

Deep sequencing of *Plasmodium falciparum* genetic crosses: a resource for the study of genome variation and meiotic recombination

Supplementary information

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1. Whole genome sequencing

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Note that typically in high throughput sequencing studies of humans or other higher eukaryotes multiple sequencing runs will be obtained for each sample, then data from each run (lane) are combined to increase coverage. However in this study a single sequencing run was sufficient to obtain ~100X coverage of the *P. falciparum* genome, so only a single sequencing run was obtained for each sample. Samples that represented biological replicates (DNA derived from the same clone but obtained from different cultures) were treated separately, with separate DNA library preparation and sequencing runs. Thus in this study there is always a one-to-one mapping from sample (biological replicate) to sequence run.

For convenience throughout this document we use a three-part identifier for each sample, e.g., “3D7/PG0051-C/ERR019061”, where the first part identifies the clone (e.g., “3D7”), the second part is our internal lab identifier for the sample (i.e., biological replicate, e.g., “PG0051-C”), and the third part is the accession for the sequencing run at the ENA (e.g., “ERR019061”). The second and third parts are redundant, because as mentioned above there is a one-to-one mapping from sample to sequencing run, however we include both for transparency. The data files available from the FTP site and the web application use the same identifier system for consistency.

2. Sequence alignment and genome accessibility

Sequence reads from each sample were aligned to the 3D7 version 3 reference genome using BWA

(Li & Durbin, 2009) version 0.6.1-r104 with the following parameter settings:

```
bwa aln -n 0.01 -k 4
bwa sampe
```

We found that the custom parameters to the `aln` command served to slightly increase the sensitivity and improve consistency of the alignment in regions with clusters of SNPs, such as the polymorphisms found at the chloroquine resistance locus (Fidock et al., 2000), however the vast majority of alignments are identical under the custom and default settings (data not shown). We recommend the custom settings for alignment of *P. falciparum* short sequence reads where possible, however the increased sensitivity does increase the runtime required by approximately an order of magnitude over the default settings, and therefore the default settings are the only practical option for large numbers of samples.

Various metrics were then calculated from the alignments of each sample. These metrics were computed per genome position based on the pileup of aligned reads, using the program `pysamstats`¹. Metrics calculated include the total depth of coverage, percentage of reads aligned in a proper pair (i.e., in correct orientation and reasonable distance apart, as defined by the aligner), average mapping quality and percentage of reads aligned ambiguously (mapping quality zero).

Alignment metrics for each of the parental samples were then plotted for each chromosome, alongside other metrics derived from the reference genome sequence, including the %GC content in a 300bp window and the non-uniqueness score (defined as the smallest k-mer size at which all k-mers overlapping a given position are unique within the genome; a high score for this metric is bad, in the sense that it indicates low uniqueness). An example plot for sample HB3/PG0052-C/ERR019054 and chromosome 4 is shown in Figure S1. The alignments themselves were also visualised using the LookSeq web application (Manske & Kwiatkowski, 2009), which can be viewed via the web application at @@URL.

From these visualisations a clear, qualitative distinction could be seen between regions of the genome with consistent coverage across all parent samples, and regions with significant alignment issues in one or more parents. To capture these large-scale qualitative differences we defined the following heuristic scheme for classifying genome regions:

- **Core** – Regions with near-continuous coverage in all samples, with a high percentage of reads mapping in a proper pair and a low proportion of reads aligned ambiguously.
- **Subtelomeric Hypervariable** – Gene-containing regions towards the sub-telomere of a chromosome, with patchy and/or highly variable coverage in one or more samples and/or a low proportion of reads aligned in a proper pair and/or a high percentage of ambiguous alignments and/or a high percentage of aligned bases mismatching the reference.
- **Internal Hypervariable** – Gene-containing regions towards the centromere of a chromosome, with patchy and/or highly variable coverage in one or more samples and/or a low proportion of reads aligned in a proper pair and/or a high percentage of ambiguous alignments and/or a high percentage of aligned bases mismatching the reference.

