Introduction

*Plasmodium falciparum* is a unicellular parasite that causes malaria in humans. It infects over 300 million people per year and is estimated to have killed 600,000–1.2 million people in 2010 [1]. One of the most remarkable biological features of *P. falciparum* is the ~60 var genes distributed across parasite chromosomes, both in the subtelomeres and in internal regions. They encode hypervariable surface proteins known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) that are critical for pathogenesis and immune evasion in *Plasmodium falciparum*. How var gene sequence diversity is generated is not currently completely understood. To address this, we constructed large clone trees and performed whole genome sequence analysis to study the generation of novel var gene sequences in asexually replicating parasites. While single nucleotide polymorphisms (SNPs) were scattered across the genome, structural variants (deletions, duplications, translocations) were focused in and around var genes, with considerable variation in frequency between strains. Analysis of more than 100 recombination events involving var exon 1 revealed that the average nucleotide sequence identity of two recombining exons was only 63% (range: 52.7–72.4%) yet the crossovers were error-free and occurred in such a way that the resulting sequence was in frame and domain architecture was preserved. Var exon 1, which encodes the immunologically exposed part of the protein, recombined in up to 0.2% of infected erythrocytes *in vitro* per life cycle. The high rate of var exon 1 recombination indicates that millions of new antigenic structures could potentially be generated each day in a single infected individual. We propose a model whereby var gene sequence polymorphism is mainly generated during the asexual part of the life cycle.
diversity observed within and between species [14]. However it is not known whether var gene recombination is sufficiently frequent to be the primary driver of var gene diversity, nor whether this recombination occurs mainly in meiosis, which takes place in the mosquito, or during mitosis, which includes the entirety of the intraerythrocytic stages within the human host. It is also unclear whether recombination obeys particular patterns, and how this might relate to var gene structure. Understanding how the parasite manages to generate such an extreme level of sequence diversity while preserving the overall architecture and biological functionality of the var gene repertoire is fundamental to understanding P. falciparum pathogenesis.

We used an experimental evolution approach to systematically investigate the mechanisms that drive var gene diversity, performing whole genome sequence analysis on >200 clonal parasites. Parasites cultured in vitro in human erythrocytes were regularly sub-cloned to isolate single infected red blood cells, so that mutations arising in asexually dividing cells could be detected using whole genome sequencing of the expanded progeny derived from these cells (Fig. 1C). We generated these ‘clone trees’ for geographically diverse P. falciparum strains, comparing parasite genomes separated by 20–30 cycles of replication (S1 Fig.). The scale of our dataset allowed us to perform a comprehensive analysis of mitotic var gene recombination using observed recombination events for the first time. This revealed how var gene sequence polymorphism – and, by inference, parasite antigenic variation – can be continuously generated during asexual division over the course of a single human infection.

Results

Structural variation is focused in and around var genes in 3D7

We began by analysing the 3D7 strain of P. falciparum as this has the most complete genome assembly of any lab isolate. 19 individual parasites were cloned from 3D7 by limiting dilution and cultured until sufficient parasite quantity was reached for whole genome sequencing. From 2 of these 19 subclones, another 3 rounds of clonal dilutions were performed over a combined culturing period of 203 days (S1 Fig.). Whole-genome sequencing of a total of 37 subclones identified 20 de novo single nucleotide polymorphisms or SNPs distributed throughout the genome, and 40 de novo structural variations comprising 10 duplications, 8 deletions and 22 translocations (Fig. 2, S2–S4 Table). Our analysis focused solely on de novo mutations, i.e. found in one or more subclone(s) but not in the parental clone. As expected, the first generation of subclones contained more mutations than subsequent generations, because they were derived from a standard 3D7 culture that had been growing for several months prior to beginning our experiment. Of the 19 structural variations that affected coding regions, all were in var genes. Of the remaining 21 structural variations, all were in telomeric regions or within internal regions of chromosomes that contained clusters of var genes. From these data we concluded that de novo structural variations of the P. falciparum genome occur relatively frequently during the mitotic intraerythrocytic life cycle and are highly concentrated in and around var genes.

