

1 Antigenic diversity in malaria parasites is maintained on
2 extrachromosomal DNA

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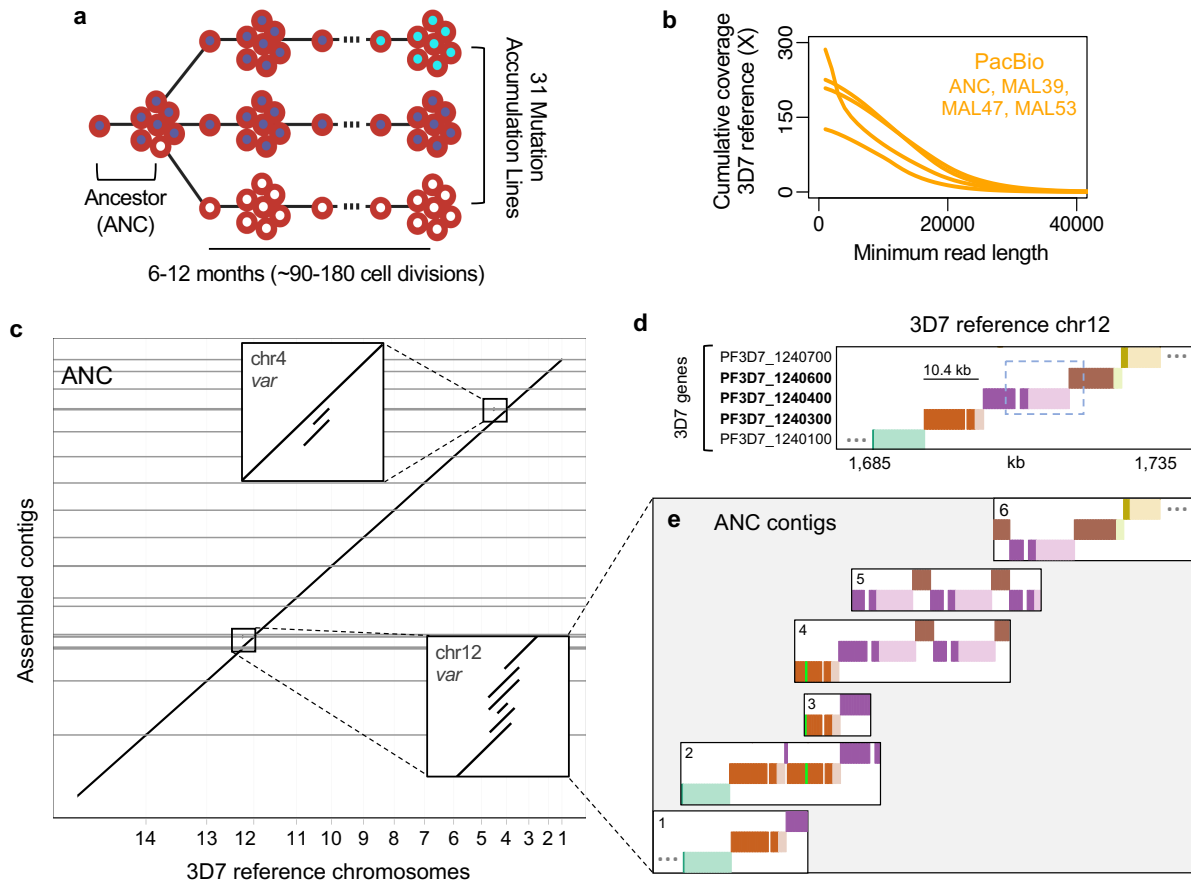
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19 **Abstract**

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21 Sequence variation among antigenic *var* genes enables *Plasmodium falciparum* malaria
22 parasites to evade host immunity. Using long sequence reads from haploid clones from
23 a mutation accumulation experiment, we detect *var* diversity inconsistent with simple
24 chromosomal inheritance. We discover putatively circular DNA that is strongly enriched
25 for *var* genes, which exist in multiple alleles per locus separated by recombination and
26 indel events. Extrachromosomal DNA likely contributes to rapid antigenic diversification
27 in *P. falciparum*.

28 Malaria caused by the parasite *Plasmodium falciparum* is a leading cause of
29 death and disease in tropical regions of the world¹. Adaptive immunity to malaria is
30 limited, even after repeated infection, by extensive variation in *P. falciparum* antigenic
31 gene families²⁻⁴. In particular, *var* genes encode PfEMP1 proteins that are exported to
32 the surface of infected red blood cells, where they mediate pathogenic cytoadherence to
33 host endothelial receptors and elicit variant-specific immunity². Each parasite genome
34 contains ~60 *var* genes distributed among 26 subtelomeric and 9 internal loci. *Var*
35 genes are named after variation in their antigenic properties⁵, driven by extreme amino
36 acid divergence⁶ relative to the rest of the genome. For example, pairs of parasites
37 sampled from the same population share almost no *var* genes with ≥96% sequence
38 identity⁷. However, *var* genes from unrelated parasites share small blocks of
39 homology^{6,8} consistent with a history of recombination or gene conversion among
40 alleles. Recent studies have reported frequent *var* recombination during asexual, mitotic
41 reproduction, which may create millions of new alleles during blood-stage infection⁹.

