoded by the *msp1* gene located on chromosome 9), block 2 is the most polymorphic region grouped under three allelic families (K1, MAD20 and RO33). For MSP-2 (encoded by the *msp2* gene located on chromosome 2), block 3 is the most polymorphic region divided into two allelic families (FC27 and IC/3D7).

In Madagascar where malaria transmission varies across the country, the routine monitoring of *P. falciparum* genotypes and the genetic diversity of parasite populations could be useful information [11]. However, very limited information on *P. falciparum* genetic diversity is available. In this context, the study presented here aims to provide recent data on the genetic diversity of *P. falciparum* populations and the MOI in malaria parasites obtained from symptomatic patients in three malaria endemic areas of Madagascar.

Methods

Study sites and blood sample collection.*P. falciparum* clinical samples were obtained from patients presenting to the local health centers enrolled in clinical drug efficacy studies conducted to assess artesunate-amodiaquine efficacy in 2013 and 2016 [12]. Included patients were individuals aged > 6 months, presenting uncomplicated *falciparum* malaria (defined as positive smear for *P. falciparum* and presence of fever ≥ 37.5°C) and residents in three different endemic zones in Madagascar: Anjoma Ramartina city located in the fringes, Tsaratanana on the eastern coast (equatorial facies) and Antanimbary on the western coast (tropical facies) (Fig. 1). After informed consent obtained from participating patients or from parents for children, blood samples were collected on day 0 of enrollment, prior treatment, by finger prick bleeding. Blood samples (100 µL) were used to make blood films and were spotted onto 3 mm Whatman 903 filter paper (Life Science), air-dried and placed individually into a plastic bag with desiccant, before being transported to the Malaria Research Laboratory (Laboratory of National Malaria Control Program in Madagascar) and stored at 4°C until DNA extraction.

Microscopy and parasite counts. Thick and thin blood film slides were stained using 10% Giemsa solution for 30 min. The stained slides were examined under a light microscope (x 100) for detection and identification of *Plasmodium* species and

for parasite count by two experimented microscopists. The parasite density was calculated per 500 white blood cells (WBC) and were recorded as the number of parasites/µL of blood, assuming an average WBC count of 8000/µL of blood.

Plasmodium species identification and msp-1/msp-2 genotyping. Genomic DNA was extracted from the dried blood spots using the QIAamp DNA Blood Mini Kit as per the manufacturer's instructions (Qiagen, CA, USA) and stored at -20°C for further use. Genus and species-specific nested-PCR targeting *18S rRNA* gene were performed as described by Snounou et *al* [13, 14]. The polymorphic regions of the merozoite surface proteins *msp-1* (block 2), *msp-2* (block 3) were amplified by nested PCR. In the first round PCR, oligonucleotide primers were used to target conserved genomic regions within *msp-1* (block 2), *msp-2* (block 3). In the second round PCR, the polymorphic allelic families of the *msp-1* (K1, MAD20, and RO33) and *msp-2* (FC27 and 3D7) genes were amplified with specific primers. The primers and the conditions used for first and second round PCR are described by Oyebola and coll. [15]. Following PCR amplification, PCR products were separated by electrophoresis on 2 % agarose gel and the fragments were visualized under scan gel (Gel Doc XR, Biorad) with ethidium bromide. The sizes of the alleles (± 20 bp) were determined using a molecular weight standard marker (100bp DNA Ladder, Invitrogen). In each run, DNA from reference *P. falciparum* strains (3D7, Dd2 and 7G8) were included as controls.

Multiplicity of infection. The Multiplicity of Infection (MOI) or number of genotypes per infection was calculated by dividing the total number of fragments detected in one antigenic marker by the number of positive samples for the same marker. The mean MOI was calculated by dividing the total number of fragments detected in both *msp-1* and *msp-2* loci by the number of positive samples for both markers. Isolates with more than one allelic family were considered as polyclonal infections while the presence of a single allelic family was considered as monoclonal infection.