Detailed inspection of these structural variations revealed that they took many forms (Fig. 3B–F) but all involved ectopic recombination between different var genes located either in the immediate vicinity on the same chromosome, or on different chromosomes. For example, Fig. 3A&B and S2 Fig. show a gene conversion event involving two subtelomeric var genes, in which the end subtelomere of chromosome 4 was duplicated and replaced the subtelomere of chromosome 9. The translocation breakpoints occurred within var genes on these two chromosomes, referred to as var4 and var9, creating a chimeric var4/var9 sequence. This gene conversion event was confirmed by capillary sequencing of PCR products, and the var4/var9 chimera was subsequently inherited by downstream parasite clones. There were also instances of two var genes recombining with multiple crossover points (Fig. 3D, S3 & S4 Fig.) and recombination between more than two var genes (Fig. 3E and Fig. S2). Recombination in subtelomeric var genes involved both reciprocal exchange and non-reciprocal gene conversion in which the entire end of one chromosome was duplicated and replaced the corresponding section of the other chromosome. In addition to recombination between subtelomeric var genes on different chromosomes, we found recombination between internal var genes on the same chromosome (Fig. 3F, S5 & S6 Fig.).

Var gene exon 1 recombination is a frequent mitotic event in diverse P. falciparum strains

To establish whether mitotic var gene recombination is a common feature among other P. falciparum strains, we generated similar clone trees for the Dd2 and W2 strains of drug-resistant P. falciparum from Southeast Asia, and for the HB3 strain of drug-sensitive P. falciparum from Honduras (S1 Fig.). The Dd2, W2 and HB3 clone trees were generated with a total of 7, 3, and 8 cloning rounds respectively, over a total of 298, 119 and 250 days. This generated 56, 20 and 81 subclones respectively, all of which were whole genome sequenced. Altogether, we observed 20, 11 and 19 SNPs in the Dd2, W2 and HB3 clone trees respectively, scattered across the genome (Fig. 2). As any mutation identified in a clone would have actually occurred in the previous generation, we can only use clones from the second generation onwards for calculating mutation rates (Fig. 1C). From these data we estimate mutation rates of 4.07×10⁻¹⁰, 3.63×10⁻¹⁰ and 3.78×10⁻¹⁰ SNPs...
On the Origin of Plasmodium falciparum Var Gene Sequence Diversity
per erythrocytic life cycle per nucleotide for 3D7, Dd2 and HB3 respectively (Table 1). The W2 clone tree did not contain enough clones for a reliable mutation rate estimate. Despite geographic and drug-sensibility differences between 3D7, Dd2 and HB3, there is no statistically significant difference in SNP mutation rate between these strains (Kruskal-Wallis, \( p > 0.7 \)); See Methods & Table 1 for rate calculations. The rates calculated here are similar to estimates for 3D7 and Dd2 by Bopp et al. 2013, suggesting that SNP mutation rates are relatively constant across \( P. falciparum \) strains.

A complete high-quality genome assembly is currently only available for 3D7, which makes mapping difficult in other strains, especially in \( var \) genes. For these strains we therefore focused on a detailed analysis of recombination events in \( var \) exon 1, which encodes the extracellular domains responsible for antigenic diversity. In Dd2 and W2, we observed 11 and 13 instances of \( var \) exon 1 recombinations in 7 and 6 pairs of recombining \( var \) genes respectively (Table 2). In contrast, there were no recombination events in HB3 clones, despite similar breadth and length of the clone trees and despite the \( var \) gene assemblies of HB3 and Dd2 being of a comparable standard.

We estimate that the rate at which pairs of \( var \) genes undergo exon 1 recombination in the Dd2 clone tree is \( 2.32 \times 10^{-3} \) per erythrocytic life cycle, compared with \( 9.48 \times 10^{-3}, 8.47 \times 10^{-3} \) and \( 8.81 \times 10^{-3} \) SNPs per life cycle in 3D7, Dd2 and HB3 respectively (see Methods for rate calculations). This means that in the Dd2 strain, for every 48-hour intraerythrocytic generation approximately 0.2% of parasites will have undergone a \( var \) exon 1 recombination event producing a new chimeric \( var \) gene.