42 Our current understanding of *var* diversification relies primarily on short-read
43 sequencing that may yield only a partial picture of *var* genetic diversity. The primary
44 hurdle is structural variation among *var* genes^{6,10}, such as copy number variation, which
45 makes it difficult to accurately align short reads to reference genomes. To achieve a
46 more complete understanding of *var* diversity and mutational mechanisms, we
47 generated long sequence reads from a mutation accumulation (MA) experiment in *P.*
48 *falciparum*¹¹ (**Fig 1A**). Specifically, 31 MA lines (MAL) were independently cloned from
49 an isogenic population of the 3D7 reference strain (the ‘Ancestor’) and propagated for 6-
50 12 months (~90-180 cell divisions). Each MAL was re-cloned to a single cell every 21±4
51 days (~10 cell divisions) to minimize selection and allow fixation of *de novo* mutations.
52 Previous Illumina analysis of the core genome of 31 MAL identified an average of 0.55
53 SNP and 3.42 small indel mutations per MAL over the course of the MA experiment¹¹.

54 To detect large structural mutations at *var* loci, we initially used long PacBio
55 reads (>16kb, **Fig 1B**) to build *de novo* genome assemblies for the Ancestor (ANC) and
56 three MAL (MAL39, MAL47, MAL53). Each of these high-quality assemblies contained
57 few gaps (0-4) and covered ≥99.5% of reference bases with ≥94.4% identity (ANC, **Fig**
58 **1C**; others, **EDF 1A**; **Supplementary Table 1**). Nonetheless, we observed that 27
59 genomic regions were represented by multiple contigs in at least one assembly (**Fig 1C**;
60 **EDF 1AB**), which could represent structural variation. These incompletely-resolved
61 regions were highly enriched for *var* genes, which comprise 9% of the genome but 69%
62 of unresolved regions ($p < 0.0001$, $\chi^2 = 228.5$). To examine the structural layout of *var*
63 genes on each assembled contig, we developed a Shiny app that draws BLAST
64 homology between contigs and reference genes. As an example, consider an internal
65 *var* locus on chr12, which was identified on multiple contigs in 3 of 4 assemblies (**Fig**
66 **1C**, **EDF 1A**). When we applied our app to this locus in the reference genome, it drew a
67 diagonal line of five sequential genes separated by color and vertical position (**Fig 1D**).



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70 **Figure 1. Unexpected clonal polymorphism in PacBio assemblies.** **a**, Mutation
71 accumulation experiment with repeated cloning. **b**, PacBio read lengths. **c**, Genome-wide dot
72 plot comparing ANC assembly with 3D7 reference. Insets are internal *var* loci with >1 contig. **d**,
73 Visualization of homology between reference genes (dark colors) and intergenic regions (light
74 colors) on 3D7 chr12 (top) and ANC contigs (bottom). *Var* gene names are bolded. The dashed
75 box outlines a region duplicated on contigs 4-6. The lime green band represents 241 bp from
76 PF3D7_0700100. Ellipses indicate continued sequence (other genes not shown).