Statistical analyses. Statistical analyses were performed using MedCalc version 12 (Mariakerke, Belgium). Mann-Whitney tests were used for non-parametric comparisons and Student's *t* tests or one-way ANOVA were used for parametric comparisons. For proportions (expressed with percentages), χ^2 or Fisher's exact tests were used. *p* values of less than 0.05 significant were deemed significant. Genetic diversity was assessed by Nei's unbiased expected heterozygosity (*He*) from haploid data and calculated as *He* = [n/ (n-1)][1-pi] (n = the number of isolates sampled; pi = the frequency of the itch allele [16]. Population genetic differentiation was measured using Wright's F statistics [17]. Population genetic parameters were computed with FSTAT software, v2. 9. 4 [18].

Ethics Approval.

The study protocol was reviewed and approved by the Ethics Committee of the Ministry of Health of Madagascar (No. 083/MSANP/CE/11-2012).

Results

Study populations. Two hundred and sixty-six *P. falciparum* isolates were obtained from patients with acute malaria, seeking antimalarial treatment at health centers in Anjoma Ramartina (n = 85), Tsaratanana (n = 79) and Antanimbary (n = 102) (Fig. 1).

The characteristics of the study populations are detailed in the Table 1.

Table 1
Characteristics of enrolled patients in Anjoma Ramartina, Tsaratanana and Antanimbary

Characteristic	Anjoma Ramartina (Fringes)	Antanimbary (Tropical)	Tsaratanana (Equatorial)	*P-value*
Population size	85	102	79	
Age, years (mean ± SD)	14.2 (10.4)	15.4 (12.8)	6.2 (3.9)	< 0.001*
<5 years	15	18	32	< 0.001**
5–15 years	39	45	47	
>15 years	31	39	-	
Sex ratio (male/female)	37/48	41/61	48/31	0.01**
Axillary temperature, °C (mean ± SD)	-	38.2 (1.4)	38.4 (1.0)	NS*
Geometric mean parasitaemia, /µL	7,297	16,740	26,282	0.003*
*ANOVA, **Chi-squared test				

Frequency and genetic diversity of msp1 and msp2 allelic families. Amongst the 266 isolates genotyped, 245 (92.1%) and 138 (56.3%) showed successful amplifications for *msp-1* or *msp-2* and both *msp-1* and *msp-2* targeted regions, respectively. For *msp-1*, 18 alleles were observed, including 10 K1-types, 7 MAD20-types and one RO33-type, with band sizes ranging from 130–270 bp. The number of detected alleles for *msp*-2 was higher. A total of 40 different alleles were found, 23 and 17 for the 3D7 and FC27 allelic families, respectively (fragment sizes between 280–700 bp). The proportions of each allele by allelic family are presented in Fig. 2.

msp-1 genotyping. The RO33 allelic family was found to be monomorphic (with an amplified fragment size of 160 bp), at a frequency of 36.7% (81/221) of the overall *msp-1* genotypes. The K1-type alleles were the most predominant with a frequency of 62.9% (139/221). The most frequent alleles were 200 bp (24%), 180 bp (19%), 220 bp (14%) and 160 bp (11%). The presence of two K1-type alleles was found in 15.8% (22/139) of the isolates. The MAD20-type alleles were less frequent (53.4%, 118/221). The 200 bp allele was the most predominant (64%) followed by 180 bp (17%) (Fig. 2). The presence of two MAD20 alleles was also observed but in low proportion (2.5%, 3/118).

msp-2 genotyping. The 3D7 and FC27 alleles were detected in 49.4% (81/164) and 69.5% (114/164) in *P. falciparum* isolates, respectively. Most of the *msp-2* alleles were observed at low frequency, except for seven alleles (3D7 allelic family: 400 bp, 15%; 350 bp, 13%; 500 bp, 10% and FC27 allelic family: 500 bp, 24%; 520bp, 13%; 460 bp, 11% and 480 bp, 11%). The presence of several alleles from the same allelic family was found in 13.6% for the 3D7 (two alleles in 10/81 and three alleles in 1/81 samples) and in 11.4% for the FC37 allelic family (two alleles). The distribution of *msp1* and *msp2* allelic families are presented in the Fig. 3. The proportions of the *msp-1* and *msp-2* alleles varied significantly between the three sites, as presented in the Table 2.

