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Characterization of Plasmodium infections and genetic diversity of Plasmodium malariae msp-1 among asymptomatic individuals in southern Benin

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Abstract

Plasmodium malariae is less widespread than *P. falciparum* but can cause prolonged and persistent infections. This study aimed to characterize asymptomatic *Plasmodium* infections and assess the genetic diversity of the merozoite surface protein 1 (*msp-1*) gene of *P. malariae* in southern Benin.

Blood samples from 484 asymptomatic participants were analysed using rapid diagnostic tests (RDTs), microscopy, and nested PCR. Samples negative by nested PCR were subsequently analysed by nested qPCR for additional detection of *Plasmodium* species. *P. malariae*-positive samples were subjected to *msp-1* genotyping and Sanger sequencing.

Microscopy and RDTs detected *Plasmodium* infections in 17% and 20% of participants, respectively, whereas nested PCR detected infections in 38% (182/484; $P < 0.0001$). Among the 302 nested-PCR-negative samples, nested qPCR identified an additional 36 infections (12%). Nested PCR detected *P. malariae* in 6.6% (32/484) of participants, with nested qPCR identifying an additional 25 *P. malariae* infections (8.3%). The nucleotide diversity (π) of the successfully sequenced *P. malariae msp-1* regions was 0.00602.

These findings demonstrate a substantial burden of asymptomatic and sub-microscopic *Plasmodium* infections in southern Benin, including *P. malariae* infections missed by conventional diagnostic methods. The sequenced *msp-1* regions showed relatively limited nucleotide diversity, providing baseline information on the genetic variation of *P. malariae* in southern Benin.

Introduction

Malaria remains a major public health challenge, with the greatest burden occurring in sub-Saharan Africa [1]. Despite substantial control efforts, malaria still caused an estimated 282 million cases and 610,000 deaths worldwide, with sub-Saharan Africa accounting for 94% of the disease burden [2]. The WHO aims for universal access to malaria prevention, diagnosis, and treatment [3]. However, a substantial proportion of infections may remain undetected because many infected individuals are asymptomatic or have parasite densities below the detection limits of routine diagnostic methods [4]. Accurate detection and characterization of these infections are therefore important for malaria surveillance and elimination, particularly in endemic communities where asymptomatic individuals may contribute to continued transmission.

Several *Plasmodium* species naturally infect humans (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* spp., and *P. knowlesi*), although *P. knowlesi* is primarily a zoonotic parasite maintained in macaques [5]. Among these species, *P. malariae* presents distinct challenges due to its unique characteristics, including a 72-hour erythrocytic cycle and the ability to maintain low parasitaemia over extended periods [6,7]. In areas where *P. falciparum* and other *Plasmodium* species predominate, conventional *diagnostic* methods may miss *P. malariae* infections, particularly when parasite densities are low [8]. However, molecular methods

such as PCR and qPCR can provide more sensitive detection of low-density infections and may therefore contribute to a more complete estimation of *P. malariae* prevalence [6,9].

Although *P. malariae* is generally detected at lower frequencies than *P. falciparum* in sub-Saharan Africa, its reported prevalence varies considerably according to the study population, geographical setting, sampling period, and diagnostic method used. Studies conducted in West Africa have reported this prevalence variability, including in Burkina Faso [10] and Benin [11]. Similarly, studies from East Africa have documented substantial variation in *P. malariae* detection over time and across study settings [12]. These findings suggest that the epidemiology of *P. malariae* remains insufficiently characterized, particularly among asymptomatic populations. In Benin, however, data on the molecular prevalence and species distribution of asymptomatic *Plasmodium i*nfections, particularly *P. malariae*, remain limited. Improved molecular surveillance is therefore needed to better characterize the contribution of *P. malariae* and other *Plasmodium* species to the asymptomatic parasite reservoir.

In addition to improving parasite detection, molecular characterization can provide information on the genetic diversity of *Plasmodium* populations. Among the genes used to study malaria parasite genetic variation, the merozoite surface protein 1 gene (*msp-1*), which encodes an immunogenic protein expressed on the surface of the merozoite, has received considerable attention [13–15]. The *msp-1* gene contains diverse allelic forms and has been widely used to investigate parasite diversity, population structure, and transmission dynamics in *Plasmodium* spp. [16–18]. In *P. falciparum*, genetic variation in *msp-1* has been extensively used to characterize parasite populations and assess epidemiological parameters such as multiplicity of infection [17,18]. However, comparatively little is known about the genetic diversity of *P. malariae msp-1*, particularly in malaria-endemic areas of West Africa. Further characterization of *P. malariae msp-1* diversity may therefore provide useful baseline information for understanding the genetic variation and epidemiology of this relatively understudied parasite species.

We hypothesized that conventional diagnostic methods underestimate asymptomatic *Plasmodium* infections, including *P. malariae* infections, and that molecular characterization of *P. malariae msp-1* would provide insights into the genetic variation of parasites circulating in southern Benin. Therefore, this study aimed primarily to characterize the prevalence and distribution of asymptomatic *Plasmodium* infections in southern Benin using microscopy, rapid diagnostic tests, and a sequential molecular testing strategy. Nested PCR was first performed on all samples, and nested qPCR was subsequently performed only on samples that tested negative by nested PCR, with the aim of identifying additional infections. In addition, samples positive for *P. malariae* were further characterized to assess the genetic diversity of the *P. malariae msp-1* gene and provide baseline information on the genetic variation of this parasite in southern Benin.