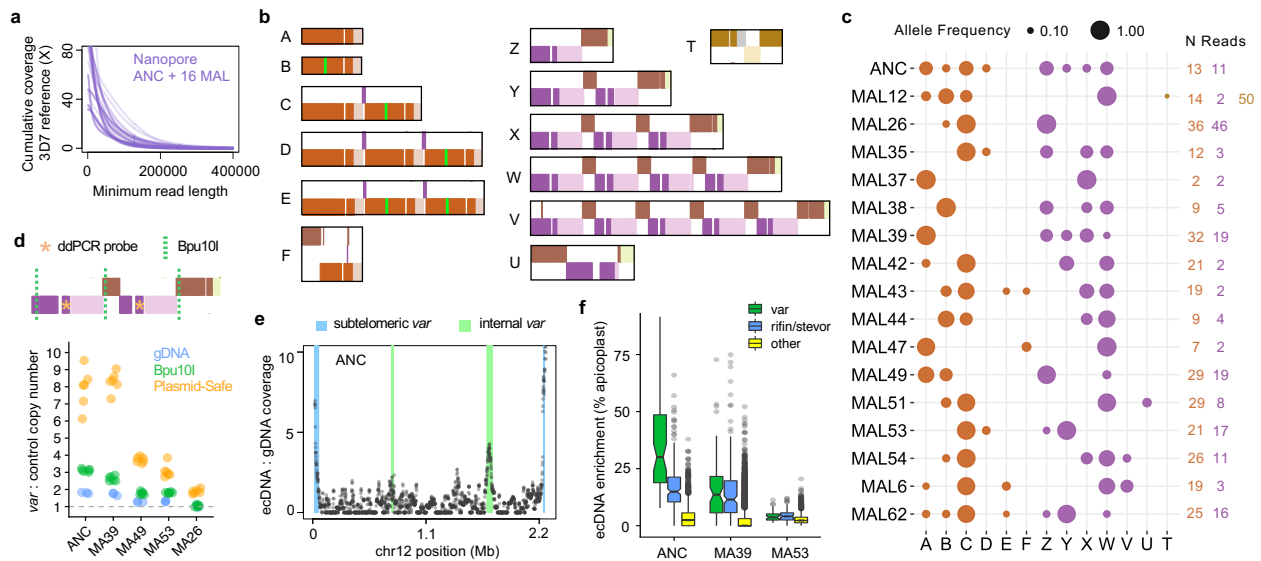
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79 In contrast, when we applied the app to ANC contigs with homology to this locus, it
80 displayed deviations from the reference diagonal that indicate structural variation (**Fig**
81 **1DE**). For example, ANC contig 1 matched the reference while contig 2 contained a
82 second copy of *var* PF3D7_1240300 (**Fig 1DE**, orange). This second copy, which was
83 also partially present on contigs 3 and 4, was differentiated from the first copy by two
84 sequence tracts of a few hundred base pairs: one homologous to an adjacent *var* (**Fig**
85 **1DE**, purple) and one homologous to a subtelomeric *var* on another chromosome (**Fig**
86 **1DE**, lime green). On contigs 4-6, tandem duplications appeared to produce novel,
87 chimeric *var* genes that were in-frame for translation and retained the upstream
88 regulatory sequence of one parent (**Fig 1D**, dashed box; **Fig 1E**, purple/brown). These

89 tandem duplication and recombination events are consistent with known mechanisms of
90 *var* mutation⁹, and we successfully confirmed specific breakpoints using PCR (**EDF 2A**).
91 We also observed similar patterns of structural variation between contigs mapping to
92 two internal *var* loci on chr4 (**EDF 1C-F**). Apparent polymorphism between contigs (1/2,
93 **Fig 1E**; 7/8, **EDF 1D**; 10/11, **EDF 1F**) was unexpected in haploid clones and could
94 reflect assembly errors or *var* polymorphism, including paralog divergence after *var*
95 locus duplication.

96 To achieve greater resolution of *var* genetic diversity and extend the analysis to
97 additional clones, we generated ultra-long Oxford Nanopore reads from ANC and 16
98 MAL (**Fig 2A**; mean coverage 11X in reads >100kb). Using the Shiny app, we examined
99 gene structure on individual Nanopore reads mapping to each *var* locus. At the chr12
100 locus, we observed extensive variation across reads in the copy numbers of *var*
101 PF3D7_1240300 and the novel *var* chimera (**Fig 2B**, **Fig 1DE**, orange and
102 purple/brown). Most Nanopore reads were not long enough to span both sets of
103 duplications at this locus while being anchored in unique sequence on either end. We
104 therefore divided the locus into two regions for genotyping: one containing the orange
105 *var*, anchored by the adjacent teal and purple genes; and one containing the purple and
106 brown *var*, anchored by the adjacent orange and dark yellow genes (see **Fig 1D** for
107 reference gene structure). Using only reads anchored by this definition, we identified 6
108 distinct orange alleles and 7 purple/brown alleles across ANC and the 16 MAL (**Fig 2B**,
109 A-F, Z-T). Variation across Nanopore alleles reflected a history of indel and
110 recombination events that altered *var* structure and copy number, consistent with the
111 PacBio contigs (**Fig 1E**). Individual reads additionally revealed rarer variation, such as
112 short tracts of recombination between adjacent *var* genes (**Fig 2B** allele U). We
113 observed similarly high levels of structural variation across reads from three other
114 internal *var* loci on chr7 and chr4 (**EDF 3**).

115 The distribution of chr12 alleles across MAL (**Fig 2C**) was inconsistent with
116 inheritance of a single chromosome from ANC, instead suggesting polyploidy at this
117 locus. Most MAL contained multiple alleles, many of which were shared across MAL
118 and present in ANC. Overall, each MAL appeared to inherit a unique subset of the many
119 alleles present in ANC. Some alleles detected at low frequency in MAL were not
120 observed in ANC reads. Nonetheless, allele F was detectable by PCR in ANC and other
121 lines (**EDF 2B**), suggesting that alleles unobserved by Nanopore may still occur at low
122 frequency. Furthermore, allele frequencies from the two parts of the locus were
123 uncorrelated across MAL (mean pairwise $R^2_{adj}=-0.01$, linear models; **Fig 2C**),
124 demonstrating that the two regions are genetically unlinked. These observations are
125 inconsistent with each MAL inheriting one DNA molecule containing the chr12 locus, no
126 matter the total number of *var* duplications. Instead, they are consistent with effective
127 polyploidy at this *var* locus (**Fig 2BC**), which is recapitulated at three more internal *var*
128 loci on chr4 and chr7 (**EDF3**).



