De novo assembly of complete *Plasmodium falciparum* isolate genomes using PacBio HiFi sequencing technology

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Abstract

Plasmodium falciparum possesses a highly structured genome with extensive sequence diversity concentrated in Variant Surface Antigen (VSA) families. These genes—*var*, *rif*, and *stevor*—play key roles in immune evasion and pathogenesis and are difficult to assemble using short-read sequencing technologies. Here, we applied PacBio HiFi long-read sequencing to generate high-quality *de novo* genome assemblies from 43 *P. falciparum* parasite cultures originating from community cases in The Gambia. Parasites were culture-adapted, cloned by limiting dilution where possible, and sequenced using high molecular weight DNA extracts. Assemblies from single-genotype lineages were constructed using hifiasm, producing complete chromosomal-length scaffolds with high base accuracy without requiring short-read polishing. We recovered full repertoires of *var*, *rif*, and *stevor* genes and classified them into known subgroups. Together, our results demonstrate that PacBio HiFi sequencing enables accurate assembly of complex *P. falciparum* genomes from natural infections. This work provides a valuable genomic resource for future studies of parasite evolution, transmission dynamics, and antigenic diversity, and suggests that VSA repertoires can serve as reliable proxies of genetic relatedness across infections.

Introduction

Malaria remains a global leading cause of morbidity and mortality, affecting millions and causing hundreds of thousands of deaths annually (*World Malaria Report 2024*, n.d.) The disease is caused by unicellular apicomplexan parasites of the genus *Plasmodium*, with *P. falciparum* responsible for most of the severe cases and fatalities. The first *P. falciparum* genome sequenced was the laboratory strain 3D7 in 2002 (Gardner et al., 2002). Since then, numerous studies have increased the number of *P. falciparum* genome sequences.

Most genome sequencing efforts have relied on Illumina short-read technologies (MalariaGen., 2021), but long-read platforms, such as Oxford Nanopore Technology or PacBio's single-molecule real-time (SMRT) sequencing have been increasingly applied to *Plasmodium* genomes (Delandre et al., 2024; Holzschuh et al., 2024; Vembar et al., 2016). Historically, the lower base-calling accuracy of long-read platforms necessitated complementary short-read sequencing to achieve assemblies that were both accurate and complete (Higgins et al., 2024). However, advances in PacBio HiFi (High Fidelity) sequencing using circular consensus sequencing (CCS) now promise long and highly accurate reads (Hon et al., 2020; Wenger et al., 2019). While this technology has been used to produce high-quality assemblies for some *Plasmodium* species (*P. yoelii* - Godin et al., 2023), it has not yet been widely applied to *P. falciparum* field isolates.

The genome of *P. falciparum* consists of a conserved 'core genome' and a hypervariable non-core genome, the latter harboring large multigene families encoding Variant Surface Antigens (VSAs) (Otto et al., 2018). The three VSA families - *var*, *rif* and *stevor* - are important for pathogenesis and immune evasion and are located in subtelomeric and internal gene clusters (Harrison et al., 2020; Jespersen et al., 2016; Rowe et al., 2009; Sakoguchi et al., 2025). Their sequences are highly polymorphic with each parasite isolate typically having a unique set of VSAs whose genetic diversity can be generated by non-homologous recombination events (Claessens et al., 2014). Their classification into biologically relevant subgroups (e.g., *var* A, B, and C; *rif* A and B) provides insights into parasite biology and virulence. For example, group A *var* genes are implicated in cerebral malaria (Claessens et al., 2012). Analyses of VSA repertoires in field isolates also bear epidemiological relevance, enabling lineage tracking across host infections (Day et al., 2025; Tonkin-Hill et al., 2021). However, assembling these hypervariable regions with short-read data has proven difficult due to repetitive sequences and limited homology between isolates, often resulting in incomplete sets of VSA sequences.

In this study, we apply PacBio HiFi sequencing to generate *de novo* assemblies of *P. falciparum* parasites from The Gambia. Using *in vitro* culture to obtain high-yield high molecular weight (HMW) DNA, we sequenced both clonal lineages (obtained via limiting dilution) and bulk field-adapted populations, resulting in 43 assemblies. We then used bioinformatics tools to assemble and annotate genomes, extract, classify and analyze VSA sequences.

Methods

Study design and sample collection

All *P. falciparum* field isolates reported here have been described previously (Collins et al., 2022; Fogang et al., 2024; Guery et al., 2025). Briefly, 5mL venous blood samples were collected from *P. falciparum* positive participants in 2016 and 2017 in The Gambia. Plasma, PBMCs and red blood cells were immediately separated by centrifugation. Red blood cells

were cryopreserved with glycerolyte in liquid nitrogen. The study protocol was reviewed and approved by the Gambia Government/MRC Joint Ethics Committee (SCC 1476, SCC 1318, L2015.50) and by the London School of Hygiene & Tropical Medicine Ethics Committee (Ref. 10982). The field studies were also approved by local administrative representatives, the village chiefs. Written informed consent was obtained from participants over 18 years old and from parents/guardians for participants under 18 years. Written assent was obtained from all individuals aged 12–17 years. Approval under the Nagoya Protocol was granted by the National Focal Point of The Gambia.

Parasite culturing

Thawed parasites were cultured in RPMI-1640 (sigma) supplemented with 25mM HEPES, 2mM L-glutamine, 0.5% Albumax II (sigma), 50µg/L gentamicin (sigma) and 5% AB^+ normal human serum (NHS) in static conditions. Parasites were cultured in 10mL volumes at 2% haematocrit in a blood gas environment of 90% N_2, 5% CO_2 and 5% oxygen (O_2).