1 <https://github.com/alimanfoo/pysamstats>

- **Subtelomeric Repeat** – Gene-free regions with repetitive sequence at the end of a chromosome, typically with highly variable coverage and a high percentage of ambiguous alignments.
- **Centromere** – Centromere as given in the GeneDB genome annotation.

Within each chromosome we defined boundaries for these regions by eye from the visualisations described above. Figure S2 shows a map of the genome regions defined, and Figure S3 gives a summary of alignment statistics for each parental clone by region class. At least 99.6% of core genome positions were covered in all parents, and at least 98.8% of the core genome was covered by unambiguously mapped reads.

Our definition of the core genome is subjective, and more sophisticated methods could be devised to partition the genome into regions with different alignment characteristics. However the contrast between these different regions of the genome is very striking, and we believe the definitions given here capture the major qualitative features in a useful way.

The genome region classification can be browsed alongside coverage, mapping quality and other metrics via the web application at the following URL:

<http://www.malariagen.net/apps/pf-crosses/#genome>

A BED file defining the region boundaries can be downloaded from the FTP site:

@@TODO

3. Variant discovery and genotype calling

3.1. Alignment-based calling method (BWA/GATK)

The alignment-based calling method used the Genome Analysis Tool Kit version 2.6-4-g3e5ff60 (McKenna et al., 2010) and followed best practice recommendations as published at the time (DePristo et al., 2011; Van der Auwera et al., 2013).

Starting from the reads aligned to the 3D7 version 3 reference genome as described above, the following steps were performed to prepare the BAM files. Using Picard tools version 1.77 the commands CleanSam, FixMateInformation, AddOrReplaceReadGroups and MarkDuplicates were run on each BAM file in that order.

Base quality score recalibration (BQSR) was then applied to the BAM files. BQSR empirically recalibrates the base quality scores reported for each base in each sequence read, by observing the correlation between mismatches in the aligned sequence reads and various covariates, including the original base quality reported by the sequencing machine, in addition to other factors like the local sequence context. BQSR thus relies on the assumption that a substantial number of bases mismatching the reference in aligned sequence reads are due to sequencing error and not true variation, alignment error or some other type of artefact. From a visual inspection of the alignments for the parental clones (see, e.g., Figure S1) it was apparent that the mismatch rate within hypervariable regions was extremely high, and given the other alignment symptoms in hypervariable regions including patchy coverage and ambiguous mapping, we assumed the vast

majority of these mismatches were due to divergence between clones and not sequencing error. To avoid hypervariable regions overwhelming BQSR we limited the building of the covariates table to the core genome. BQSR also requires a set of known variant positions to exclude when building the covariates table. To bootstrap BQSR we created an initial set of variant calls for each cross from the raw BAM files using UnifiedGenotyper, then filtered these calls to exclude any that had less than 2 confident (GQ = 99) ALT calls, contained Mendelian errors, had more than 2 missing calls or were part of a homopolymer run of length 5 or more.

We then applied INDEL realignment to the recalibrated BAMs. Each BAM file was realigned separately, but to improve the sensitivity of INDEL realignment we provided as input the set of bootstrap INDEL calls obtained from the previous BQSR step, which has the effect of sharing information about possible INDEL alleles between samples. All other settings were default.

We then generated a raw variant callset using UnifiedGenotyper run under a haploid model (-ploidy 1).

The next step was to empirically recalibrate variant quality scores (VQSR). VQSR requires at least a positive training set of known true variants, and optionally one or more negative training sets of sites where variant calls are likely to be spurious. We defined a positive training set for each cross by selecting variants from the raw callset that segregated within the cross according to Mendelian inheritance (i.e., parents had different genotypes, progeny had no Mendelian errors) and also produced highly parsimonious patterns of inheritance (i.e., did not induce an unrealistically high rate of recombination). Specifically, the positive training sets included only SNP and INDEL variants within the Core genome, with no missing calls, no non-Mendelian calls, and no calls inducing an apparent double-crossover at a single variant. We also created two negative training sets for each cross, the first containing variants with Mendelian errors, the second containing variants inducing single-variant double-crossovers in one or more samples.