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Figure 2. Structural variation at *var* loci maintained on extrachromosomal, circular DNA (ecDNA). **a**, Nanopore read lengths. **b**, Alleles from single reads mapping to the second internal *var* locus on chr12. **c**, Allele frequencies in independent clones, scored from reads spanning local duplications. **d**, Elevated copy number of PF3D7_1240400. Bpu10I is a control for DNA fragmentation. **e**, Nanopore enrichment of ecDNA over genomic DNA on chr12. **f**, Nanopore enrichment of ecDNA genome-wide.

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In light of these patterns, we hypothesized that common *var* polymorphisms were not generated *de novo* during the MA experiment, but instead maintained on extrachromosomal DNA (ecDNA) inherited from ANC with stochastic loss (**Fig 2C**). ecDNA is ubiquitous in eukaryotes¹² and has been reported in one *P. falciparum* study to date, where it was implicated in selective amplification of a drug-resistance gene¹³. We performed molecular experiments to quantify *var* polymorphism and test the ecDNA hypothesis, again focusing on the most polymorphic internal locus on chr12. First, we used Southern blot to confirm that DNA fragments containing one or two copies of *var* PF3D7_1240300 (**Fig 2B**, orange) were simultaneously present in clonal MAL (**EDF 2C**). Next, using droplet digital PCR (ddPCR), we found that DNA molecules containing the *var* gene Pf3D7_1240400 (**Fig 2B**, purple) were up to 1.8X more abundant than a control gene on the same chromosome (**Fig 2D**; all $p \leq 0.003$ except MA26 $p = 0.93$, t-tests). The copy number of DNA fragments containing this *var* gene within each MAL was strongly correlated with the number of alleles detected with Nanopore ($R^2 = 0.987$, $p = 0.0004$, linear model). Finally, we treated DNA with Plasmid-Safe exonuclease, which efficiently degrades linear but not circular DNA. Plasmid-Safe treatment prior to ddPCR elevated the copy number of Pf3D7_1240400 relative to the control gene in all tested samples (**Fig 2D**, all $p < 9.3 \times 10^{-5}$, t-tests; **Supplementary Table 2**), suggesting that extrachromosomal alleles of this *var* locus are maintained on circular DNA.

158 To obtain a genome-wide estimate of ecDNA, we performed Nanopore
159 sequencing of ANC DNA digested with Plasmid-Safe and aligned the reads to the 3D7
160 reference genome. After normalization to genomic DNA coverage, ecDNA coverage
161 displayed clear intra-chromosomal peaks at the four internal loci with many common
162 alleles (**Fig 2E**, chr12; **EDF 4**, others). Genome-wide, *var* genes were strongly enriched
163 for ecDNA despite variation in total ecDNA levels across clones (**Fig 2F**; all $p < 2.2 \times 10^{-16}$,
164 KS-tests). Besides *var*, the largest gene categories enriched for ecDNA were rifin,
165 STEVOR, and “conserved *Plasmodium* protein unknown function.”

166 All *P. falciparum* telomeres and subtelomeres were also highly enriched for
167 ecDNA, except on chr14, which contains no *var* (**Fig 2E**; **EDF 4**). Visual inspection of
168 Nanopore reads and PacBio contigs from subtelomeric *var* loci failed to identify any
169 common polymorphisms shared across lines. Nonetheless, we detected 17 instances of
170 subtelomeric recombination private to individual lines, including 13 found only on single
171 reads (**Supplementary Table 3**). In 7 of these events, sequence from one telomere
172 was copied onto another, creating a chimeric *var* in the recipient locus without altering
173 the donor. Two similar events, along with a duplication of four rifins on chr10, were fixed
174 or nearly fixed in ANC and all MAL and likely occurred prior to the MA experiment.
175 Within individual MAL, we observed six additional instances of telomere replacement
176 that did not interrupt coding genes, including a fixed, reciprocal exchange of non-coding
177 DNA between the second telomeres of chr2 and chr3 in MAL42. Four single reads
178 displayed smaller recombination events, in which 3-7.5 kb of a *var* gene from one
179 subtelomere was overwritten with sequence from another. Together, these rare
180 polymorphisms are consistent with recombination events that occurred during MAL
181 expansion for DNA extraction. We used Luria-Delbruck fluctuation analysis to estimate
182 a *de novo* subtelomeric recombination rate of 6.67×10^{-4} per genome per cell division,
183 which is 3.8-fold lower than a previous estimate from Illumina reads⁹.