Results

Demographic Profile of the Study Cohort:

A cohort of 484 individuals participated in this study, with 392 (81%) enrolled in 2022 and 92 (19%) in 2023. The overall age ranged from 3 to 90 years. The median age was 25 years (range: 3–90) in 2022 and 37 years (range: 6–72) in 2023. The proportion of females exceeded that of males in both years, comprising 62% (n = 241) in 2022 and 69% (n = 63) in 2023. Among the three defined age groups, individuals older than 15 years constituted the largest segment in both sampling years, comprising 58% (2022; n = 227) and 80% (2023; n = 73) of participants, respectively. Additionally, 69% (n = 334) were recruited from the Ouidah district, while 31% (n = 150) were from the Kpomasse district. All socio-demographic characteristics of participants in both 2022 and 2023 are provided in Table 1.

Table 1
Socio demographic characteristics of the study population

Characteristics	DS 2022 (392)	DS 2023 (92)	Total (484)
Age range (years)	3–90	6–72	3–90
Median (IQR)	25 (31.3)	37 (27.3)	27.5 (33)
BMI (Body mass Index)	5.3–49.1	12.4–37.3	5.3–49.1
Median (IQR)	18.4 (7.2)	20.5 (7.6)	18.7 (7.4)
Gender n (%)			
Female	241 (61.5)	63 (68.5)	304 (62.8)
Male	151 (38.5)	29 (31.5)	180 (37.2)
Temperature (°C) n (%)			
≤ 37.5°C	334 (85.2)	79 (85.9)	413 (85.3)
> 37.5°C	58 (14.8)	13 (14.1)	71 (14.7)
Haemoglobin (g/dl) range	6.5–18.3	6.2–16.9	6.2–18.3
Median (IQR)	11.9 (1.8)	11.7 (1.7)	11.9 (1.7)
Age groups (year) n (%)			
< 5	14 (3.6)	1 (1.1)	15 (3.1)
5–15	151 (38.5)	18 (19.6)	169 (34.9)
> 15	227 (57.9)	73 (79.3)	300 (62)
Location n (%)			
Ouidah	308 (78.6)	26 (28.3)	334 (69)
Kpomasse	84 (21.4)	66 (71.7)	150 (31)

DS: Dry Season, n: represents the number of participants and the corresponding percentage value is indicated in brackets, IQR: Interquartile Range

Prevalence of Plasmodium infections in the study population:

The prevalence of *Plasmodium* infections varied according to the diagnostic method used. A total of 484 samples were analysed by microscopy, RDT, and nested PCR, while 302 nested PCR-negative samples were further analysed using nested qPCR.

The overall prevalence of *Plasmodium* infections determined by microscopy was 17% (82/484), while rapid diagnostic tests (RDTs) indicated a slightly higher prevalence of 20% (96/484). However, this

difference was not statistically significant (P = 0.07). In contrast, nested PCR demonstrated a significantly higher prevalence of 38% (182/484) in comparison to microscopy and RDT (P < 0.0001).

Furthermore, nested qPCR identified additional *Plasmodium* spp. infections among samples that were negative by nested PCR, corresponding to 12% (36/302) of the samples analysed by nested qPCR (**Table 3**).

Species-specific Plasmodium infection prevalence

Microscopy detected two *Plasmodium* species (*P. falciparum* and *P. malariae*). Overall, *P. falciparum* infections were identified in 16.7% (81/484) of participants, while *P. malariae* was observed in 0.8% (4/484). Among these infections, 16.1% (78/484) were *P. falciparum* mono-infections and 0.2% (1/484) were *P. malariae* mono-infections (**Table 2**).

RDTs detected *Plasmodium* spp. in 19.8% (96/484) of participants, with *P. falciparum* accounting for 19.4% (94/484) and Pan-positive results for 0.4% (2/484). The two Pan-positive cases were further investigated: one was confirmed as *P. malariae* by both microscopy and nested PCR, while the second was identified as a mixed infection (*P. falciparum/P. malariae*) by nested PCR.

Nested PCR identified three *Plasmodium* spp. (*P. falciparum*, *P. malariae*, and *P. ovale* spp.). *P. falciparum* and *P. malariae* were detected in 35.5% (172/484) and 6.6% (32/484) of the study population, respectively **(Table 2**). This represents a significant increase compared to microscopy (P < 0.0001).

Among the 302 PCR-negative samples analyzed by nested qPCR, additional infections were identified: 11 *P. falciparum* mono-infections (3.6%), 16 *P. malariae* mono-infections (5.3%), and 9 (3%) mixed *P. falciparum/P. malariae* infections (**Table 3**). Two participants were found to be triply infected with *P. falciparum*, *P. malariae*, and *P. ovale wallikeri*. Furthermore, no isolates were found to be positive for *P. vivax*.