Table 2
Distribution of msp-1 and msp-2 allelic families by study sites.

Gene	Allelic families	Study sites				*P-value*
		Overall	Anjoma Ramartina (Fringes)	Antanimbary (Tropical)	Tsaratanana (Equatorial)	
msp-1	N	221	67	80	74	-
	K1	29.4%	34.3%	32.5%	21.6%	< 0.0001*
	MAD20	17.2%	7.5%	26.3%	17.6%	
	RO33	9.0%	17.9%	3.8%	6.8%	
	K1 + MAD20	16.7%	1.5%	8.8%	20.3%	
	K1 + RO33	8.1%	7.5%	25.0%	8.1%	
	MAD20 + RO33	10.9%	22.4%	0.0%	12.2%	
	K1 + MAD20 + RO33	8.6%	9.0%	3.8%	13.5%	
msp-2	N	164	50	31	83	-
	3D7	30.5%	20.0%	40.7%	30.9%	0.0006*
	FC27	50.6%	67.3%	27.8%	56.4%	
	3D7 + FC27	18.9%	12.7%	31.5%	12.7%	

N = population size; *= Chi-squared test

Monoclonal vs. polyclonal infections. The proportion of monoclonal infections. defined by *msp-1* genotyping was estimated to 55.6% (123/221). The presence of a single allele was most frequent in the K1 allelic family (29.4%), followed by MAD20 (17.2%) and RO33 (9.0%). The combination of RO33, MAD20 and K1 allelic families was identified in 98/221 samples (44.4%). The most frequent combination was K1/MAD20 (16.7%). The proportion of polyclonal infections was significantly more frequent in Tsaratanana (Equatorial facies) (60.8%. $p = 0.02$, Chi-squared test) compared to the two other sites (40.3% and 42.5%). By *msp-2* genotyping. the presence of a single allele (monoclonal infection) was highly frequent (81.1%. 133/164). The presence of a single allele was most frequent in the FC27 allelic family (50.6%. 83/164). The combination of 3D7 and FC27 allelic families was identified in 31/164 samples (18.9%) (Table 3).

Table 3
Proportion of multiclonal infections defined by msp-1 and msp-2 genotyping according to the study sites, age groups and parasite density groups

Variable		multiclonal infections					
		msp-1	*P-value**	*msp-2*	*P-value**	*Combined msp-1 and msp-2*	*P-value**
Overall		106/221 (48.0%)	-	48/164 (29.3%)	-	87/138 (63.0%)	-
By site	Anjoma Ramartina (Fringes)	27/67 (40.3%)	**0.02**	15/55 (27.3%)	NS	25/43 (58.1%)	NS
	Antanimbary (Tropical)	34/80 (42.5%)		21/54 (38.9%)		26/43 (60.5%)	
	Tsaratanana (Equatorial)	45/74 (60.8%)		12/55 (21.8%)		36/52 (69.2%)	
By age group	< 5 years	31/58 (53.4%)	NS	10/41 (24.4%)	NS	24/38 (63.2%)	NS
	5–15 years	53/108 (49.1%)		24/86 (27.9%)		44/71 (62.0%)	
	> 15 years	22/55 (40.0%)		14/37 (37.8%)		19/29 (65.5%)	
By parasite density group	< 5.000	20/48 (41.7%)	NS	9/39 (23.1%)	NS	19/31 (61.3%)	NS
	5.000–50.000	54/115 (47.0%)		26/84 (31.0%)		44/70 (62.9%)	
	> 50.000	31/57 (54.4%)		12/39 (30.8%)		23/36 (63.9%)	