184 *P. falciparum* *var* loci are known to be enriched for sequence motifs predicted to
185 form G4-quadruplex secondary structures¹⁴, which are associated with *var*
186 recombination¹⁵ and replication stalling¹⁶ that might potentiate ecDNA formation. We
187 noticed that three hypervariable internal *var* loci on chr12, chr7, and chr4 share at least
188 one conserved copy ($\geq 85\%$ identity) of a 7-kb sequence containing six predicted
189 motifs¹⁷ for G4-quadruplexes. In PlasmidSafe-treated DNA, we also observed many
190 Nanopore reads with central breakpoints for large, inverted duplications (**EDF 5**), which
191 have been implicated in ecDNA formation^{18,19}. As expected from Nanopore sequencing
192 of true inverted duplications²⁰, the inverse, repeated sequence on the second half of
193 these reads was degraded in sequence quality (**EDF 5C**). Although many questions
194 remain regarding the full mechanism of ecDNA formation in *P. falciparum*, these
195 observations provide intriguing hints about the roles of repetitive DNA and secondary
196 structure.

197 ecDNA provides an intuitive mechanism for the maintenance of *var* diversity
198 through population bottlenecks, such as mosquito bites that transmit 1-25 *P. falciparum*
199 cells to humans²¹. ecDNA is also consistent with previous observations of gene
200 exchange among *P. falciparum* cells via exosomal vesicles²². Nonetheless, since
201 parasite populations express only one *var* gene at a time²³, most *var* alleles on ecDNA
202 are unlikely to be immediately functional. Instead, we propose that the production and
203 maintenance of ecDNA enables rapid diversification of *var* gene sequence. This
204 function is thought to be under strong selection in response to the host immune system
205 and relevant to vaccine efficacy^{2-4,24}. Future clinical work could assess whether *var*
206 diversification through ecDNA impacts parasite evasion of host immune responses and
207 malaria severity. More broadly, these findings add to growing evidence from yeast and
208 cancers implicating ecDNA as a mechanism of rapid adaptation^{12,25}.

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283 Methods

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285 **Parasite culture and DNA extraction**

286 *P. falciparum* mutation accumulation lines (MAL) were generated as previously
287 described¹¹. Briefly, 31 independent MAL were cloned from an isogenic population of
288 the 3D7 reference strain and propagated in red cell culture. Clonal dilution of MAL was
289 performed every 10.5 parasite cycles to reach a theoretical concentration of 0.25
290 parasites per well. MAL were cryopreserved after 114-267 days of culture, including 11-
291 25 single-cell bottlenecks.

292 To generate DNA for PacBio sequencing, cryopreserved aliquots of MAL were
293 cultured in 182 cm² flasks at 4% hematocrit. When parasites reached 10% parasitemia,
294 red blood cell pellets were lysed with saponin at a final concentration of 0.01%. Parasite
295 pellets were washed with 1X PBS and used as input for the Genomic-tip DNA extraction
296 kit (Qiagen). DNA was further cleaned with the PowerClean Pro kit (MoBio) and
297 concentrated by ethanol precipitation.

298 To generate high-molecular-weight (HMW) DNA for Nanopore sequencing,
299 cryopreserved aliquots of MAL were cultured in 10 mL and 40 mL plates at 2%
300 hematocrit, as previously described²⁶. When 80-120 mL of culture reached 4-10%
301 parasitemia at schizont stage, red blood cell pellets were lysed with saponin at a final
302 concentration of 0.014%. Parasite pellets were washed in PBS and suspended in 379
303 μ L extraction buffer (0.1M Tris-HCl pH 8.0, 0.1M NaCl, 20 mM EDTA) with 10 μ L of 20
304 mg/mL Proteinase K (Thermo Fisher Scientific), 10 μ L SDS (10% w/v), and 2 μ L of 10
305 mg/mL RNase A (Millipore Sigma). Tubes were incubated at 55°C for 2-4 hr and gently
306 inverted every 30–60 min to minimize DNA shearing, as previously described²⁷. DNA
307 was purified from the lysates with an equal volume of 25:24:1 v/v phenol chloroform
308 isoamyl alcohol (Thermo Fisher Scientific) in a 2 mL light phase lock gel tube
309 (Quantabio), followed by an equal volume of chloroform (Millipore Sigma). HMW DNA in
310 the aqueous layer was poured into a fresh tube and precipitated by adding 0.1 volume
311 3M sodium acetate and 2-2.5 volumes of cold 100% ethanol. A wide-bore tip was used
312 to transfer visible strings of DNA to a low-retention tube, where it was washed with 70%
313 ethanol and partially air-dried. HMW DNA was resuspended by adding 100-150 μ L of 10
314 mM Tris and incubating at 55°C for up to one hour. To achieve homogenous
315 concentration in the viscous and highly concentrated sample of HMW DNA, samples
316 were gently sheared 1-5 times with a 26G blunt-end needle and incubated for at least 2
317 weeks at 4°C before proceeding to library preparation. DNA was quantified with Qubit
318 (ThermoFisher) using the dsDNA BR kit.