Table 2. Prevalence of *Plasmodium* infections in the Southern Benin population detected by microscopy and Nested PCR

***Plasmodium spp.* infections**	**Microscopy (n = 484)**		**Nested PCR (n = 484)**	
N	Prevalence (%)	N	Prevalence (%)	
All Plasmodium Positive	**82**	**17**	**182**	**37.6**
Pf infections	81	16.7	172	35.5
Pm infections	4	0.8	32	6.6
Po infections	0	0	5	1
Mono-infections	**79**	**16.3**	**157**	**32.4**
Pf	78	16.1	147	30.4
Pm	1	0.2	10	2.1
Po	0	0	0	0
Co-infection	**3**	**0.6**	**25**	**5.2**
Pf + Pm	3	0.6	20	4.1
Pf + Po	0	0	3	0.6
Pf + Pm + Po	0	0	2	0.4

Pf: P. falciparum, Pm: P. malariae, Po: P. ovale

N: Number of participants positive for each *Plasmodium* spp., Pf *infections*: mono and mixed *P. falciparum* infections, *Pm* infections: mono and mixed *P. malariae* infections, *Po* infections: mono and mixed *P. ovale* infections. Mixed infections are counted in each infecting species category; therefore, species-specific totals are not mutually exclusive and may exceed the total number of *Plasmodium*-positive samples.

Table 3. *Plasmodium* spp. infections detected by nested qPCR in samples negative by nested PCR

***Plasmodium spp.* infections**	**Overall (n = 302)**	
N	Prevalence (%)	
All Plasmodium Positive	**36**	**11.9**
Pf infections	20	6.6
Pm infections	25	8.2
Po infections	0	0
Mono-infections	**27**	**8.9**
Pf	11	3.6
Pm	16	5.3
Po	0	0
Co-infection	**9**	**3**
Pf + Pm	9	3
Pf + Po	0	0
Pm + Po	0	0
Pf + Pm + Po	0	0

Pf: P. falciparum, Pm: P. malariae, Po: P. ovale

N: Number of participants positive for each *Plasmodium* spp., *Pf* infections: mono and mixed *P. falciparum* infections, *Pm* infections: mono and mixed *P. malariae* infections, *Po* infections: mono and mixed *P. ovale* infections. Mixed infections are counted in each infecting species category; therefore, species-specific totals are not mutually exclusive and may exceed the total number of *Plasmodiu*m-positive samples.

Sub-microscopic Plasmodium infections prevalence

Among the 402 microscopy-negative participants, 142 (35.3%) were positive by molecular testing, including 106 detected by nested PCR and an additional 36 detected by nested qPCR

Sub-microscopic *P. malariae* infections were detected in 11.2% of participants, of which 6% were mono-infections and 5.2% were mixed infections (**Supplementary Table 4**).

P. malariae msp-1 fragments genetic diversity.

The genetic diversity of *P. malariae* was evaluated using a set of 57 samples that were positive for either *P. malariae* mono-infections or co-infections. This diversity was investigated by splitting the *msp-1* gene

into five fragments to cover more than half of the gene.

In our samples, fragment 1 encodes a peptide of 177 residues, spanning amino acids 180 to 356 in the unaligned MM1 sequence (GenBank #FJ824669). Notably, amino acid polymorphism was concentrated within the region spanning residues 231–262 of the MM1 reference sequence (Fig. 2). Multiple amino acid substitutions were observed across the sequenced isolates within this region, with individual isolates carrying up to approximately 15 substitutions relative to the MM1 reference sequence. Due to the presence of a microsatellite and numerous non-specific bands after amplification, fragment 2 could not be sequenced. Fragment 3 corresponds to amino acids 970 to 1179 (210 aa) in the MM1 sequence. An insertion of 9 amino acids (PQPQAALPA) relative to the MM1 isolate was detected in ten of our sequences at position 997 (Fig. 2). For fragment 4, aligned to amino acids 1380 to 1526 (147 aa) in the MM1 sequence, a deletion of PIVG amino acids at positions 1381 to 1384 and two other substitutions (E1416D in two samples and K1510I in one sample) were observed (Fig. 2).

In the C-terminal region, fragment 5 was amplified, corresponding to amino acids 1548 to 1747 (200 aa) in the MM1 sequence. Its variability was limited to 8 amino acid substitutions (Fig. 2). Phylogenetic analysis showed that all *P. malariae* sequences generated in this study clustered within the *P. malariae* lineage, together with the MM1 reference sequence (FJ824669), and were clearly separated from reference sequences representing other *Plasmodium* species (Fig. 3**).** The results of the nucleotide diversity metrics, including θ (0.008053), π (0.006015) per site, and Tajima's D (-1.094775), provide quantitative measures of genetic diversity and indicate variation within the *P. malariae* parasite in the study population.

Discussion

The dominance of *P. falciparum* within *Plasmodium* species in sub-Saharan Africa has led to its prioritization in malaria research, often overshadowing the study of non-falciparum species, particularly in endemic areas like Benin. Comprehensive spatial and temporal surveillance of all *Plasmodium* species, particularly *P. malariae*, among asymptomatic populations, alongside an understanding of its genetic diversity, is crucial for unravelling various facets of malaria research, including epidemiology, transmission dynamics, and evolutionary processes. This study aimed to monitor asymptomatic *Plasmodium* infections and characterize the polymorphisms of the *msp-1* of *P. malariae* in Beninese individuals. These findings may help inform malaria surveillance and control strategies that better account for non-falciparum infections.