*Chi-squared test

Population genetic measures: Multiplicity of infection (MOI), expected heterozygosity (He) and genetic differentiation between sites. The multiplicity of infection (MOI) estimated in the three study sites are summarized in Table 4. The number of *msp-1* and *msp-2* genotypes per isolate ranged from 1 to 4 and 1 to 3, respectively. The mean MOI calculated by *msp-1* or combined *msp-1* and *msp-2* genotyping were found to be significantly higher in isolates from Tsaratanana (Equatorial facies) (1.92, $p = 0.001$ and 2.52, $p = 0.04$, respectively) compared to the MOI detected in isolates from the two other sites (1.50 and 1.51 and 2.02 and 2.25, respectively). These trends were confirmed according to the age groups: the mean MOI (*msp-1* and combined *msp1/msp-2*) in isolates obtained from patients aged from 5–15 years were higher in Tsaratanana (Equatorial facies) compared to Anjoma Ramartina (Fringes) ($p = 0.004$ and $p = 0.007$, Mann-Whitney test) and Antanimbary (Tropical facies) ($p = 0.02$ for *msp-1*, Mann-Whitney test).

Table 4
Multiplicity of infection (MOI) estimated by msp-1. msp-2 and combined msp-1/msp-2 genotyping by study sites, age groups and parasite density groups.

Variable		No. of isolates		No. of genotype		MOI (SD)					
		msp1	*msp2*	*msp1*	*msp2*	*msp1*	*p-value**	*msp2*	*p-value**	*msp1 + 2*	*p-value**
Overall		221	164	1–4	1–3	1.64 (0.77)	-	1.34 (0.57)	-	2.28 (1.54)	-
By site	Anjoma Ramartina (Fringes)	67	55	1–3	1–2	1.51 (0.68)	**0.001**	1.27 (0.45)	NS	2.02 (1.18)	**0.04**
	Antanimbary (Tropical)	80	54	1–3	1–3	1.50 (0.63)		1.50 (0.69)		2.25 (1.51)	
	Tsaratanana (Equatorial)	74	55	1–4	1–3	1.92 (0.90)		1.25 (0.52)		2.52 (1.81)	
By age group	< 5 years	58	41	1–4	1–3	1.74 (0.81)	NS	1.27 (0.50)	NS	2.10 (1.08)	NS
	5–15 years	108	86	1–4	1–3	1.68 (0.80)		1.34 (0.58)		2.35 (1.75)	
	> 15 years	55	37	1–3	1–3	1.47 (0.63)		1.43 (0.60)		2.34 (1.56)	
By parasite density group	< 5000	48	39	1–3	1–2	1.50 (0.65)	NS	1.23 (0.43)	NS	1.90 (1.07)	NS
	5000–50000	115	84	1–3	1–3	1.63 (0.80)		1.39 (0.64)		2.4 (1.78)	
	> 50000	57	39	1–4	1–3	1.77 (0.80)		1.33 (0.53)		2.33 (1.37)	

* ANOVA test; in bold font are present significant p-values

The expected heterozygosity's (*He*) of isolates from the three sites are presented in the Table 5. In all sites, the *He* were higher by *msp-2* genotyping (0.823 to 0.892) compared to those defined by *msp-1* genotyping (0.413 to 0.489). However, the *He* were found to be similar between study sites, age groups and parasite density groups.

Table 5
Expected heterozygosity (He) estimated by msp-1, msp-2 and combined msp-1/msp-2 genotyping by study sites.

Site	*He (SD)*		
	msp-1	*msp-2*	*Combined msp-1/msp-2*
Anjoma Ramartina (Fringes)	0.489 (0.425)	0.892 (0.010)	0.629 (0.363)
Antanimbary (Tropical)	0.440 (0.434)	0.823 (0.129)	0.612 (0.367)
Tsaratanana (Equatorial)	0.413 (0.400)	0.859 (0.04)	0.579 (0.379)

The estimation of the fixation index (*Fst*), measuring the population differentiation due to genetic structure in each site was not significant (Table 6).

Table 6
Estimation of the fixation index (Fst) between study sites

Fst	Anjoma Ramartina (Fringes)	Antanimbary (Tropical)
Tsaratanana (Equatorial)	0.04755	0.05824
Anjoma Ramartina (Fringes)		0.05036

This measure was concordant with the proportion of shared genotypes between the study sites as shown in the Table 7. The highest proportion of shared genotypes (6%) was observed between Anjoma Ramartina and Tsaratanana.