Cloning by limiting dilution

Once field isolates were culture-adapted, typically after 2 to 3 weeks, clones were isolated by limiting dilution in 96-well plates at a theoretical density of 1 parasite per well (ppw) – a 3ppw was included as positive control – and maintained at 1.25% haematocrit. Plates were kept in a modular chamber with blood gas and incubated at 37ºC. On day 7, exhausted media was removed and replaced with fresh media containing uninfected RBCs (uRBCs) at 1.25% haematocrit. Culture media was changed every two days from this point onwards. From day 14, 3ppw wells were screened by microscopy for viable parasites. If parasites were absent, plates were screened every two days until day 21, at which point the cloning assay was considered failed and discarded. If viable parasites were present, the entire plate was screened for parasites in the 1ppw wells. Positive wells were initially transferred into 6-well plates with 100µL uRBCs and 3mL media, and grown until parasitaemia was visible by microscopy (usually 2 to 4 replication cycles). Parasites were then genotyped by MSP1 and MSP2 (see details in supplementary methods, Supplementary Figure 1) to assess the number of parasite genotypes present in cultures. Wells with confirmed unique single clones were selected for expansion in 25mL cultures at 2% haematocrit, while all other wells were terminated. Parasites were then grown to higher parasitaemia (~10%) and harvested at the schizont stage for high molecular weight DNA extraction.

High molecular weight DNA extraction and PacBio sequencing

High molecular weight (HMW) DNA was extracted from both cloned parasites and 'bulk' populations (parasite cultures directly adapted from field isolates) using the Monarch® HMW DNA Extraction Kit for Tissue (New England Biolabs). The tissue kit was chosen over the blood kit due to the protein separation step being able to remove residual cell debris and haemozoin contaminants. Several modifications were made to the extraction procedure. Briefly, RBCs were initially lysed with RBC Lysis Buffer from the Monarch HMW DNA Extraction Kit for Cells & Blood. Free parasite pellets were then washed with 1X PBS until the supernatant was clear. All subsequent steps followed the tissue kit's protocol. The agitation speed for lysis was reduced to 1500 rpm and in special cases where parasite material was limited, precipitation enhancer was added prior to binding DNA to the glass beads. DNA was solubilised in 200µL of elution buffer, incubated at 37ºC for 1 hour to enhance dissolution, stored at 4ºC overnight and then frozen at -20ºC until sequencing. Prior to sequencing, DNA was cleaned using SPRI (Solid Phase Reversible Immobilization) beads, sheared with g-TUBE

134 (Covaris) aiming for a size of 10kb and assessed for integrity using FEMTO Pulse (Agilent).
135 Samples were sequenced using the Pacific Biosciences (PacBio) high fidelity (HiFi) circular
136 consensus sequencing technology (Wenger *et al.*, 2019) on the Sequel IIe plaform, with up to
137 10 samples per SMRT cell.

138 *Genome assemblies and annotation*

139 We assembled genomes from PacBio HiFi reads datasets using Hifiasm (H. Cheng et al., 2021),
140 using the -l0 option to assemble haploid genome. Contigs were split, oriented and arranged to
141 the 3D7 chromosome configuration using RagTag, with default parameters (Alonge et al.,
142 2022). Assemblies coming from polyclonal isolates were left in the post-Hifiasm assembly
143 state, with no re-arrangements to the 3D7 chromosome configuration. Companion (Steinbiss
144 *et al.*, 2016) was used to annotate clone and bulk assemblies with the following options:
145 pseudogene detection activated, AUGUSTUS (Stanke et al., 2004) for structural annotation
146 and RATT (Otto et al., 2011) for transfer of highly conserved genes from 3D7.

147 *VSAs extraction*

148 *Var* genes were retrieved from all assemblies by screening for genes annotated as potentially
149 encoding PfEMP1 by the Companion pipeline and filtering for a nucleotide length higher than
150 2500bp. Each *var* gene had its different domains annotated by submitting its amino acid
151 sequences to the varDOM server (Rask et al., 2010). Copies of *var2csa* were extracted from
152 the list of *var* genes by screening for genes with DBLpam domains. Upstream group affiliation
153 of the remaining *var* genes was performed as in the method described in Pangilinan et al., 2025.
154 Briefly, a phylogenetic tree was constructed using the 500bp upstream nucleotide sequences of
155 newly assembled *var* genes plus reference upstream sequences previously annotated as group
156 A, B or C (Lavstsen et al., 2003; Rask et al., 2010). Sequences of all *var* genes were aligned
157 using MAFFT v7.520 and a phylogenetic tree was inferred from the alignment using FastTree
158 v2.1.11. Upstream sequences were annotated based on the clade of the reference upstream
159 group. The HMM-based software STRIDE (Zhou et al., 2022) was used to retrieve *rif* and
160 *stevor* from the list of Companion proteins, and further annotate the *rif* into *rif* groups A or *rif*
161 group B.

162 *Estimation of complexity of infection across bulk parasite populations*

163 To infer whether bulk parasite populations were single- or poly-genotype we looked at average
164 heterozygosity levels over a panel of 72,020 core-genome variable loci. This set of variable
165 loci was generated by mapping PacBio HiFi reads to the reference 3D7 genome using
166 minimap2 v2.28 (Li, 2018). SNP calling and generation of VCF files were performed with
167 GATK v4.1.0.5.1 (Van der Auwera et al., 2020), using the HaplotypeCaller pipeline. Variants
168 were filtered based on their quality using bcftools v1.9 (Danecek et al., 2021) and imported in
169 an R environment with the R package vcfR (Knaus & Grünwald, 2017). Genome assemblies
170 were considered to be originating from a single-genotype population if their average
171 heterozygosity level was lower than 0.02 over the entire set of 72,020 variants, which
172 corresponded to the highest heterozygosity level found in clone genome assemblies.

173 *Estimation of base-calling error rates in hifiasm assemblies*

174 To estimate base-calling error rates level in newly-generated assemblies, we used two *ex vivo*
175 isolates (i.e. without any culturing), DC03m1 and DC10m3, sequenced with Illumina in a
176 previous study (Guery et al., 2025). Illumina reads were mapped to their respective long read
177 assembly using bwa MEM v0.7.17 (Li & Durbin, 2009). The output sam files were then
178 converted and sorted using samtools v1.10 (Li et al., 2009). GATK v4.1.0.5.1 (Van der Auwera
179 et al., 2020) was used again for SNPs calling and generation of VCF files, using the
180 HaplotypeCaller pipeline. Additionally, nucleotide level accuracies in assemblies were also

181 estimated by comparing the k-mer decomposition of PacBio HiFi assemblies to illumina reads
182 datasets via the software merqury (Rhie et al., 2020). Here we used the quality value (qv) score
183 to assess base-calling accuracy, a metric that is generated by looking at k-mers present in high-
184 quality reads dataset (Illumina) but not in the PacBio assemblies.