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320 **PacBio sequencing and genome assembly**

321 SMRTbell libraries were prepared by the Genomics Core at Washington State
322 University and sequenced on a PacBio RS II sequencer. Genomes were assembled *de*

323 *novo* with HGAP3²⁸ using the longest 25X of reads (equivalent to a minimum read
324 length of 16.5-24.7 kb), with additional reads used for polishing. Lowercase (low-quality)
325 bases were removed from the ends of assembled contigs, and contigs containing ≥ 1000
326 basepairs with $\geq 80\%$ identity to the human genome (GRCh37) were identified with
327 BLAST and removed. Remaining contigs were aligned to the *P. falciparum* reference
328 genome (3D7) using minimap2²⁹. Dot plots were generated using dotPlotly³⁰.

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330 **Nanopore sequencing**

331 Nanopore libraries were prepared from 3-5 μg gDNA with the ONT Ligation
332 Sequencing Kit (SQK-LSK109), with the following modifications to the official protocol to
333 optimize recovery of ultra-long (>100 kb) reads. After the end-prep/repair step, size
334 selection was performed with Short Read Eliminator (SRE) buffer (Circulomics) instead
335 of magnetic beads. After adapter ligation, DNA was isolated by centrifuging the sample
336 at $10,000 \times g$ for 30 minutes without the addition of any reagents. This DNA pellet was
337 washed twice with 100 μL SFB or LFB from the ligation sequencing kit and resuspended
338 into 30 μL 10mM Tris pH 8.0. After library preparation, the SRE buffer was used for a
339 final round of size selection, with two washes of SFB or LFB instead of ethanol.
340 Approximately 350 ng of prepared library was loaded onto the Nanopore flow cell for
341 each sequencing run. To improve throughput, flow cells were flushed every 8-16 hours
342 with the ONT Flow Cell Wash Kit (EXP-WSH003) and reloaded with fresh library. Raw
343 Nanopore data were basecalled with Guppy v3.2.4, using the high-accuracy caller
344 (option: -c dna_r0.4.1_450bps_hac.cfg)

345 To generate Nanopore reads from ecDNA, 15-20 μg of genomic DNA (gDNA)
346 was digested with ~ 95 U Plasmid-Safe ATP-Dependent DNase (Lucigen), 25 mM ATP
347 solution, and Plasmid-Safe 10X buffer for 16 hours at 37°C. The remaining DNA was
348 purified via ammonium acetate precipitation. This treatment eliminated 95-99% of the
349 input DNA, as measured by Qubit (ThermoFisher) and confirmed with gel
350 electrophoresis. Putative ecDNA was gently sheared 10 \times with a 26G blunt-end needle,
351 and library preparation and sequencing were performed with the standard LSK109 kit
352 protocol (Oxford Nanopore). Library preparation inputs (25-100 ng) and yields (10-25
353 ng) were far below Nanopore minimum recommendations due to small quantities of
354 DNA remaining after Plasmid-Safe digestion.

355

356 **Visual genotyping of *var* loci**

357 Thirty-five *var* loci were defined as 10-110 kb regions of the 3D7 reference
358 genome containing at least one 1 *var* gene or pseudogene and a few surrounding
359 genes. 3D7 sequences at these loci were divided into 500-bp segments and compared
360 with BLAST against PacBio contigs longer than 5 kb and Nanopore reads longer than
361 30 kb. To visualize the BLAST output, each contig and read was assigned to at most
362 one *var* locus using minimap2 alignment to 3D7. For each locus, a custom Shiny app

363 (available at <https://github.com/emily-ebel/varSV>) was used to visualize the orientation
364 of the 500-bp reference segments on each individual contig or read. In this visualization,
365 wild-type (3D7) reads or contigs appear as a continuous diagonal line of blocks, similar
366 to a dot plot. Duplications are visible as blocks that deviate from the diagonal line, while
367 deletions appear as truncated or missing genes. Recombination and translocation
368 events are detectable by gaps or truncations in the series of blocks on the diagonal line
369 (due to lack of homology between the read and assigned locus). For putative
370 recombination events >2 kb, we used BLAST and minimap2 to search for donor
371 sequence from elsewhere in the 3D7 reference genome. We did not investigate
372 common, smaller runs of missing segments (<2 kb) after determining that the vast
373 majority were explained by very simple repeats or low-quality sequence. Reads were
374 generally considered wild-type if they contained at least two genes from the assigned
375 locus without structural variation >2 kb. When the same structural variant was detected
376 on >1 read in a sample, we limited the definition of wild-type to reads that were long
377 enough to span the entire variant. Large inverted duplications ('triangle reads') were not
378 included in visual allele counts but were quantified from PAF alignments using a custom
379 R script.