Table 7
Estimated proportions of shared *msp-1 + 2* genotypes between study sites.

Sites	Anjoma Ramartina (Fringes)	Antanimbary (Tropical)	Tsaratanana (Equatorial)
Anjoma Ramartina (Fringes)	91%	3%	6%
Antanimbary (Tropical)	3%	95%	2%
Tsaratanana (Equatorial)	6%	2%	92%

Discussion

The PCR genotyping analysis, using *msp-1* and *msp-2* polymorphic markers, was performed to improve our knowledge on the genetic diversity of *P. falciparum* parasite populations in three regions with different malaria transmission pattern in Madagascar. So far, this information is currently lacking. Until now and excluding genotyping data obtained from clinical drug efficacy studies (performed to distinguish recrudescence from reinfections in enrolled patients presenting recurrences during their follow-up), only two studies, performed in 2000 and 2008, have reported such analysis [11, 19].

Overall, the number of different alleles for *msp-1* and *msp-2* found in the three sites (18 and 40, respectively) confirms a high level of malaria transmission in Madagascar. These numbers are comparable to those found in Africa such as in Nigeria, the Republic of Congo, the Central African Republic, the Equatorial Guinea or Senegal [20–24].

The K1-type for *msp-1* and FC27-type for *msp-2* were the most predominant alleles. These findings are consistent with data previously reported in Madagascar [11] and in different settings such as Africa (Nigeria [15, 22], Congo Brazzaville [25], Mauritania [26], Benin [27], Gabon [28, 29], Ivory Coast [30], Cameroon, [31], Ethiopia [32–35]), India [36] or Southeast Asia [37]. However, they contrast with recent reports from Myanmar [38] that showed that MAD20 and 3D7 were the most prevalent alleles.

The distribution of the *msp-1* and *msp-2* allelic families varied significantly between sites (Table 2). By *msp-1* genotyping, half of the individuals had *P. falciparum* isolates with a single *msp-1* allele. The proportion of isolates with more than one *msp-1* allele was significantly higher in patients living in the Equatorial facies site, likely reflecting higher malaria transmission in this setting. This association was confirmed by a significantly highest proportion of polyclonal infections in Tsaratanana (Equatorial facies, 60.8%) compared to the two other sites (40.3% and 42.5%) (Table 3). *Msp-2* allelic diversity was found to be more contrasted between sites: the type FC27 was largely more frequent than the 3D7 type in the Tropical zone, while in the Equatorial and the fringes facies, the 3D7 allele type was more predominant.

The number of *msp-1* and *msp-2* genotypes per isolate ranged from 1–4 and 1–3, respectively. Again, the means MOI calculated by *msp-1* or combined *msp-1* and *msp-2* genotyping were found to be significantly higher in isolates from patients living in Tsaratanana (Equatorial facies) (1.92 and 2.52) compared to the means MOI in isolates from patients living in the two other sites (1.51 and 2.02 in Anjoma Ramartina; 1.50 and 2.25 in Antanimbary, respectively). These values are similar to those reported in some African countries, like Ghana [39], Congo Brazzaville [24], Ethiopia [32–35], but lower

compared to Nigeria [22] and Gabon [40]. An association between the increase of the MOI and the age group was also observed. Particularly, the mean MOI (*msp-1* and combined *msp1/msp-2*) of isolates obtained from patients aged from 5–15 years were higher in Tsaratanana (Equatorial facies) compared to Anjoma Ramartina (Fringes) and Antanimbary (Tropical facies). However, no such association was found between the mean MOI and the parasite density, counter to several reports [41].

However, these data confirm that the assessment of the MOI is a good measure of the malaria transmission intensity and this metric can be considered in Madagascar as a useful tool to evaluate the impact of vector control measures (Long-lasting bed nets and insecticides indoor sprayings) currently implemented over the country.

The genetic population measures did not reveal significant differences between sites. First, the *He* was found to be similar between study sites, age groups and parasite density groups. Second, the estimation of the fixation index (*Fst)* and the low proportion of shared genotypes between the study sites indicated that the circulation of the parasite population remains limited between sites.