185 *Estimation of pairwise Identity By Descent (IBD) and VSAs sharing amongst samples*
186
187 To estimate core genome relatedness between parasites genomes we calculated the pairwise
188 IBD values between all genomes. Chromosomal segments are in IBD state if they descend from
189 a recent common ancestor, and a pairwise IBD value indicate the average proportion of two
190 genomes that are in IBD state. In practise, PacBio HiFi reads were mapped to the reference
191 3D7 genome using minimap2 v2.28 (Li, 2018). SNP calling was performed as described above.
192 From this, VCF files were parsed with the R package vcfR to generate a tab-delimited table
193 containing SNPs position and status for each genome. We used hmmIBD v2.0.0 (Schaffner et
194 al., 2018) to calculate the fraction of the genome in an IBD state, from here referred as pairwise
195 IBD values, between each pair of genomes.

196 In parallel, we assessed the similarity of VSAs repertoires between each single-genotype
197 genome pair by clustering *var*, *rif*, and *stevor* sequences using CD-HIT (Fu et al., 2012).
198 Nucleotide sequences in different isolate genomes were considered identical if their sequence
199 similarity was higher than 99%. After counting the number of shared VSAs between two
200 genomes we calculated the three normalised pairwise VSA sharing indexes *var* sharing, *rif*
201 sharing and *stevor* sharing using the following formula:

202

203 $$VSA\ sharing\ index = \frac{2 * Number\ of\ shared\ VSA\ genes}{Number\ of\ VSA\ genes\ in\ genome\ 1 + Number\ of\ VSA\ genes\ in\ genome\ 2}$$

204

205 To account for the non-independence of pairwise estimates, overall correlations between core
206 genome IBD values and VSA sharing indexes were then evaluated using Mantel tests, as
207 implemented in the R package vegan (v2.6-10). Distance matrices of pairwise IBD values and
208 normalized *var, rif* and *stevor* sharing were compared via the mante() function, with 9999
209 permutations and p-values computed using the Spearman rank correlation method.
210
211
212 **Results**
213
214 Blood isolates were collected in four neighboring villages from The Gambia, following the
215 sample design presented in Collins et al., 2022 and Fogang et al., 2024. Briefly, samples were
216 collected during the wet season (October 2016 – November 2016) from both asymptomatic
217 and symptomatic cases, and from asymptomatic carriers at monthly timepoints during the
218 following dry season (December 2016 to May 2017). From this sampling design, isolates were
219 separated into three infection groups: the Wet season Symptomatics (WS), corresponding to
220 parasites collected from symptomatic cases in the wet season; the Wet season Asymptomatics
221 (WA), corresponding to parasites collected from asymptomatic cases in the wet season and the
222 Dry season asymptomatic Cohort samples (DC), corresponding to parasites collected at
223 monthly timepoints from asymptomatic cases during the dry season. Sample identifiers
224 incorporate group, timepoint, and sequencing type (e.g., bulk 'b' vs clone 'c'; e.g., WA01b,
225 DC01m1c). Metadata and group assignments are detailed in Table 1.
226

227 ***Culture adaptation, cloning, and high molecular weight DNA extraction***
228
229 Parasites were culture-adapted to generate the high DNA yields necessary for long-read
230 sequencing. Cloning by limiting dilution allowed the isolation of single-genotype populations
231 (Supplementary Figure 1). Two extraction kits (Qiagen Genomic-tip 100/G and Monarch
232 HMW DNA extraction kit) were tested for high molecular weight (HMW) DNA recovery, with
233 the Monarch kit performing better (Supplementary Figure 2). Overall, a total set of 43 DNA
234 extractions derived from 30 isolates were all successfully sequenced with PacBio HiFi (Table
235 1). Twenty three (23) genomes were derived from cloning dilutions and were strictly single-
236 genotype parasite populations. The other 20 DNA samples were bulk populations (not cloned)
237 with an unknown number of parasite genotypes. Based on their level of heterozygosity over a
238 set of 72,020 informative loci, we inferred 8 bulk genomes to be single-genotype and 12 to
239 consist of multiple parasite genotypes (Table 1). Across infection groups, we sequenced 17
240 genomes from asymptomatic infections in the dry season (DC group), 15 genomes from
241 symptomatic infections in the wet season (WS group) and 11 genomes from asymptomatic
242 infections in the wet season (WA group).
243
244 ***Pacbio HiFi enables chromosome-scale genome assemblies***
245
246 Raw assemblies from the 43 Pacbio HiFi reads datasets contained between 17 and 185 contigs
247 (Table 2), with a median number of 54. The N50 statistics in all genomes (range 1,198,427
248 to 1,693,536 bp, median = 1,571,854 bp) were indicative of the presence of large chromosome-
249 size contigs, which was further supported by syntenic analyse comparing newly assembled
250 genomes to the 3D7 reference genome (Figure 2). The number of complete telomere-to-
251 telomere contigs was however systematically lower than the 14 expected *P. falciparum*
252 chromosomes (range 2 to 9, median = 4), demonstrating difficulties in assembling
253 hypervariable sub-telomeric regions and complete telomeric contigs.
254
255 Across the 31 single-genotype genomes, some of the Hifiasm assemblies contained contigs
256 with regions syntenic to more than one chromosome. Mapping PacBio HiFi reads onto their
257 assemblies revealed that the apparent chromosome breaks coincided with regions of low
258 coverage or low sequence complexity, with no evidence of breaks supported by high read
259 depth. Accordingly, raw contigs were split and reorganized into the 3D7 chromosome structure
260 using ragtag (Alonge et al., 2022). After the re-contiguation step, all single-genotype
261 assemblies had 14 large contigs corresponding to the 14 *P. falciparum* chromosomes, alongside
262 2 contigs for the mitochondrial and apicoplast genomes and a variable number of small extra
263 contigs (median = 32, range 4 to 171; Figure 2, Table 2).
264
265 ***Pacbio Hifi P. falciparum genome assemblies showed high base-calling accuracy***
266
267 To assess base-calling accuracy of genome assemblies, we aligned Illumina reads from two ex
268 vivo isolates, DC03m1 and DC10m3, (Guery et al., 2025) to their respective Pacbio HiFi
269 assemblies from clonal genomes. We found only 55 SNPs over the entire DC03m1 genome
270 (total length 23.6 Mbp) and 40 SNPs over the entire DC10m1 genome (total length 23.5 Mbp).
271 We also computed k-mer-based quality scores using Merqury (Rhie et al., 2020), which yielded
272 quality value (qv) scores of 64.5 for DC03m1 and 65.5 for DC10m3, indicating error rates
273 ranging between 1 in 1,000,000 bp and 1 in 10,000,000 bp for both genomes. Those results
274 confirmed the high accuracy and quality of genome assemblies generated by hifiasm for Pacbio
275 HiFi reads, and their use in generating reliable *P. falciparum* genome assemblies.
276