380

381 **Mutation rate calculation**

382 Mutation rate was estimated using Luria-Delbruck fluctuation analysis via
383 maximum likelihood on the FALCOR web server³¹. This approach assumes exponential
384 growth from a single cell to the large population used for DNA extraction, analogous to a
385 plating experiment on selective media. Rates were calculated for all *var* loci excluding
386 the four hypervariable internal loci on chr12, chr4, and chr7. Each MAL was treated as
387 an independent replicate. The total number of genotyped Nanopore reads was
388 considered the number of viable cells. Reads with the least abundant genotype were
389 considered mutants; i.e., each MAL was assumed to have inherited its most common
390 allele from ANC, which then mutated to produce additional alleles. Deletions on single
391 reads were excluded from the calculation, since they are the most common form of
392 Nanopore sequencing error³². Fixed variants within MAL were also excluded, since
393 they are unlikely to have occurred during the final population expansion.

394

395 **Coverage analysis**

396 Nanopore reads ≥ 30 kb from untreated gDNA and ≥ 10 kb from Plasmid-Safe-
397 treated ecDNA were aligned to the 3D7 reference genome using minimap2. Alignments
398 with MAPQ<10, including multiply mapped reads with MAPQ=0, were removed using
399 samtools³³ view -q 10. Coverage was calculated in 1-kb windows using
400 bedtoolscoverage and normalized to 1 via division by the genome-wide mean,
401 excluding the circular apicoplast. For analysis by gene category, coverage was

402 calculated in windows corresponding to Ensembl gene annotations (ASM276v2.54) and
403 normalized by the ecDNA:gDNA ratio for the apicoplast, which ranged from 12.0-35.9.

404

405 **PCR and Southern Blot**

406 PCR primers were designed to amplify over novel junctions and corresponding
407 reference junctions in the second internal *var* locus on chr12 (**EDF 2A**; **EDT 1**). PCR
408 was performed using Phusion High-Fidelity DNA Polymerase (NEB) according to
409 manufacturer instructions. PCR products were visualized on 1% agarose gels with a 1
410 kb plus ladder (NEB).

411 For a Southern Blot probe, primers were designed to amplify a 522-bp segment
412 of PF3D7_1240300 (**EDF 2C**; **EDT 1**). Amplification was performed on MA53 DNA
413 using Phusion High-Fidelity DNA Polymerase (NEB). The PCR product was purified
414 using the QIAquick PCR Purification Kit (Qiagen) and labeled using the North2South
415 Biotin Random Prime Labeling Kit (ThermoFisher), with the addition of 1.5 uL Glycoblue
416 Precipitant (ThermoFisher). Restriction digests of *P. falciparum* DNA were performed
417 using 10 U SacI-HF (NEB), 5 U StuI (NEB), CutSmart buffer (NEB), and ~1 ug DNA for
418 16 hours at 37°C. Digested DNA was run on a gel containing 0.4% UltraPure agarose
419 (Invitrogen) for 7 hours with a current of 3V/cm. TAE buffer was replaced every 2 hours
420 to keep bands crisp. GeneRuler High Range DNA Ladder (ThermoScientific) was used
421 to quantify DNA migration. Gel processing and blotting was performed with the
422 Amersham ECL Direct Nucleic Acid Labeling and Detection Systems Kit (Cytiva) and
423 Amersham Hybond-N+ Nylon Membrane (Cytiva). Probe hybridization was performed
424 using the North2South Chemiluminescent Hybridization and Detection Kit
425 (ThermoScientific). The final blot was visualized with Odessey-XF Imaging System (Li-
426 Cor).

427

428 **ddPCR**

429 ddPCR was performed using the QX200 Droplet Digital PCR system (Bio-Rad)
430 and ddPCR Supermix for probes (Bio-Rad). Briefly, primers were designed (**EDT 1**) to
431 amplify a 120-bp fragment of the *var* gene PF3D7_1240400 and a 130-bp fragment of
432 the control gene PF3D7_1212500, which encodes glycerol-3-phosphate 1-O-
433 acyltransferase. Oligonucleotide probes for these amplicons (**EDT 1**) were labeled with
434 HEX/ZEN/IBFQ and FAM/ZEN/IBFG reporter fluorophores (IDT), respectively. For
435 Plasmid-Safe treatments, 400 ng of DNA was incubated with 5 U Plasmid-Safe ATP-
436 Dependent DNase (Lucigen), 25 mM ATP solution, and Plasmid-Safe buffer for 16
437 hours at 37°C. For restriction enzyme treatments, 200 ng of DNA was incubated with
438 7.5 U Bpu10I (NEB) in r3.1 buffer (ThermoFisher) for 4 hours at 37°C. ddPCR was
439 performed in five replicates per sample on DNA diluted to 0.05 ng per well. The copy
440 number of *var* and control fragments in each well was calculated using QuantaSoft
441 software (Bio-Rad).