Despite providing recent data on *P. falciparum* genetic diversity, this study presents several limitations. First, the main limitation was the use of *msp-1* and *msp-2* markers for genotyping that, as other markers based on DNA fragment size, could reduce the estimation of the genetic diversity. Nevertheless, *msp-1* and *msp-2* are usually considered as robust polymorphism markers and genotyping protocols are easy to perform in low-equipped laboratory such as those available in Madagascar. Second, the selected sites for sample collection was not designed for this study (but for assessing the efficacy of artesunate-amodiaquine in clinical drug efficacy study) which limits the opportunity to extrapolate the data. Third, as no direct malaria transmission measures such as the Entomological Inoculation Rate (EIR) were available in the selected sites, no opportunity to investigate association between these metrics and the genetic diversity data was possible.

Conclusions

Despite several limitations, this study provides recent genetic diversity data from *P. falciparum* isolates collected in three regions with different transmission patterns. The obtained information remains valuable for policy makers to improve strategies implemented against malaria. Particularly, a continue evaluation of these metrics could be useful to evaluate the impact of the control measures implemented in Madagascar, such as vector control measures. In addition, the detection of these two markers can be used to investigate malaria outbreaks, which have been frequent over the last years [42] in the southern low transmission areas by investigating whether outbreaks are due to the clonal expansion of local or imported *P. falciparum* clones and by designing more adapted strategies to prevent outbreaks.

Declarations

Authors' contributions

AR conceived, designed, coordinated the study, and supervised the enrolment and follow up of the patients. FR, TR, AR and DM analysed the data and map. FR and TR performed the molecular assay. FR, JV, AR, and DM composed the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

The study was approved by the 'Comité d'Éthique de la Recherche Biomédicale de Madagascar'.

Consent for publication

All authors approved the manuscript's submission for publication.

Availability of data and materials

Data are available from the National Control Programme of Madagascar.

Competing interests

The authors declare that they have no competing interests.