We further analysed the \( var \) gene recombination rate by comparing the ratio of total de novo \( var \) recombinations to SNPs observed in the clone trees. Our mutation rate calculations given above exclude mutations found in the first generation of each clone tree because these mutations occurred prior to our beginning the experiment after an unknown length of time in culture (Fig. 1C). However, mutations identified in this first clone tree round must have arisen during the intraerythrocytic stage, at some point between the original cloning of 3D7/HB3/Dd2 in the 1980s and the start of our clone tree experiments. As demonstrated in this study and in Bopp et al. [9], the SNP mutation rate appears to be relatively constant between strains. Therefore, the number of

![Fig. 1. Var gene nomenclature and generation of a clone tree. (A) Var gene nomenclature, as defined in [8]. From top to bottom: schematic \( var \) gene locus, schematic PfEMP1 protein with its DBL & CIDR classes followed by subclasses. Note that the minor domain classes CIDRt4 to s6 and the \( var2csa \) specific domains are not represented here. Abbreviations: DBL = Duffy-binding like; CIDR = Cys rich inter-domain region; NTS = N-terminal segment; ATS = Acidic terminal segment. (B) Partial alignment of 3D7 PfEMP1 sequences, representative of the juxtaposition of conserved and variable stretches of amino acids. (C) Principle of generating a clone tree: each blue circle represents a parasite genome, a coloured oval is a mutation, Single Nucleotide Polymorphism (SNP) or Structural variant. Cloning by limiting dilution randomly selects one individual parasite, possibly with one or more mutations. Note that mutations observed in a progeny sample occurred within the parental generation, at some point after the preceding clonal dilution.

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![Fig. 2. Chromosomal location of structural variants in the 3D7 clone tree and SNPs in the 3D7, Dd2, W2 and HB3 clone trees. SNPs from 3D7, Dd2, W2 and HB3 clone trees were scattered around the genome. In contrast, structural variants (deletions, duplications and translocations) were solely in subtelomeric and internal \( var \) gene regions. Structural variants in Dd2, W2 and HB3 \( var \) genes are not shown here as their \( var \) gene location in these strains is unknown.

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SNPs found in first generation subclones becomes a proxy for the time of culture of the parent, i.e. the more SNPs identified, the longer the parent had been in culture since the original cloning step. Because of this constant SNP mutation rate, we can use all subclones, including the first generations subclones, to measure the ‘var recombination/SNP ratio’, which indicates the number of var recombinations over a period of time. The overall var exon 1 recombination to SNP mutation ratio is 0.25, 0.35, 0.54 and 0 in the 3D7, Dd2, W2 and HB3 clone trees respectively (Table 2).

Thus, while the SNP mutation rate is constant between different P. falciparum strains, the structural variant rate in HB3 var genes is significantly lower than in Dd2 (p = 0.03, Fisher’s exact test), at least with the specific HB3 isolate used in our laboratory.

The dynamic nature of var recombination in Dd2 is illustrated by a pair of internal var genes referred to here as var34 and var45 (Fig. 4). Recombination between var34 and var45 was first observed in a first generation subclone of the Dd2 clone tree (Dd2var34var45). This was a double crossover recombination which created a new chimeric var gene named var34-45-34 (see main text and Fig. 4). (D) As depicted by this cartoon, the majority of recombining pairs of var genes showed multiple crossover points (S4 Table). (E) Triple reassortment var gene, composed of three ‘parent’ var genes (S2 Fig.). (F) ‘Duplication chimera’ in internal var genes. Duplicated sections of internal var genes recombine to form new chimeras. TLR = Trans-locus reads. PCR and capillary sequencing data validating these chimeric sequences can be found in S2–S7 Fig.