IBD analysis reveals genotypic relatedness

To investigate genetic relatedness, we calculated identity-by-descent (IBD) fractions using core-genome SNPs mapped to the 3D7 reference genome (Supplementary Figure 3). In some cases, the same parasite genotypes were observed in different hosts (e.g. DC03m1c and WS11b), highlighting the presence and persistence of certain parasite genotypes across infection groups and time. In addition to shared parasite genotypes, we also observed groups of parasites with intermediate pairwise IBD values ranging from 0.1 to 0.9, highlighting groups of inter-related parasites that share a common recent ancestry (*e.g.* DC07m1c and DC10m3c).

Full VSAs repertoires retrieved from PacBio HiFi assemblies

We extracted 3,288 *var* genes from all genomes (1,504 unique sequences) using Companion annotations. Clonal assemblies contained 56–84 *var* genes per genome (median = 69), consistent with expectations for single-genotype isolates. Bulk assemblies had 65–130 *var* genes, suggesting up to two co-infecting strains in some samples. The number of extracted *var* genes strongly correlated with the number of protein-coding DNA sequences (CDS) containing the LARSFADIG PfEMP1 motif ($p < 2e-16$), consistent with earlier findings (Otto et al., 2019).

Using the information on shared genotypes across samples deduced from pairwise IBD values (Supplementary Figure 3), we defined an unbiased dataset of unique single-genotype isolates (N = 17). Within this dataset, the numbers and proportions of each *var* upstream groups were relatively stable (Figure 3 and Table 2). Group A *var* genes ranged from 8 to 16 genes per genome with a median number of 11 per genome, group B *var* genes ranged from 29 to 46 per genome with a median number of 37 genes per genome and group C *var* genes ranged from 8 to 19 per genome with a median number of 12 per genome. In genomes sequenced from poly-genotype isolates the proportions of groups A, B and C were similar to the ones observed in clones, with a larger overall number of *var* genes (Table 2). Some *var* genes could not be reliably classified into any upstream group. This included genes with very divergent upstream sequences and *var* genes sequences located in small contigs where their 500bp upstream sequence could not be isolated.

As previously reported (Benavente et al., 2018; Sander et al., 2009), multiple copies of the conserved *var* gene *var2csa* were observed in multiple genomes (Table 2). Across the 17 unique single-genotype, 11 assemblies had a single *var2csa* copy and 12 assemblies had two or more *var2csa* copies (median = 2, range 1 to 6). In single-genotype genomes where copies of *var2csa* could be localised there was always a copy on chromosome 12, as in 3D7 and other reference genomes. Extra copies of the gene were found on sub-telomeric regions of chromosomes 1, 3, 8, 12, 13 and 14.

Similar to the pattern observed with *var* genes, the numbers and proportions of *rif* and *stevor* stayed relatively constant across different isolates (Figure 2). In the dataset of unique single-genotype genomes (N = 17) the total number of *rif* varied from 117 to 196 per genome with a median number of 168 genes. From those, *rif* group A ranged from 85 to 139 per genome with a median number of 116 genes and *rif* group B ranged from 32 to 65 with a median number of 48 genes per genome. The *stevor* were less numerous, ranging from 26 to 51 with a median number of 39 genes per genome.

There was no correlation between the number of *var* genes and the number of *rif* ($p = 0.61$), or the number of *stevor* ($p = 0.83$), and the numbers of *rif* and *stevor* were mildly correlated ($p = 0.03$; see Supplementary Figure 4).

Pairwise core genome IBD and VSAs sharing indexes are highly correlated

We quantified pairwise sharing indexes for *var*, *rif*, and *stevor* gene repertoires in the single-genotype dataset (N = 31, excluding technical replicates). The pairwise sharing indexes reflects a measure of genetic similarity for non-core *P. falciparum* genomes. Overall, sharing patterns closely paralleled core-genome IBD values—genome pairs with high IBD exhibited similar VSA repertoires (Figure 4A). Matrices permutation tests revealed significant correlations between core-genome IBD and VSA sharing for all three families (Figure 4B) ($p < 0.01$). Of note, we also observed most pairs of genomes seemed to share some *stevor* sequences, with an average *stevor* sharing value of 0.03 in pairs of samples with IBD < 0.25, against a lower average sharing value of 0.008 for *rif* and 0.003 for *var*. This is in line with previous observations that reported *stevor* as the least hypervariable of the three VSAs families (Q. Cheng et al., 1998; Lawton et al., 2025).

Discussion

In total, we successfully culture-adapted and long-read sequenced parasite genomes from 43 parasite cultures, originating from 30 distinct field isolates. To the best of our knowledge this is the largest dataset of *P. falciparum* long read assemblies generated from natural infections to date. With PacBio HiFi reads and using the hifiasm assembler, we demonstrated long and accurate assembly contigs could be generated without the need for complementary illumina libraries, as is necessary with other long reads sequencing technologies (Delandre et al., 2024; Higgins et al., 2024). Particularly, our method proved useful in assembling *P. falciparum* sub-telomeric regions and complete sets of VSA families which are highly polymorphic and hard to assemble using short-read datasets (Andradi-Brown et al., 2024).