Community surveillance in Ouidah and Kpomasse, southern Benin, revealed a *Plasmodium* infection prevalence of 38% in the study area, using molecular methods. This prevalence, while high, is consistent with previous studies from the region [11,19,20], albeit lower than infection rates reported in other West African countries such as Nigeria and Ghana [21,22]. Historically, southern Benin has been classified as a high malaria transmission area, with several contributing factors, including a high entomological inoculation rate (EIR) and suboptimal coverage of prevention measures. The large proportion of

asymptomatic carriers in these communities poses a major challenge to malaria control efforts, as asymptomatic infections may contribute to the human parasite reservoir and sustain transmission [23–25]. This underscores the need for community-based strategies that specifically target asymptomatic carriers across all *Plasmodium* species, which are essential for malaria elimination [26,27].

Our findings confirm that *P. falciparum* remains the dominant *Plasmodium* species in the study area [11,20,28]. However, the detection of *P. malariae* varied markedly according to the diagnostic method used. While microscopy detected fewer than 1% of infections and RDTs identified only two non-falciparum cases, nested PCR and, particularly, nested qPCR detected substantially more *P. malariae* infections, highlighting the greater detection yield of molecular approaches, particularly for low-density infections, in agreement with previous reports [9].

These observations can largely be explained by the biological characteristics of *P. malariae*, including its typically low parasitemia and its frequent occurrence in mixed infections with *P. falciparum*, which can mask its detection by microscopy and RDTs [8,29]. Consequently, a substantial proportion of infections are likely to remain undiagnosed using routine diagnostic tools. Because persistent sub-microscopic infections may contribute to malaria transmission and contribute to chronic clinical outcomes, their detection is of considerable public health importance. In Benin, where malaria surveillance remains primarily focused on *P. falciparum*, our findings support broadening diagnostic and surveillance strategies to routinely include non-falciparum species.

In addition to improving parasite detection, this study provides new insights into the genetic diversity of *P. malariae*. Analysis of *msp-1* revealed limited diversity across the successfully sequenced fragments. Although five gene regions were targeted, one fragment could not be reliably characterized because of non-specific amplification, resulting in incomplete coverage of the *msp-1* gene. This limitation suggests that certain genomic regions of *P. malariae* may present technical challenges for molecular characterization and may require optimization of primer design or alternative sequencing approaches. Nevertheless, the successfully sequenced regions contained amino acid substitutions and insertions, confirming the presence of genetic variation among circulating *P. malariae* isolates in southern Benin.

Despite the polymorphisms identified, phylogenetic analysis showed that all *P. malariae* isolates clustered within the *P. malariae* lineage, together with the MM1 reference sequence, and were separated from reference sequences representing other *Plasmodium* species, suggesting relatively limited genetic diversity across the *msp-1* regions successfully sequenced in our study population. Although our analysis was limited to Beninese isolates and did not include a formal global population comparison, the low genetic diversity observed is consistent with previous studies [30–32] and with reports for *P. ovale* spp. [33]. In contrast, *P. falciparum msp-1* exhibits substantially greater genetic diversity across Africa, including in our study region [34]. The low nucleotide diversity observed in our dataset ($\theta = 0.008053$; $\pi = 0.006015$ per site) suggests relatively limited nucleotide diversity across the *msp-1* regions successfully analyzed. This level of diversity appears lower than that reported for highly polymorphic regions of *P. falciparum*

msp-1, although direct comparisons should be interpreted with caution because different *msp-1* regions and parasite populations have been analyzed across studies [14,15].

This study has some limitations that should be considered when interpreting the findings. First, it was conducted during two dry-season sampling periods (2022 and 2023), with unequal sample sizes (392 participants in 2022 and 92 in 2023) due to logistical and budget constraints. This limits the comparability of prevalence estimates between the two sampling periods. Second, the cross-sectional design does not allow assessment of infection dynamics over time, including infection duration or differentiation between persistent and newly acquired infections, particularly for *P. malariae*. Third, only 302 of the 484 samples were analyzed by nested qPCR, as this assay was applied selectively to nested PCR-negative samples within a sequential diagnostic strategy designed to enhance detection of low-density infections. While this improves sensitivity, it limits direct comparability across the full study population and may influence overall prevalence estimates. In addition, amplification and sequencing of fragment 2 of the *msp-1* gene failed due to non-specific amplification, resulting in incomplete gene coverage and potentially limiting full characterization of genetic diversity. Finally, the relatively small number of *P. malariae*-positive samples, particularly in subgroup analyses, reduced statistical power and may have affected the robustness of stratified estimates.

In conclusion, molecular testing revealed a substantial burden of asymptomatic and submicroscopic *Plasmodium* infections in southern Benin, including a considerable proportion of *P. malariae* infections that were not detected by microscopy or RDT. The limited genetic diversity observed across the successfully sequenced regions of *P. malariae msp-1* provides an initial molecular characterization of *P. malariae* genetic variation in southern Benin. These findings highlight the value of sensitive molecular surveillance for characterizing the asymptomatic parasite reservoir and support the inclusion of non-falciparum species in malaria surveillance and elimination efforts.