We then applied VQSR to each cross separately. VQSR was run for SNPs with the following options:

- -an QD -an DP -an MQ -an UQ -an HaplotypeScore -an ReadPosRankSum -an FS
--target_titv 1.0 --percentBadVariants 0.1 --stdThreshold 10.0 --maxGaussians 6

VQSR for INDELs was run with the following options:

- -an QD -an DP -an MQ -an UQ -an HaplotypeScore --target_titv 1.0 --percentBadVariants 0
--stdThreshold 10.0 --maxGaussians 6

“UQ” is the non-uniqueness score define above and the other annotations are standard INFO annotations produced by GATK.

To verify that the VQSR runs had been effective, we plotted the rate of Mendelian error against the number of variants for different thresholds of the VQSLOD score (similar to an ROC curve). For all three crosses and for both SNPs and INDELs we observed a strong inflection point in these curves, corresponding to a Mendelian error rate of approximately @@X% (Figure @@SX).

We generated a final, analysis-ready VCF from the raw VCF by applying the following filters:

@@TODO

3.2. Assembly-based calling method (Cortex)

@@TODO

4. Recombination analyses

4.1. Determination of maximal block length for conversion tracts

@@TODO

5. Figures and tables

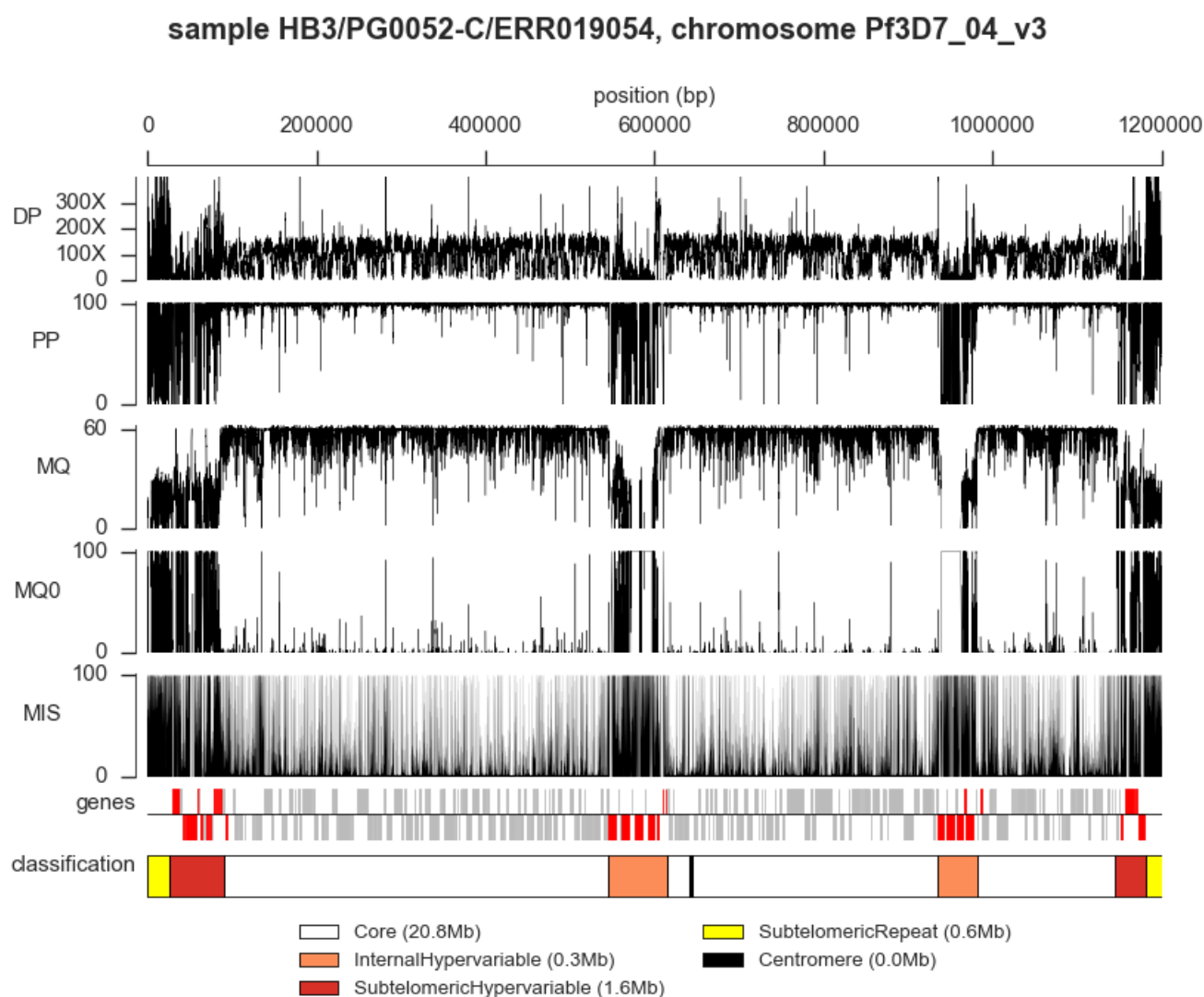


Figure S1: Example of alignment metrics for an individual sample and relationship to genome region classification. The sample shown is HB3/PG0052-C/ERR019054 (parent of 3D7xHB3) and data are shown for the entirety of chromosome 4. DP = total depth of coverage, PP = percent of reads aligned in a proper pair; MQ = root mean square mapping quality of aligned reads; MQ0 = percent of reads aligned ambiguously (mapping quality zero); MIS = percent of reads aligned with a base mismatching the reference. Genes tracks shows forward strand above the line, reverse strand below the line; genes in red are var/rif/stevor. Genome region classification is shown in the bottom track, colours as in the legend.