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Var gene exon 1 recombination is structured such that overall var gene architecture is preserved

The clone trees demonstrate that var gene recombination during intraerythrocytic growth is extensive and complex, and in at least three strains occurs nearly as frequently as SNPs. To investigate the patterns and mechanisms of var gene recombination, we collated all available data on chromosomal crossovers in var exon 1 (Table 1) based on our clone tree experiments, together with data from previous smaller studies [9,15] as well as data obtained by sequencing the progeny of three genetic crosses (Dd2xHB3, 3D7xHB3 and 7G8xGB4) [16–18] (Pearson & Miles, in prep) (discussed in more detail below). From these combined datasets we identified 110 crossovers in 46 pairs of var gene exon 1s, generating a mean of 2.4 (range 1–12) crossover points per recombining pair of exons (Table 2, S4 and S8 Fig.).
### Table 1. Calculation of the Single Nucleotide Polymorphism mutation rate.

| Column | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|--------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|        | Generation | Number of clones | Number of SNPs | SNP/clone | Culturing (days) | Culturing (hours) | Assumed lifecycle time (hours) | Number of lifecycles | SNP/lifecycle (D/H) | Genomic size (Mb) | SNP/lifecycle/bp (I/J) | Weighting* | Weighted SNP/lifecycle/bp (K/L) | Weighted SNP/lifecycle (L/N) |
| **3D7** | 1 | 19 | 15 | 0.794 | unkn. | 37 | 0 | 23.3 | 0.278 | 0 | 23.3 | 8.58E-10 | 0.278 | 4.069E-10 | 9.480E-03 |
|        | 2m | 5 | 0 | 0 | 74 | 1776 | 48 | 23.3 | 3.10E-10 | 0.389 | 3.10E-10 | 6.67E-03 | 2.81E-03 |
|        | 2o | 7 | 2 | 0.29 | 79 | 1896 | 48 | 23.3 | 3.10E-10 | 0.389 | 3.10E-10 | 6.67E-03 | 2.81E-03 |
|        | 3 | 6 | 3 | 0.5 | 50 | 1200 | 48 | 23.3 | 3.10E-10 | 0.389 | 3.10E-10 | 6.67E-03 | 2.81E-03 |
| **Total** | 37 | 20 | 203 | 4.069E-10 | 9.480E-03 |
| **Dd2** | 1 | 5 | 7 | 1.40 | unkn. | 34.8 | 0.07 | 23.3 | 0.34 | 0 | 23.3 | 1.21E-03 | 0.34 | 1.11E-03 | 6.67E-03 |
|        | (A)_2 | 10 | 5 | 0.5 | 64 | 1536 | 44.1 | 34.8 | 0.0144 | 23.3 | 6.16E-10 | 0.196 | 1.21E-10 | 2.81E-03 |
|        | (B)_2 | 17 | 4 | 0.24 | 64 | 1536 | 44.1 | 34.8 | 0.0068 | 23.3 | 2.90E-10 | 0.333 | 9.66E-11 | 2.25E-03 |
|        | (A)_3 | 3 | 1 | 0.33 | 47 | 1128 | 44.1 | 34.8 | 0.0130 | 23.3 | 5.59E-10 | 0.059 | 3.29E-10 | 7.67E-04 |
|        | (B)_3 | 2 | 0 | 0 | 47 | 1128 | 44.1 | 34.8 | 0.0068 | 23.3 | 2.90E-10 | 0.333 | 9.66E-11 | 2.25E-03 |
|        | (A)_4 | 14 | 3 | 0.21 | 41 | 984 | 44.1 | 34.8 | 0.0130 | 23.3 | 5.59E-10 | 0.059 | 3.29E-10 | 7.67E-04 |
|        | (B)_4 | 5 | 0 | 0 | 35 | 840 | 44.1 | 34.8 | 0.0068 | 23.3 | 2.90E-10 | 0.333 | 9.66E-11 | 2.25E-03 |
| **Total** | 56 | 20 | 298 | 3.635E-10 | 8.470E-03 |
| **HB3** (Two clone trees) | 1 | 7 | 0 | 0 | unkn. | 1400 | 0 | 23.3 | 0.119 | 0 | 23.3 | 5.64E-10 | 0.119 | 1.32E-03 |
|        | 2 | 8 | 0 | 0 | 29 | 696 | 49.7 | 1400 | 0 | 23.3 | 5.64E-10 | 0.119 | 1.32E-03 |
|        | 3 | 22 | 5 | 0.23 | 32 | 768 | 49.7 | 1545 | 0.0147 | 23.3 | 6.31E-10 | 0.328 | 2.07E-10 | 4.83E-03 |
|        | 4 | 15 | 1 | 0.067 | 50 | 1200 | 49.7 | 2414 | 0.0028 | 23.3 | 1.99E-10 | 0.224 | 2.65E-11 | 6.18E-04 |
|        | 5 | 8 | 2 | 0.25 | 47 | 1128 | 49.7 | 2270 | 0.0110 | 23.3 | 4.73E-10 | 0.119 | 5.64E-11 | 1.32E-03 |
|        | b1 | 7 | 8 | 1.143 | unkn. | 1128 | 49.7 | 2270 | 0.0110 | 23.3 | 4.73E-10 | 0.119 | 5.64E-11 | 1.32E-03 |
|        | b2 | 8 | 1 | 0.125 | 48 | 1152 | 49.7 | 2318 | 0.0054 | 23.3 | 2.31E-10 | 0.119 | 2.76E-11 | 6.44E-04 |
|        | b3 | 6 | 2 | 0.33 | 44 | 1056 | 49.7 | 2125 | 0.0157 | 23.3 | 6.73E-10 | 0.090 | 6.03E-11 | 1.40E-03 |
| **Total** | 81 | 19 | 250 | 3.782E-10 | 8.812E-03 |