Methods

Study population:

Two independent community-based cross-sectional investigations were conducted within the rural regions of Ouidah and Kpomasse municipalities, situated in the southern region of Benin, during the dry seasons of January to March 2022 and March 2023. Different participants were recruited during each survey round. Detailed descriptions of the study areas have been previously documented [20,28]. Selection of the rural sites was based on preliminary findings regarding *P. malariae* prevalence in the study region [11]. Eligible asymptomatic residents were recruited through community-based surveillance in the selected villages. No household-cluster sampling design was applied, and participants were enrolled individually according to predefined inclusion criteria. Individuals were eligible if they (i) had no clinical symptoms suggestive of malaria at enrolment, (ii) were permanent residents of the study villages, and (iii) were older than one year of age. A structured questionnaire was administered to collect socio-demographic and anthropometric data, including age, gender, weight, and height. Additionally, approximately 2 mL of

venous blood was collected from each participant in an EDTA tube. This blood was used for various measurements, including haemoglobin levels, rapid diagnostic tests (RDTs) for *Plasmodium* spp. detection, preparation of thick and thin blood smears for microscopy, and preparation of filter paper samples for further molecular analysis.

Diagnostic and genotyping procedures:

All samples were initially analysed by microscopy, malaria RDT, and nested PCR. Samples that tested negative by nested PCR were subsequently analysed using a highly sensitive nested qPCR protocol to improve the detection of low-density infections. All *P. malariae*-positive samples (mono- and mixed infections) were subsequently subjected to *msp-1* genotyping and Sanger sequencing (Fig. 1).

Rapid diagnostic test (RDT): Malaria RDTs were performed using the SD Bioline Malaria Ag Pf/Pan test (Abbott/Standard Diagnostics) according to the manufacturer's instructions. Briefly, 5µL of whole blood was added to the sample well, followed by the assay buffer supplied by the manufacturer. Test results were read after 15 minutes.

Plasmodium spp. detection using Microscopy

Thin and thick blood smears were prepared, stained with Giemsa, and examined under a microscope for parasite identification and density determination following WHO recommendations [35].

Nested PCR for *Plasmodium* species identification

DNA was extracted from filter paper samples using commercial kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA underwent nested PCR targeting the 18S rRNA gene region of *Plasmodium* spp., as described previously [36]. Species-specific primers were used in nested PCR to identify *P. falciparum*, *P. malariae*, *P. ovale* spp., and *P. vivax* as described previously. The PCR products were visualized by gel electrophoresis on a 1.5 %agarose gel stained with SYBR Green. Details of the primers used and their expected size are provided in **Supplementary Table 1.** Each PCR run included a positive control consisting of confirmed *Plasmodium*-positive DNA and a negative control consisting of nuclease-free water.

Nested qPCR for *Plasmodium* species identification

All 484 samples were analysed by microscopy, RDT, and nested PCR. Samples negative for nested PCR were subsequently analyzed using a previously validated highly sensitive nested qPCR protocol in a two-step reaction to detect very low-density *Plasmodium* infections [37]. Amplification was performed on a LightCycler480-II (Roche, Mannheim, Germany). In the first step, a conventional PCR amplification using conserved primers for all *Plasmodium* species[36] increased the template DNA available for downstream species-specific detection. The resulting amplicons were subsequently used as templates for species-specific primers and probes for five human malaria species, including *P. falciparum*, *P. malariae*, *P. vivax,*

P. ovale curtisi and *P. ovale wallikeri*. Each sample was analysed in triplicate, with positive and negative controls included in each run. Ct values generated for each sample by the LightCycler 480 software were recorded, and the amplification curves of the three technical replicates were examined. Samples were considered positive when the Ct value met the positivity threshold established in the validated assay, and consistent amplification was observed across the technical replicates [37]. Details of the primers and probes used are provided in **Supplementary Table 2**.

P. malariae msp-1 genotyping: Genotyping of the *P. malariae msp-1* gene was performed according to the protocol described by Guimarães et al. [31] using the MM1 reference strain (GenBank accession number FJ824669). Five fragments were amplified, covering more than 50% of the *msp-1* coding sequence to enable robust assessment of genetic diversity. The *P. malariae msp-1* partial gene amplification was performed on *P. malariae*-positive samples using a nested PCR approach [31]. The first-round PCR was carried out in a 25µL reaction volume containing 3µL (~ 20ng) of DNA, 300nM of each primer, 2.5 mM $MgCl_2$, 1X PCR buffer, 0.2 mM mixed dNTPs, and 0.5 units of Taq DNA polymerase (Qiagen, Hilden, Germany). The resulting PCR product served as template for the nested PCR performed in a 25µL reaction, which amplified *P. malariae msp-1* gene-specific fragments of various sizes. The details of the primers, PCR conditions, and amplicon sizes obtained for subsequent sequencing are provided in **Supplementary Table 3**. Expected PCR products were visualized on a 1.5% agarose gel stained with SYBR Green under UV transillumination. PCR products were purified using Exo-SAP-IT PCR (Applied Biosystems, Beverly, MA, USA), and purified amplicons were used as a sequencing template using the BigDye Terminator v.1.1 Cycle Sequencing Kit on an ABI 3130XL DNA sequencer (Applied Biosystems, Beverly, MA, USA). Both mono- and mixed *P. malariae* infections were subjected to direct Sanger sequencing. Sequence quality was assessed by visual inspection of the chromatograms, and sequences with clear and interpretable chromatographic profiles were retained for downstream genetic diversity analyses.