Using cloning by limiting dilutions, parasite lineages could be delimited to ensure sequences were retrieved from single-genotype populations. Bulk populations (without the cloning step) also proved useful in retrieving complete VSA sequences, regardless of the number of parasite genotypes present.

The risk of culture-adaptation–induced mutations, although well established for parasites subjected to extended *in vitro* passages (Claessens et al., 2017, 2023; Kemp et al., 1992), is minimal here, as all genomes were derived from parasites maintained in culture only for the few weeks required for DNA extraction. This makes the generated assemblies a reliable genomic resource for analysis of circulating West African *P. falciparum*—across both symptomatic and asymptomatic infections.

The analysis of VSA variation among genome assemblies confirmed patterns found in previous studies, with ratios of each *var* and *rif* groups stable across isolates. The presence of multiple copies of *var2csa* was observed in approximately half of the genomes generated here, which seems to be common in field isolates (Sander et al., 2009). There however seem to be an evolutionary constraint on maintaining at least one copy of the gene at the 5' telomeric end of chromosome 12, as this copy was found in all genomes.

Contrary to a previous study which sampled *P. falciparum* reference genomes from widespread worldwide locations (Otto et al., 2018) we have generated a set of genomes originating from infections that were both close in collection dates and geographical locations. With this sampling design, we collected parasites with a wide range of genetic relatedness, from completely unrelated parasites to identical parasite genotypes sampled across different hosts or timepoints of a chronic infection. The relationship between core genome and non-core genome estimates of relatedness have seldom been studied (Neafsey et al., 2021), and the generation of genetic diversity in these two genomic regions stems from different biological processes. In the core genome, homologous recombination events occurring during meiosis lead to an uneven repartitioning of genetic variants and SNPs in offspring, with the proportion of the genome regions containing similar genetic variants tightly linked to the kinship value between parasites (Guo et al., 2025; Schaffner et al., 2018). In the non-core genome, variability is additionally generated through non-homologous recombination events, leading to sequence hypervariability and rapid generation of new VSAs (Claessens et al., 2014). Despite these differences, we observed a remarkably close relationship between core genome pairwise IBD values and the three sharing indexes for *var*, *rif*, *stevor*. These results indicate that in epidemiological settings similar to the one observed in The Gambia, measures of VSA sharing could effectively be used as a proxy for core genome relatedness, and vice-versa. These results support the validity of the growing number of studies relying on mass amplification of conserved *var* domains (e.g. DBLα) for population genetic analyses, and highlight VSA sharing as valid measure of genetic relatedness (Day et al., 2025).

Overall, the genome and VSA sequence datasets provided in this study presents a great resource for future research. Increasing the number of long read sequences generated from field isolated parasites will enable analyses of large structural variants occurring in *P. falciparum* genomes at a large scale, including regions with high repeats or high AT content. Furthermore, the complete and high-quality *var* gene sequences extracted here will prove a useful resource and training dataset for algorithm linking partial *var* sequences to full *var* domain composition (Mackenzie et al., 2022), or upstream group (Pangilinan et al., 2025). Finally, obtaining full-length VSA sequences will enable robust analyses of *var* gene expression in samples coming from The Gambia, and will enable differentiating *var* expression between asymptomatic and symptomatic infections.

Data availability statement

All PacBio HiFi reads datasets are publicly available through the NCBI accession number indicated in Table 1. A collection of refined genome assemblies, annotation, VSA sequences and classification are available at the public repository https://zenodo.org/records/17551160. A series of codes and scripts used for this paper can be found on the github repository https://github.com/MQuenu/Falcigenomes-toolkit.

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Figures

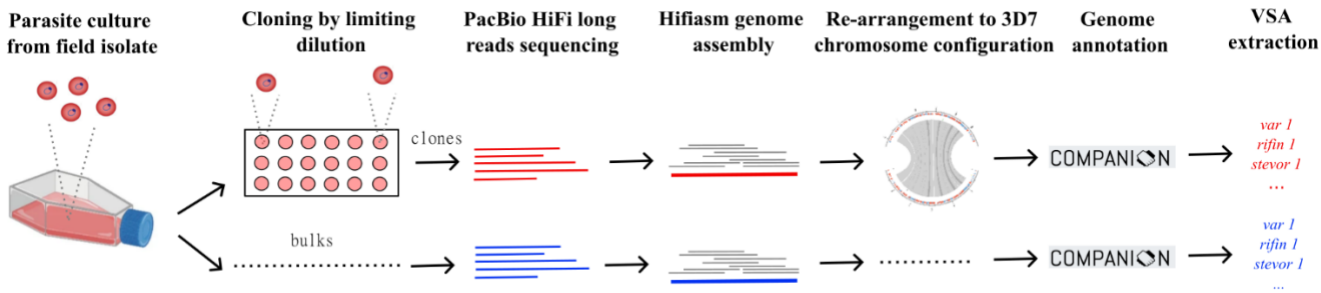

Figure 1: Schematic overview of the genome assembly method used to generate high quality genomes and extract Variant Surface Antigens (VSAs) from parasite cultures.