Each column is labelled to indicate the steps leading to the mutation rate numbers (see Methods for more details). We first calculate the number of SNPs per sub-clone detected in each clone tree generation. This represents the average number of \( \text{de novo} \) mutations in each clonal population (e.g. if the SNP/clone is 0.2, then approximately 1 in 5 clones will have a SNP). We divide this by the number of intraerythrocytic life cycles the parasites have undergone prior to the most recent cloning step, counting from the previous clonal dilution. The number of intraerythrocytic life cycles is reached by dividing the time in culture by the time per intraerythrocytic life cycle. This gives the SNPs/intraerythrocytic parasite life cycle for each clone tree generation. To derive the SNPs/life cycle/nucleotide, we divide by the haploid nuclear genome of \( P. falciparum \) (23,300,000 base pairs). The final mutation rate for each strain is an average from all the generations in the clone tree. We weight this average based on the number of subclones analysed in each generation, so that generations with more subclones have proportionally more representation in the average. Note that the first generation of subclones cannot be used to calculate the SNP mutation rate because the length of time in culture prior to our starting the experiment is not known. The W2 clone tree was too small to accurately determine the SNP mutation rate. The same process was used to calculate the exon 1 var recombination rates (S5 Table).
These data revealed a highly structured pattern of ectopic recombination. We found that all of the crossovers were in frame and in the vast majority of cases (109/110) the recombinating sequences were of the same domain class, e.g. CIDR\(\alpha\) almost invariably recombined with CIDR\(\alpha\) (Fig. 5). Every var gene begins with a DBL\(\alpha\) and CIDR\(\alpha\) domain, followed by a variable number and order of other domains. Given that recombination only occurs between the same domain classes, this mechanism would preserve the overall architecture of chimeric var genes. Consistent with this, all observed chimeric var genes possessed a typical number of var domains. We observed only one exception to this, between 7G8var49 and 7G8var56, in which recombination took place in an area of shared homology between a DBL\(\alpha\) and a DBL\(\delta\) domain [8], producing a truncated gene with only two domains (S8 Fig.).

The new gene sequences generated by this structured process were extremely diverse, as var genes did not necessarily recombine with the most highly homologous var genes to themselves (S9 Fig.). The average sequence identity of the two recombining domains was 68\%, and that of the two recombining exons was only 63\%. In the terminology used by Rask et al, 65\% of recombinations were between domains of different sub-classes (Fig. 6). But despite this overall diversity, we observed that crossovers invariably took place within a short section of perfect sequence identity ranging in length from 4 to 48 bp (median 15 bp). This section of sequence identity, which we refer to here as an identity block (S10 Fig.), was typically located within a broader section of elevated sequence homology (median 80 bp) relative to the average for the two genes concerned (Fig. 6). When exon 1 var sequences were aligned by BLAST analysis, 94\% of crossovers were found to occur in a region of high similarity between the two recombining genes (S11 Fig.), whereas only 59\% would be expected if the point of recombination was random \(\left( P < 10^{-3}\right)\) by chi-squared test.