Statistical and phylogenetic analysis:

Data were analysed using R software version 4.3.2 (http://www.r-project.org), with variables expressed as proportions for categorical data and medians (with range) for continuous data. The study participants were stratified into three groups: <5, 5–15, and > 15 years. Prevalence of *Plasmodium* spp. was calculated and compared across diagnostic methods using appropriate statistical tests. Participants with an axillary temperature of 37.5°C or higher were classified as febrile. A p-value < 0.05 was considered statistically significant.

Sequences were aligned using SeqMan software (https://www.dnastar.com/software), and the resulting sequence contigs were aligned to the published *P. malariae msp-1* (FJ824669) obtained from GenBank database (http://www.ncbi.nlm.nih.gov/GenBank) using the multiple alignment algorithm and MAFFT version 7 using the G-INS-i model. Genetic variants were identified according to nucleotide polymorphisms observed within the sequenced msp-1 fragments relative to the MM1 reference sequence. Phylogenetic trees were reconstructed using MEGA version 11 (www.megasoftware.net) [38],

employing the Maximum Likelihood method and the General Time Reversible (GTR) plus Gamma Distribution model. The statistical robustness and reliability of the branching order were confirmed via bootstrapping with 1000 replicates. The nucleotide diversity (π) and Watterson's estimator (θ) were calculated using MEGA version 11. Tajima's D was also calculated to assess departures from neutral evolution. The resulting phylogenetic tree was annotated and visualized using the online tool iTOL v6 (https://itol.embl.de/) [39]. The representative sequences of *P. malariae msp-1* sequenced in this study have been deposited in the NCBI GenBank database and can be retrieved with accession numbers PX662751-PX662813.

Declarations

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Author contributions:

RA conceptualized the study design and contributed to the study materials for experimental investigations, performed experimental procedures, statistical and phylogenetic analysis, wrote the first draft and acquired the funding. TPV and AAA supervised RA associated study objectives. SR, VMN, and MMN supported RA and performed partial experimental procedures. RAk was involved in the sampling and microscopy procedures. RAk, SR, VMN, MMN, AAA and TPV reviewed the circulated draft. All authors have read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

Data Availability: All data generated or analysed during this study are included in the article. A total of 63 high-quality sequences corresponding to the amplified fragments of *P. malariae msp-1* gene were submitted to the NCBI GenBank database under accession numbers PX662751-PX662813

Ethics approval and consent to participate

Ethical approval for this study was obtained from the Ethics Committee of the Faculty of Sciences and Health of Cotonou, Benin, Cer_ISBA (No. 115/2018/CER-ISBA/FSS/UAC, 29 October 2018). Written and signed informed consent was obtained from all participating residents and household heads. The study

was conducted in accordance with the principles outlined in the Declaration of Helsinki, ensuring the protection of the rights, safety, and well-being of the study participants. For participants under 18 years of age, the consent form and survey were completed and signed by a parent or legal guardian. Residents who tested positive for malaria were treated with artemisinin-based combination therapies (ACTs) in accordance with the National Malaria Control Programme (NMCP) guidelines.