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Figures

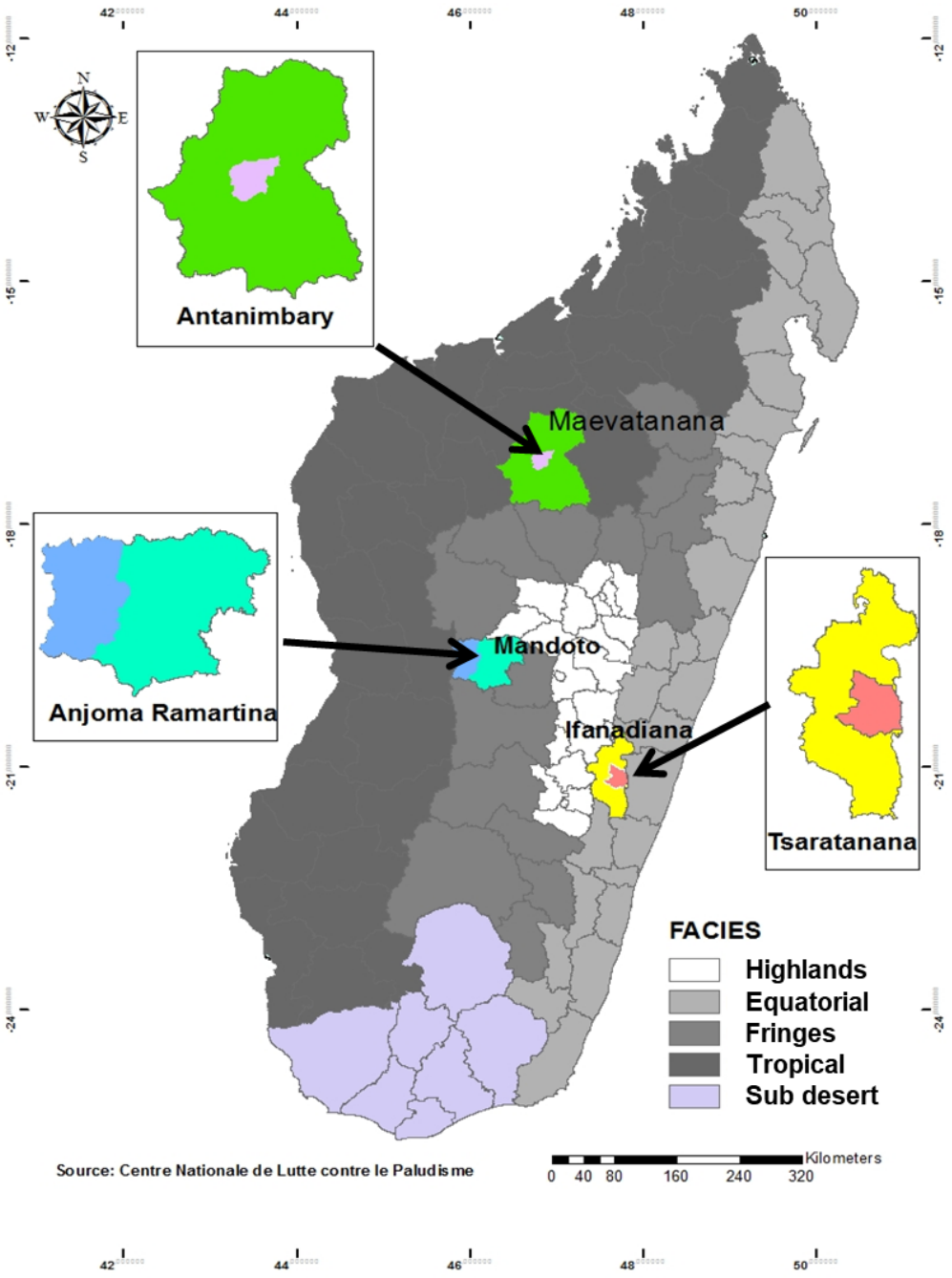

Figure 1

Location of the study sites according to the epidemiological facies, Madagascar Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

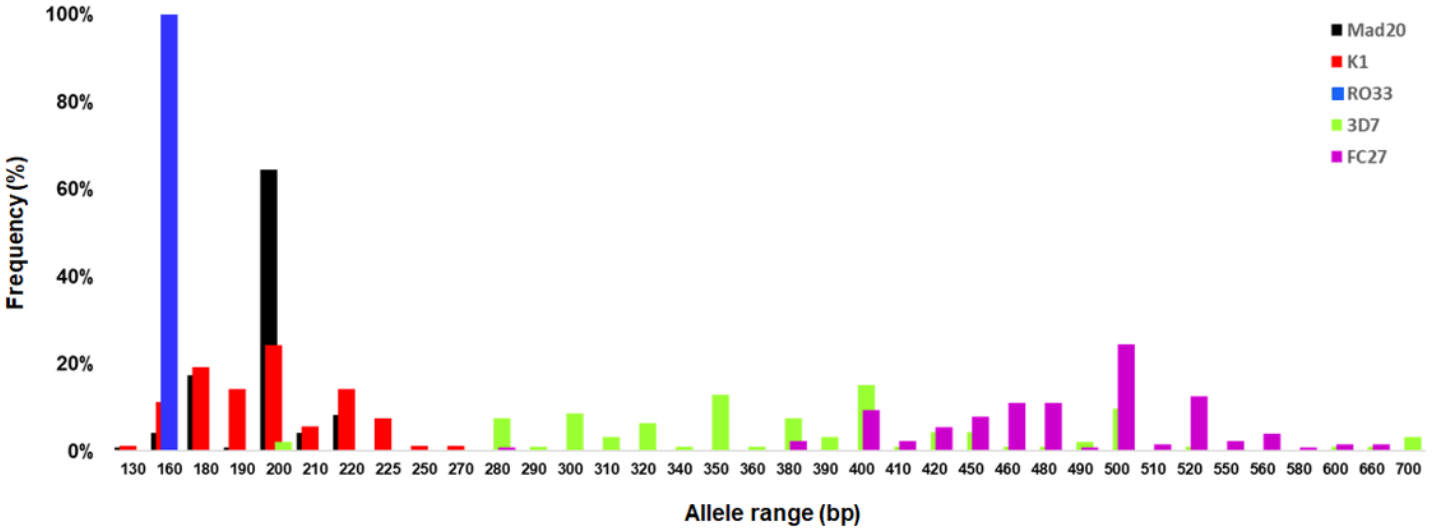

Figure 2

Distribution and proportions of msp-1 and msp-2 alleles.