We compared the recombination rates found in group A, B and C var genes from our pooled var exon 1 recombination dataset. In total, we observed 19 instances of B\(\times\)B recombination, 12 instances of C\(\times\)C recombination and 2 instances of B\(\times\)C recombination. In contrast we observed no instances of recombination involving group A var genes. The 3D7 reference genome contains 10 group A, 37 group B and 13 group C var genes, so these observations depart significantly from what would be expected if the pairing of ectopic sequences was random across all var genes (\(P = 0.0028\), Fisher’s exact test). These data suggest that group A var genes have a lower recombination rate than group B or C.

### Table 2. Summary of var gene exon 1 recombinations.

| Clones analysed | Total number of SNPs | Pairs of var genes involved (of which are internal var\(\delta\)) | Total number of recombinations in var exon 1 | var/SNP ratio** |
|-----------------|----------------------|----------------------------------------------------------|------------------------------------------|----------------|
| 3D7 clone tree  | 31                   | 20                                                       | 5 (4)                                     | 17             | 0.27          |
| Dd2 clone tree  | 56                   | 20                                                       | 7 (5)                                     | 11             | 0.35          |
| W2 clone tree   | 20                   | 11                                                       | 6 (6)                                     | 13             | 0.54          |
| HB3 clone tree  | 81                   | 23                                                       | 0                                         | 0              | 0             |
| 3D7xHB3         | 16                   | 6                                                        | 6 (3)                                     | 14             | 1             |
| HB3xDd2         | 30                   | 12                                                       | 6 (2)                                     | 24             | 0.50          |
| 7G8xGB4         | 27                   | 12                                                       | 16 (unknown)                              | 28             | 1.33          |
| Bopp et al[9]** | 15                   | /                                                        | 2 (0)                                     | 2              | /             |
| Guler et al[15] | 5                    | /                                                        | 1 (1)                                     | 1              | /             |
| Total           | 281                  | 46                                                       | 110                                       |                |

* Excluding the parental samples. ** var/SNP ratio is the number of pairs of recombining var genes divided by the number of SNPs.

**Although Bopp et al reports 4 pairs of recombining var genes, one is in the exon2, a region that was not considered in this analysis; another one did not correlate with our own analysis and was discarded. All but one chimeric var genes reported in Sander et al were also discovered in our analysis (the remaining one was in a progeny sample not available to us).

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The prevailing view of var recombination is that it occurs primarily during meiosis, based on the observation of recombination in the progeny of experimental crosses [10,19]. An alternative model is that var recombination in these progeny actually occurred during mitosis while the parasites were cultured in *in vitro* prior to and after the cross itself [20]. To test these alternatives we identified and characterised var recombinations in genome sequence data from progeny of all three experimental genetic crosses, and compared them with analogous data from progeny of our clone trees. This comparison strongly supported the latter model – that the vast majority of var recombination occurs during mitosis – for three main reasons (Fig. 7). First, the recombination in both the (purely mitotic) clone trees and the crosses progeny share key features and appear to have been produced through the same process. Recombined var genes were always in frame without SNPs or InDels; crossovers took place in small regions of sequence identity; overall var exon 1 domain architecture was preserved because domains only recombined with other domains of the same class (with one exception), and recombination often involved multiple crossovers between the same two var genes, with an average of 2.6 and 2.4 crossovers per recombining var pair in the clone trees and crosses progeny respectively (S7 Table). Second, within the crosses progeny we could find only two cases where the var genes in a recombining var pair were derived from different parents. This is not what would be expected if recombination occurred during meiosis when the parasite was diploid. During meiosis, each progeny inherits on average half its chromosomes and associated var genes from each parent. If heterologous chromosomes only recombined at that stage, we should observe chimeric var sequences derived from var genes of different parents or from the same parent in an approximately 50:50 ratio (Fig. 7). In contrast, we found that 26 out of 28 recombinations were ‘intra-strain’, i.e. involving pairs of var genes.
from the same parent. The simplest explanation is that these recombination events occurred during mitosis while the parental strains were in culture prior to the cross.