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Figures

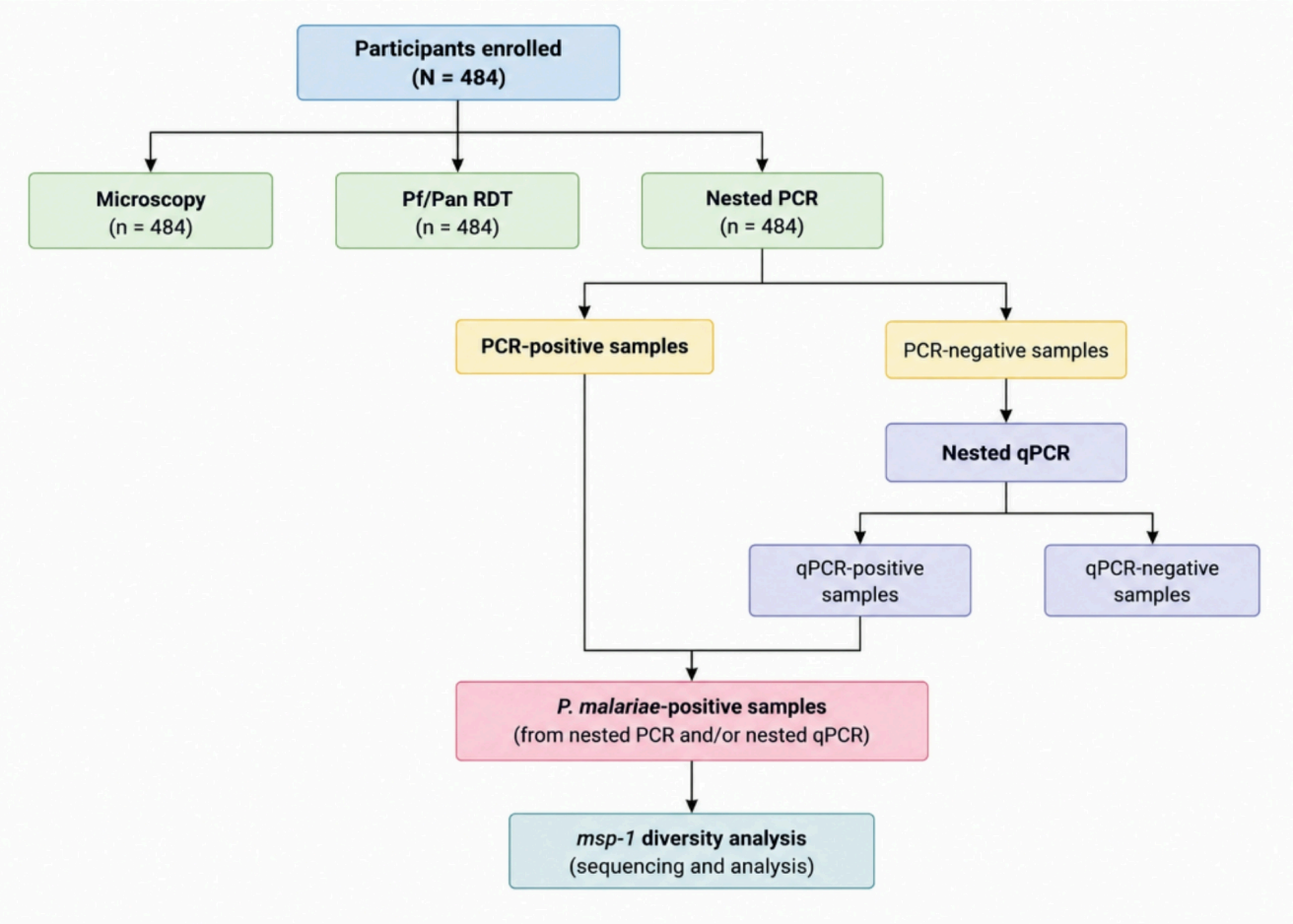


Figure 1

Flowchart of *Plasmodium* spp. screening using microscopy, rapid diagnostic tests (RDT), nested PCR, nested qPCR, and *msp-1* genetic diversity analysis