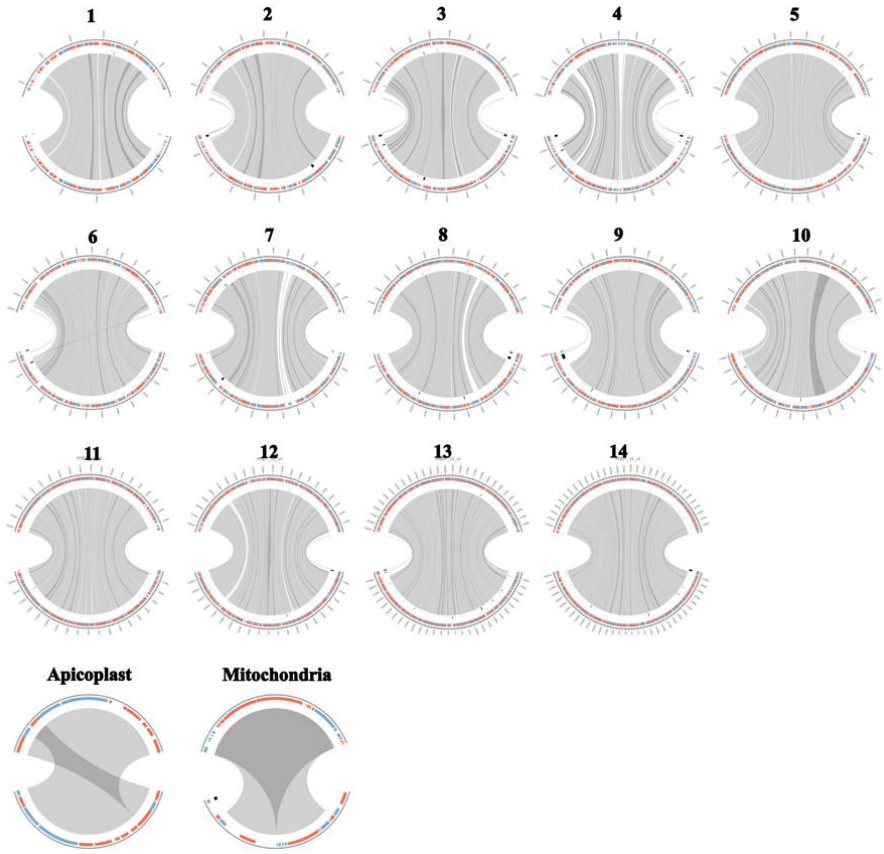

Figure 2: PacBio HiFi sequencing and hifiasm assembly allows chromosome-scale genome assemblies. Each circos plot represents chromosomes of the reference 3D7 genome

(top) and the DC01m1 contigs (bottom) as a representative genome generated from single-genotype parasite lineage. Grey lines between top and bottom sequence assemblies indicate syntenic regions of high sequence similarity. The outer ring indicates position of genes annotated by the companion pipeline, genes in the forward strand are indicated in blue and genes in the reverse strand indicated in red. Gaps in synteny can be seen over internal *var* clusters on chromosomes 4, 7, 8 and 12.

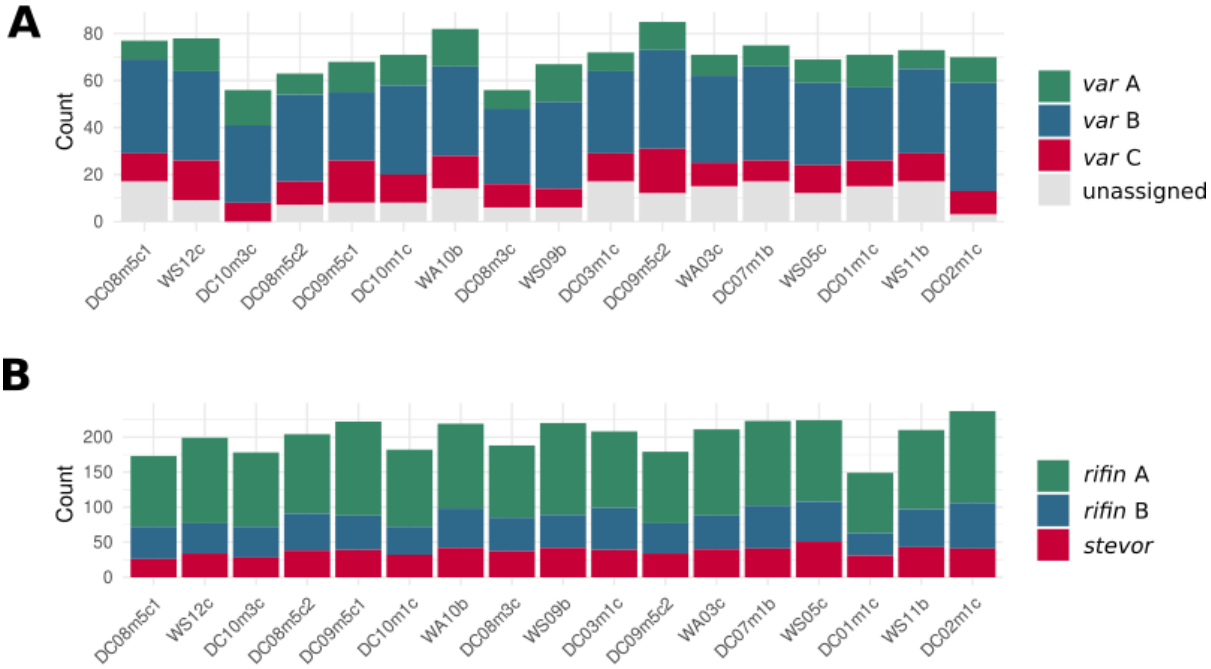

Figure 3: Numbers and proportions of VSA groups are stable across unique single-genotype assemblies (N = 17). The upper panel indicates the number of *var* gene per genome assembly, with colours indicating the different *var* groups. The lower panel indicates the number of *rif* and *stevor* per genome assembly, with colours indicating the different groups.