442 Methods References

443

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463

464 Legends for Extended Data Figures and Supplementary Tables

465

466 **Extended Data Fig. 1: Structural variation among assembled PacBio contigs. a,**
467 Genome-wide dot plots. Insets are internal *var* with >1 contig. **b,** Summary of loci with
468 >1 contig across assemblies. **c,** Visualization of indel polymorphism across contigs at
469 two internal *var* loci on chr4.

470

471 **Extended Data Fig. 2: Molecular confirmation of long-read polymorphism. a,** PCR
472 of breakpoints observed in contigs that map to the second internal *var* locus on chr12.
473 Colored bands represent amplicons. Asterisks indicate the amplicons expected in each
474 sample, based on assembled PacBio contigs. In the left diagram, the pink amplicon is
475 expected to be 562 bp in the reference allele (e.g. allele A) and 667 bp with the gene
476 conversion from PF3D7_0700100 (e.g. allele B). These data confirm the existence of
477 breakpoints detected with PacBio in ANC and MA53 but undetected in MA39 and
478 MA47. **b,** PCR detection of allele F. Asterisks indicate the amplicons expected in each
479 sample, based on Nanopore reads. **c,** Southern blot of copy number variation in
480 PF3D7_1240300. Teal asterisks mark the bands expected in each sample, based on
481 Nanopore reads.

482

483 **Extended Data Fig. 3: Structural polymorphism across Nanopore reads mapping**
484 **to internal *var* loci from all clonal MAL.**

485

486 **Extended Data Fig. 4: Genome-wide coverage of extrachromosomal, circular DNA**
487 **relative to genomic DNA.**

488

489 **Extended Data Fig. 5: Large inverted duplications on ecDNA reads. a,** Example
490 read from MA54 containing a large inverted duplication. **b,** Plasmid-Safe-treated DNA is
491 strongly enriched for “triangle reads”. **c,** Signal degradation consistent with single-strand
492 annealing after passing through Nanopore.

493

494 **Supplementary Table 1. Summary of PacBio assemblies including PAF**
495 **alignments to 3D7.**

496

497 **Supplementary Table 2. ddPCR count data from QuantaSoft.**

498

499 **Supplementary Table 3. Structural variation on Nanopore reads assigned to non-**
500 **hypervariable *var* loci.** The three events fixed in ANC and all MAL are considered wild-
501 type.

502

503 **Extended Data Table 1. Probe and primer sequences.** Colors refer to diagrams in
 504 EDF 2.

Experiment	Item	Forward primer/probe	Reverse Primer
PCR	Pink amplicon	TCAACCCAGACGACAACATC	AAAGTGCCTCGGTTGAGAC
PCR	Black amplicon	CAGATCCATGCAGACTTGTAGAGGATTA	GTATAGGCGCAACAGTTCCCAC
PCR	Red amplicon	CATCCGTGCGGAATAGGAAA	CTCACACAGGCATGTAACCA
PCR	Tan amplicon	GAAGAACTCTCCACAGAC	AGAGTGGTGACAAAGATATGT
PCR	Blue amplicon	ACCAAGTCATACCACAAGTGAA	GGTAACAAAGAACCTAGTGACGA
PCR	Gray amplicon	AAACTACGGTTGGAGGTGTG	AAGAGGAAACACAAGGACAGG
PCR	Yellow amplicon	AGATGACGACAACGAAGAAGAG	TGGCTTCAGCACCACCTT
Southern Blot	<i>var</i> probe	TGCCACGTTGTGAGTGGTAA	ATCAAGGCCCCCTTCAGGTA
ddPCR	<i>var</i> amplicon	CGCTTGGAAAGTCAGGAAA	GTGGTGGTACAGTCGTTG
ddPCR	control amplicon	CGGCTCTTCGCATAGATT	GTGCCCTTGTATGGATCTG
ddPCR	<i>var</i> probe	AAATTGGTGAGTGCAACCGCTTCC	-
ddPCR	control probe	TGCTATCAATACACACGCATCAATAAACT	-

505

506 **Data Availability Statement**

507 Read data and genome assemblies generated by this study are deposited at NCBI SRA
508 and GenBank under NCBI BioProject: PRJNA894225.

509

510 **Code Availability Statement**

511 Code used to identify structural variants is available at [https://github.com/emily-](https://github.com/emily-ebel/varSV)
512 [ebel/varSV](https://github.com/emily-ebel/varSV).

513

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527

528 **Author contributions**

529 ERE, DAP, and TJCA conceptualized the study. MMW, ERE, and ESE cultured the
530 parasites. ERE and MMW isolated DNA. BYK performed Nanopore sequencing. ERE
531 performed the other experiments. ERE and BYK analyzed sequencing data. ESE,
532 TJCA, and DAP contributed resources and supervision. ERE and DAP wrote the
533 manuscript with input from all authors.

534

535 **Competing interest declaration**

536 The authors declare no competing interests.