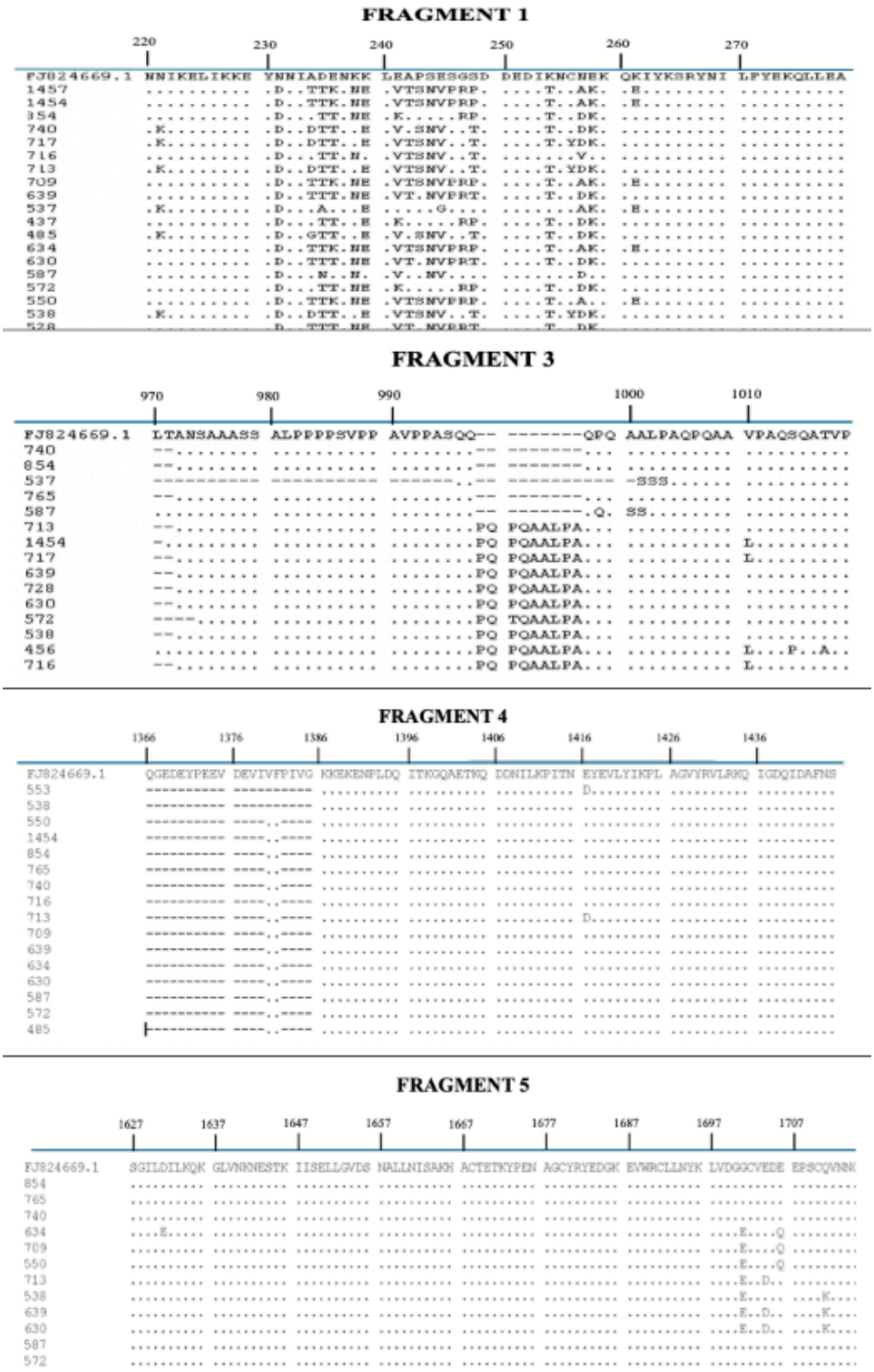


Figure 2

Amino acid polymorphism in the *P. malariae* merozoite surface protein 1 sequences of fragments 1, 3, 4, and 5

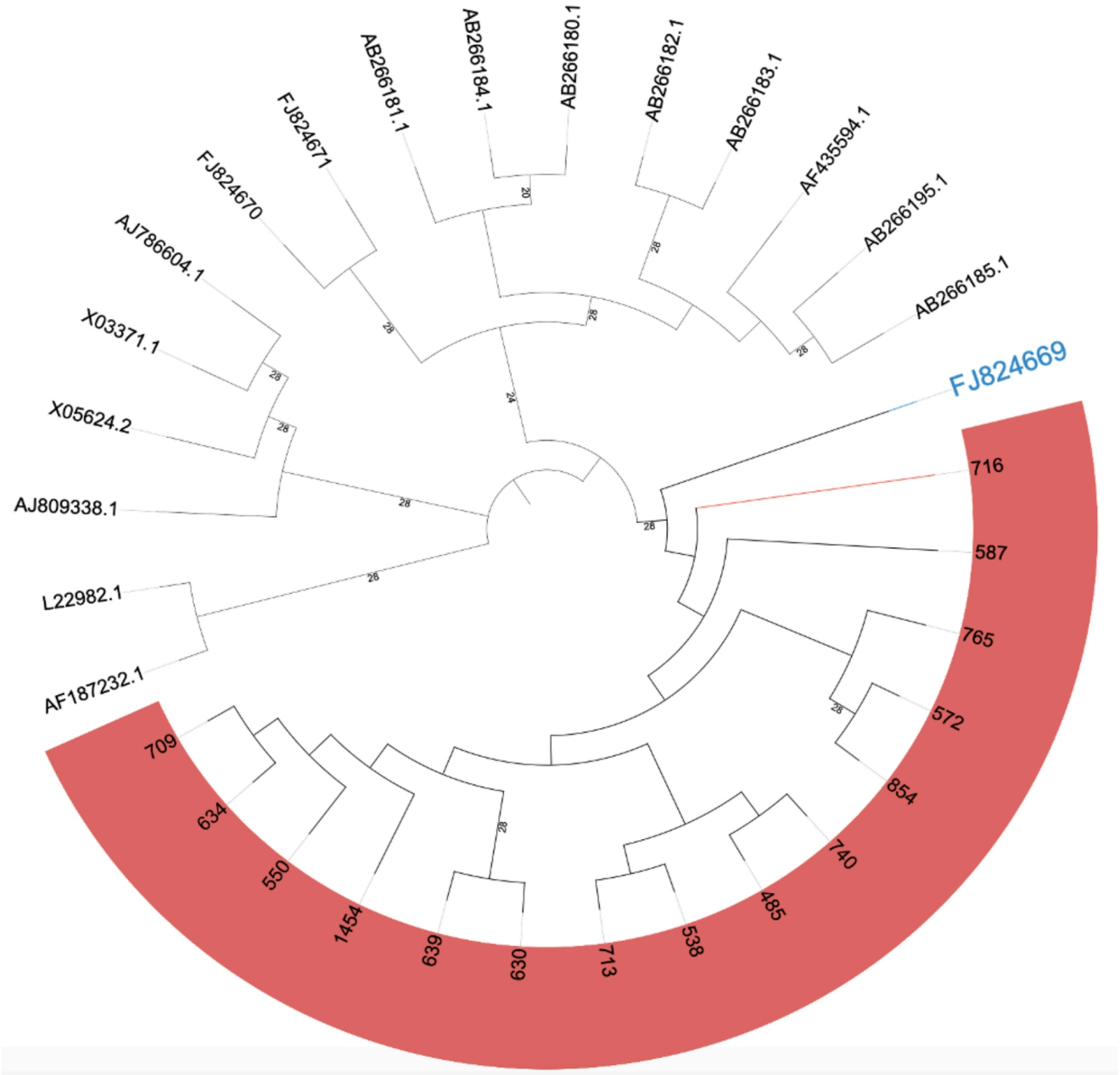


Figure 3

Phylogenetic tree of the *msp-1*gene using the GTR+G+I model, with 17 sequences from 16 different *Plasmodium* species. The *P. malariae* sequences from this study are shown in pink, and the *P. malariae* reference sequence is shown in blue. The remaining *Plasmodium* species were included as outgroups for phylogenetic rooting and are not part of the diversity analysis of *P. malariae*. Bootstrap support values (based on 1000 replicates) are indicated at the internal nodes.

Supplementary Files

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