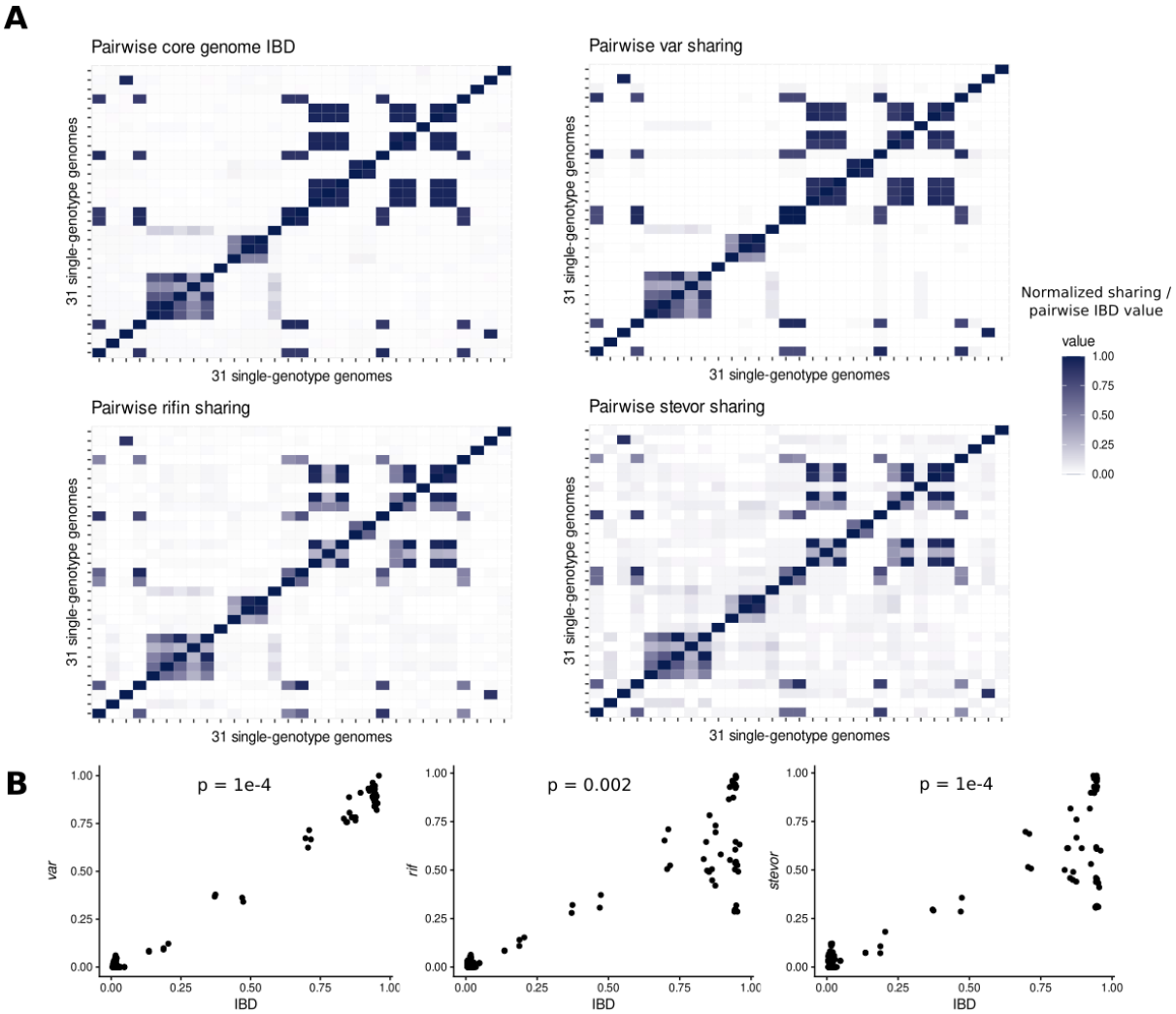

Figure 4: Groups of related parasites share common *var*, *rif* and *stevor* genes.
(A) Pairwise matrices of IBD values and three normalized sharing indexes (*var*, *rif*, *stevor*) in a dataset of 31 single-genotype genomes (B) Correlations between pairwise IBD values and the three VSAs sharing indexes for the same dataset. P-values were calculated by a mantel test.

Tables

Table 1: Genome accession numbers and metadata for 43 *P. falciparum* genome assemblies

Genome_ID	Isolate_ID	Group	Cloning dilution	Complexity of infection	Collection date	Host age	Host sex	NCBI accession number
DC01m1c	DC01m1	Dry Cohort	clone	single-genotype	24/12/2016	9	male	ERR9731534
DC02m1c	DC02m1	Dry Cohort	clone	single-genotype	24/12/2016	11	male	ERR9731535
DC03m1c	DC03m1	Dry Cohort	clone	single-genotype	22/12/2016	13	female	ERR9731539
DC04m2c	DC04m2	Dry Cohort	clone	single-genotype	26/01/2017	15	female	ERR9731540
DC07m1b	DC07m1	Dry Cohort	bulk	single-genotype	23/12/2016	23	female	ERR12263817
DC07m1c	DC07m1	Dry Cohort	clone	single-genotype	23/12/2016	23	female	ERR12263821
DC08m3b	DC08m3	Dry Cohort	bulk	poly-genotypes	13/02/2017	10	male	ERR12263802
DC08m3c	DC08m3	Dry Cohort	clone	single-genotype	13/02/2017	10	male	ERR12263818
DC08m5b	DC08m5	Dry Cohort	bulk	poly-genotypes	19/04/2017	10	male	ERR12263832
DC08m5c1	DC08m5	Dry Cohort	clone	single-genotype	19/04/2017	10	male	ERR12263826
DC08m5c2	DC08m5	Dry Cohort	clone	single-genotype	19/04/2017	10	male	ERR12263801
DC09m5b	DC09m5	Dry Cohort	bulk	poly-genotypes	20/04/2017	12	male	ERR12263810
DC09m5c1	DC09m5	Dry Cohort	clone	single-genotype	20/04/2017	12	male	ERR12263806
DC09m5c2	DC09m5	Dry Cohort	clone	single-genotype	20/04/2017	12	male	ERR12263805
DC10m1c	DC10m1	Dry Cohort	clone	single-genotype	23/12/2016	12	male	ERR9731536
DC10m2c	DC10m2	Dry Cohort	clone	single-genotype	12/01/2017	12	male	ERR9731537
DC10m3c	DC10m3	Dry Cohort	clone	single-genotype	12/02/2017	12	male	ERR9731538
WA01b	WA01	Wet Asymptomatic	bulk	single-genotype	23/11/2016	15	female	ERR12263816
WA01c	WA01	Wet Asymptomatic	clone	single-genotype	23/11/2016	15	female	ERR12263812
WA03b	WA03	Wet Asymptomatic	bulk	poly-genotypes	21/11/2016	27	female	ERR12263820
WA03c	WA03	Wet Asymptomatic	clone	single-genotype	21/11/2016	27	female	ERR12263808
WA04b	WA04	Wet Asymptomatic	bulk	single-genotype	21/11/2016	11	male	ERR12263815
WA04c	WA04	Wet Asymptomatic	clone	single-genotype	21/11/2016	11	male	ERR12263823
WA10b	WA10	Wet Asymptomatic	bulk	single-genotype	21/11/2016	8	male	ERR12263827
WA10c	WA10	Wet Asymptomatic	clone	single-genotype	21/11/2016	8	male	ERR12263807
WA11c	WA11	Wet Asymptomatic	clone	single-genotype	23/11/2016	9	male	ERR12263813