Finally, knowing the average mitotic \textit{var} recombination rate calculated using our clone trees allows us to test whether the number of \textit{var} recombination events observed in the crosses progeny is consistent with the culturing time before and after the cross. In our clone trees and other experiments [9], the SNP mutation rate is similar across strains (see above). Thus, the number of SNP mutations in a subclone is directly proportional to the number of intraerythrocytic life cycles that occurred before the last cloning step. The number of days of culture can then be inferred from the number of SNP mutations observed. Based on the number of \textit{de novo} SNPs identified in the crosses progeny and our mitotic SNP mutation rate, we calculated that the \textit{P. falciparum} strains used for the crosses had undergone 124 and 133 days of \textit{in vitro} culturing for 3D7xHB3 and HB3xDd2 respectively. i.e. there had been 124 and 133 days in culture from when the original isolates were first sub-cloned (from NF54 for 3D7, H1 for HB3, etc), to the day the crosses’ progeny were cloned out. For 3D7xHB3 and HB3xDd2, these estimates could be validated by direct comparison with the actual known culturing
Fig. 5. Var gene recombination occurs within domain classes and between domain subclasses. Each var gene typically comprises 4 to 7 DBL and CIDR domains, each domain being subdivided in various classes (α, β,...) and subclasses (α0.1, α0.2,...). In all but one of the recombining var pairs, the crossover breakpoint was predicted between domains of the same class in both genes. In contrast, recombination was indiscriminate of subclass within each domain class (outside wheels). Subclassification data are not available for 7G8 and GB4 strains. For clarity only recombining domain subclasses are shown here. Inter = Interdomain region. Numbers in the central wheel indicate the relative frequency of that domain class in group B and C var genes. DBLx domains showed the highest number of recombinations (S13 Fig.) but within that domain we found no evidence for a hotspot (S14 Fig.).

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Fig. 6. Recombination occurs in shared var domains within identity blocks. (A) and (B) refer to two example var genes, Dd2var34 and Dd2var45. For all var gene pairs, see S8 and S12 Fig. (A) All recombination crossovers observed between the two var genes shown schematically, from the Dd2 clone tree and HB3xDd2 cross. (B) Sequence homology using overlapping 50 bp windows following a ClustalW alignment of var exon 1. Triangles indicate coordinates of crossover midpoints within the alignment. (C) Median percentage identity for all recombinations between sequences of 10–800 bp upstream (‘ups’) and downstream (‘down’) from the recombination breakpoint, defined as the identity block midpoint. Homology peaks at the point of recombination.

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data of the parental clones, provided by Tom Wellem (NIH) and Lisa Ranford-Cartwright (University of Glasgow). The total recorded culture time, from isolation to progeny sub-cloning, was 89.5 and 99.5 days for the 3D7 and HB3 parasites used in the 3D7xHB3 cross – an average of 94.5 days. The equivalent time for the HB3 and Dd2 parasites used in the HB3xDd2 cross was 89 and 138 days respectively – an average of 113.5 days. These actual dates are very similar to the dates predicted by our mutation analysis, which suggested that the 3D7xHB3 and HB3xDd2 isolates used for the crosses had been cultured for 124 and 133 days, where the real culture times were reassuringly close at approximately 94.5 and 113.5 days respectively (S7 Table). If we assume that the average mitotic var recombination rate is 6.44×10⁻⁴ recombinations per life cycle (the mean from the recombination rates calculated in the 3D7, Dd2, W2 and HB3 clone trees), then we would expect to find about 0.34 var recombinations per progeny sample. Again, these predictions, which are based on purely mitotic recombination, are very similar to what we observe: 0.38, 0.20 and 0.59 in 3D7xHB3, HB3xDd2 and 7G8xGB4, respectively. Therefore, the number of var recombinations found in the crosses progeny is consistent with what is expected from the number of mitotic events that the parasites have gone through both before and after the cross. No additional input of recombination from meiosis is required to explain the data.