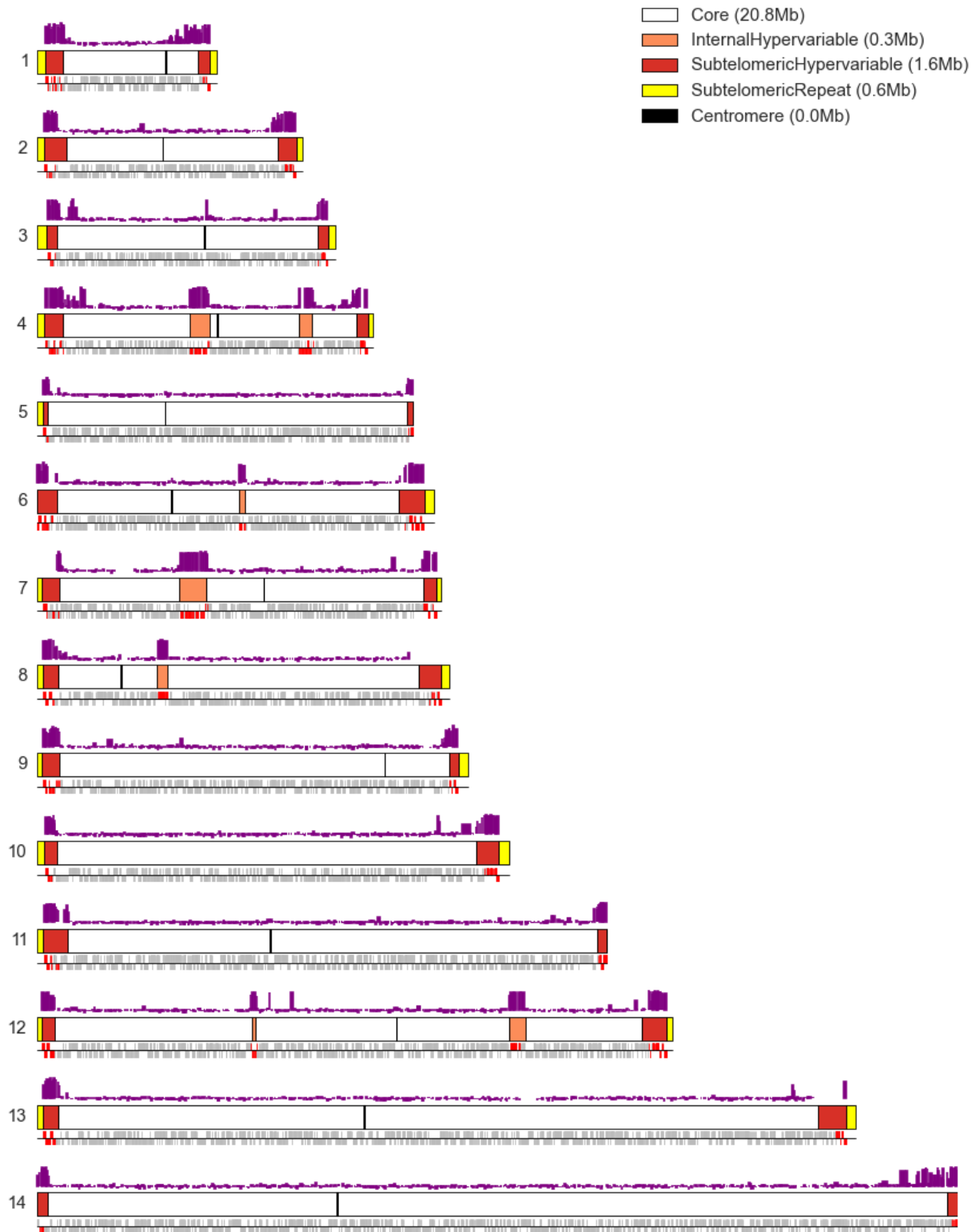


Figure S2: Genome region classification. Each sub-plot corresponds to one of the fourteen nuclear chromosomes. The central bar in each sub-plot shows the genome region classification coloured according to the legend. Above the central bar in purple are levels of heterochromatin protein 1 (HP1) per gene from (Flueck et al., 2009). Below in grey are genes, with positive and negative strands plotted above and below the line respectively; genes in the *rif*, *stevor* and *var* families are shown in red.