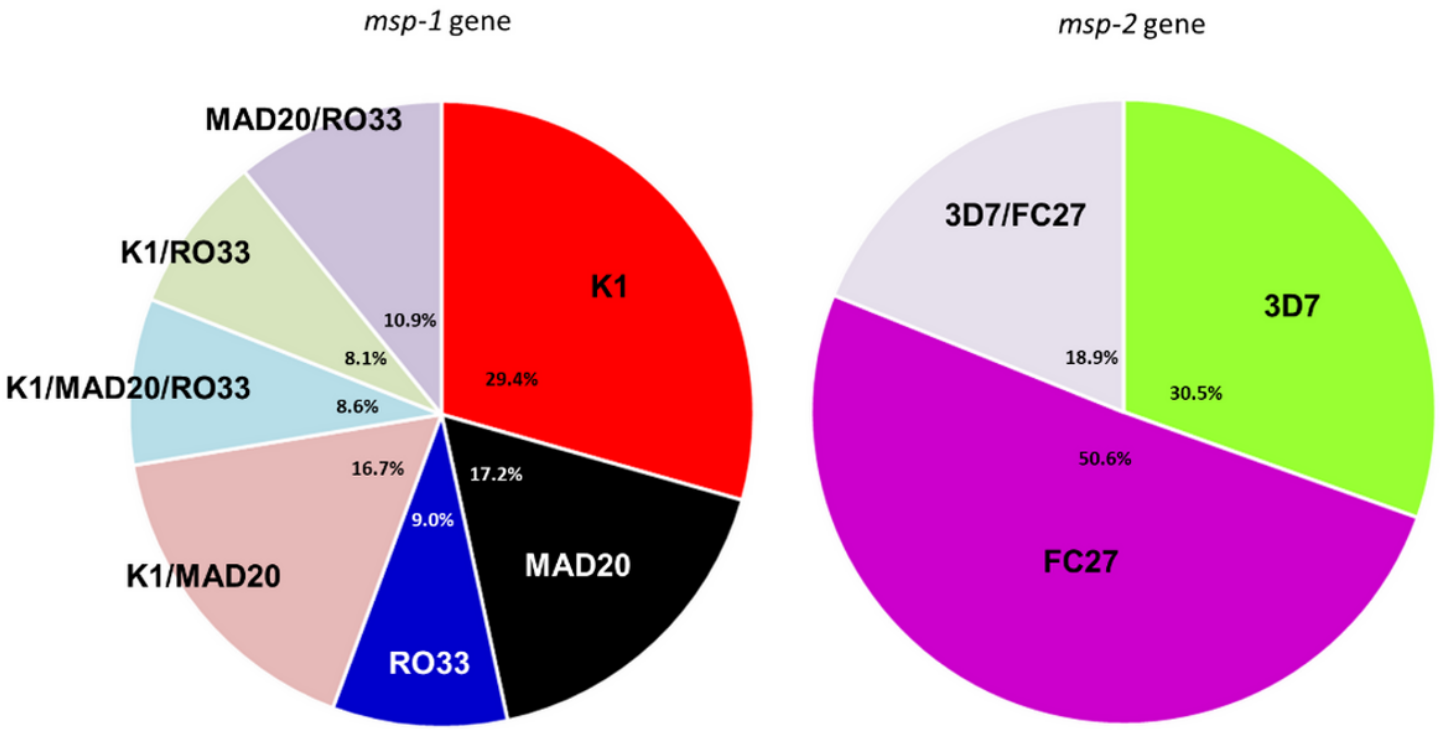

Figure 3

Overall distribution of msp-1 and msp-2 genotypes.