WA47b	WA47	Wet Asymptomatic	bulk	single-genotype	21/12/2016	11	male	ERR12263803
WA47c	WA47	Wet Asymptomatic	clone	single-genotype	21/12/2016	11	male	ERR12263833
WS01b	WS01	Wet Symptomatic	bulk	poly-genotypes	08/11/2016	NA	male	ERR12263825
WS03b	WS03	Wet Symptomatic	bulk	poly-genotypes	31/10/2016	13	female	ERR12263830
WS04b	WS04	Wet Symptomatic	bulk	poly-genotypes	31/10/2016	45	male	ERR12263837
WS05b	WS05	Wet Symptomatic	bulk	poly-genotypes	17/10/2016	54	female	ERR12263811
WS05c	WS05	Wet Symptomatic	clone	single-genotype	17/10/2016	54	female	ERR12263828
WS06c	WS06	Wet Symptomatic	clone	single-genotype	17/10/2016	22	female	ERR12263800
WS07c	WS07	Wet Symptomatic	clone	single-genotype	01/11/2016	10	male	ERR12263814
WS08b	WS08	Wet Symptomatic	bulk	single-genotype	07/11/2016	41	male	ERR12263804
WS09b	WS09	Wet Symptomatic	bulk	single-genotype	07/11/2016	49	female	ERR12263834
WS10b	WS10	Wet Symptomatic	bulk	poly-genotypes	08/11/2016	16	female	ERR12263838
WS11b	WS11	Wet Symptomatic	bulk	single-genotype	08/11/2016	8	male	ERR12263824
WS12b	WS12	Wet Symptomatic	bulk	poly-genotypes	08/11/2016	28	male	ERR12263831
WS12c	WS12	Wet Symptomatic	clone	single-genotype	08/11/2016	28	male	ERR12263822
WS13b	WS13	Wet Symptomatic	bulk	poly-genotypes	18/11/2016	64	female	ERR12263836
WS15b	WS15	Wet Symptomatic	bulk	poly-genotypes	18/11/2016	37	female	ERR12263835

Table 2: Summary statistics for 43 *P. falciparum* genome assemblies.

Genome_ID	Genome length	Total contigs	Telomere to telomere contigs	N50	L50	Total genes	*var*	LARSFADIG motifs	*var2csa*	*rif*	*stevor*
DC01m1c	23.4	20	3	1622224	5	5864	70	61	2	107	24
DC02m1c	23.7	28	5	1693155	5	6056	70	64	1	178	34
DC03m1c	23.6	24	7	1627210	5	6081	68	64	1	156	34
DC04m2c	23.4	24	4	1636287	5	5781	66	62	2	118	25
DC07m1b	25.07	85	6	1433249	7	6222	73	70	4	172	35
DC07m1c	26.7	187	5	1507801	6	7229	60	58	2	154	34
DC08m3b	36.34	113	4	1198427	12	8298	116	115	4	230	43
DC08m3c	24.28	64	5	1631162	5	6157	55	55	2	134	32

DC08m5b	27.88	62	5	1421197	8	6427	105	103	4	234	47
DC08m5c1	24.18	52	6	1606191	5	6085	73	71	2	133	22
DC08m5c2	25	98	4	1630462	5	6324	62	62	2	150	32
DC09m5b	27.99	164	3	1405453	9	6714	116	114	2	236	46
DC09m5c2	24.22	121	2	1661132	5	6669	84	79	1	130	28
DC09m5c1	25.61	58	6	1543257	6	6046	67	66	1	161	33
DC10m1c	23.5	23	7	1556504	5	5725	70	68	1	134	26
DC10m2c	23.7	20	9	1571854	5	5781	71	69	2	154	29
DC10m3c	23.3	27	5	1672417	5	5684	52	63	2	137	23
WA01b	25.85	127	3	1423884	7	6566	65	64	2	116	25
WA01c	23.67	25	2	1637256	5	5756	69	67	2	120	25
WA03b	24.26	49	3	1519022	5	5689	72	69	3	164	37
WA03c	23.79	46	4	1693536	5	5812	70	69	6	154	36
WA04b	24.92	81	2	1446723	7	5656	75	73	4	165	35
WA04c	23.98	44	2	1613267	5	5852	83	76	4	170	37
WA10b	24.19	36	4	1487219	6	5680	82	80	5	162	35
WA10c	26.6	182	2	1468285	6	6799	79	77	5	161	33
WA11c	23.65	24	4	1621859	5	5807	73	70	2	105	25
WA47b	29.85	328	3	1408774	8	8042	91	86	9	177	39
WA47c	23.97	48	2	1693146	5	5681	71	66	4	160	36
WS01b	26.09	104	4	1517931	6	6024	124	119	1	193	39
WS03b	24.11	38	5	1658341	5	5954	79	76	4	132	33
WS04b	30.49	144	7	1440087	8	7081	131	130	5	293	54
WS05b	25.02	56	3	1435358	6	6002	72	70	1	166	45
WS05c	23.81	42	3	1641247	5	5853	68	66	1	153	42
WS06c	24.27	65	2	1560362	5	6050	64	62	4	149	34
WS07c	24.34	73	2	1612653	5	6117	69	66	2	158	37
WS08b	24.05	51	5	1638922	5	5944	67	66	2	116	25
WS09b	24.45	54	2	1653278	5	6160	65	64	1	163	35
WS10b	32.57	123	5	1386579	7	9291	87	85	1	165	39
WS11b	25.63	108	2	1479817	6	6759	69	66	1	152	33

WS12b	33.39	113	6	1356076	10	7836	130	130	2	274	51
WS12c	24.22	45	2	1649284	5	5959	75	75	1	152	27
WS13b	24.09	40	5	1564570	5	5926	75	75	4	174	23
WS15b	24.3	41	5	1592718	5	6051	74	74	4	173	23