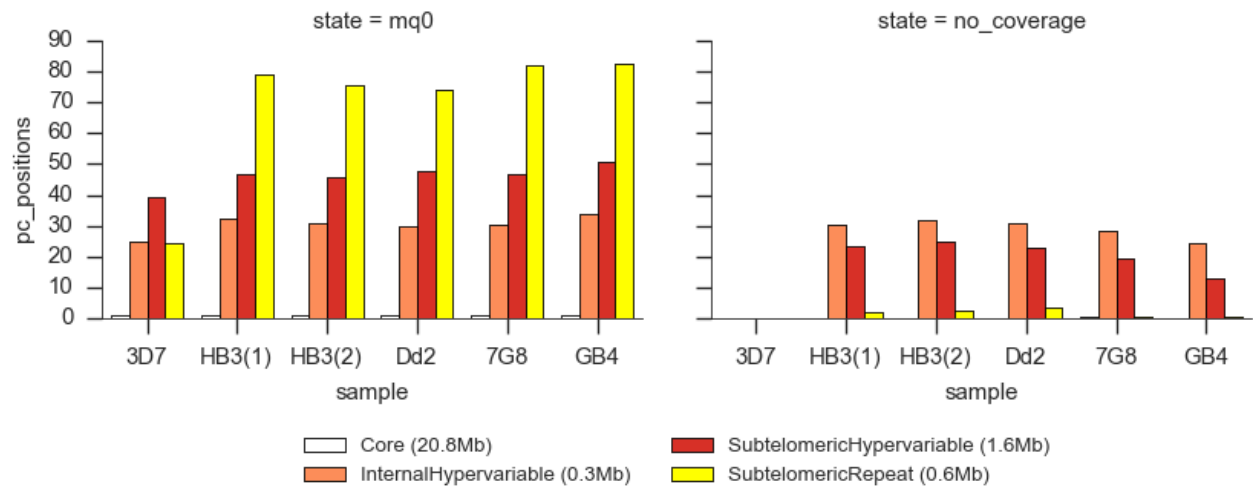


Figure S3: Summary of alignment characteristics for different genome region classes. The left-hand sub-plot shows the percentage of positions with more than 10% of reads aligned ambiguously (mapping quality zero). The right-hand sub-plot shows the percentage of positions without any coverage whatsoever.

6. References

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