**Discussion**

By analysing whole genome sequences from hundreds of subclones derived from asexually replicating parasites, we have generated a large dataset of mitotic mutations in the *P. falciparum* genome. We found that var genes of groups B and C undergo ectopic recombination at a rate which greatly exceeds that of structural variation in other parts of the *P. falciparum* genome, and is not dissimilar to the SNP mutation rate. Recombining var genes often have low levels of sequence homology, and a pair of var genes can recombine at different positions on different occasions, so the new var sequences generated by this process are extremely diverse. However, the recombinant sequence is invariably in frame and free of errors, and var gene domain architecture is preserved. This process has presumably evolved to generate antigenic diversity in *P. falciparum* during the course of a single infection to evade the human immune response.

Our SNP and structural variant detection method by whole-genome sequencing was validated by PCR, capillary sequencing or both (S2–S4 Table). Sander et al recently published an analysis of var gene recombination in progeny of 3D7xHB3 and HB3xDd2 [19]. They reported that recombination breakpoints are concentrated near low folding free energy DNA 50-mers, with a minimal required sequence identity of around 20 bp with 10% mismatch. Three out of the four progeny samples in which they identified var recombinations were available to us. We independently found the exact same var recombination breakpoint coordinates, and came to the same conclusion that crossover events take place within short regions of high homology.

Generating diversity by structured recombination of regional gene segments is somewhat analogous to the way in which the vertebrate immune system generates diversity by V(D)J recombination of immunoglobulin and T cell receptor genes. However, our data show that the molecular mechanism of var gene recombination is markedly different from that of V(D)J recombination in two respects. First, the site of recombination was not
marked by any specific sequence motif equivalent to the ‘Recombination Signal Sequence’ that targets recombines in V(D)J recombination (S15 Fig.), and we observed instances of the same pair of var genes recombining at different positions on separate occasions (S8 Fig.). Second, V(D)J recombination depends on repair of DNA double-strand breaks (DSBs) by non-homologous end joining (NHEJ), a molecular pathway that appears to be missing or atypical in *P. falciparum* [21,22]. All the instances of ectopic recombination we observed were achieved without errors or indels, indicating that the mechanism of DSB repair is more similar to homologous recombination (HR) than NHEJ, as this is generally high fidelity whereas NHEJ is error-prone. It is interesting to note that, lacking a typical NHEJ system, *P. falciparum* is presumably reliant on the machinery of HR for DSB repair. This is somewhat paradoxical given that the parasite is haploid for the majority of its life cycle, including the entirety of the mitotic intraerythrocytic stages. It therefore lacks homologous chromosome copies to use as templates for HR-mediated DSB repair. These conditions may be conducive for non-allelic homologous recombination during mitosis, with non-allelic templates being employed in the absence of homologous chromosomes.

The while the pattern of var gene recombination therefore differs from well-known methods for generating diversity, it is also strikingly different from conventional HR in which crossovers take place in a region of extremely high sequence homology (>92%), typically extending over hundreds of base pairs [23]. Without extensive homology as a cue for pairing sequences from different regions of the genome, some alternative mechanism must exist for co-localisation of var genes before initiation of the recombination-inducing lesion [24]. Var genes are located in more than 30 regions of the genome, i.e. in most subtelomeric regions and in internal regions of four chromosomes. *P. falciparum* telomeres are tethered to the nuclear periphery where they form several distinct clusters [10] and it has been demonstrated by FISH that both subtelomeric and internal var genes aggregate within these clusters [25]. This nuclear clustering is believed to play an important role in the transcriptional activation and silencing of var genes [25] and it might also be a mechanism of co-localising sequences from different regions of the genome prior to ectopic recombination [10].

Our genome-wide approach in 3D7 subclones identified structural variants in and around var genes, indicating that these regions are more recombinogenic. In these samples, we did not observe any recombination in other variant surface antigen families such as rifin or stевой, despite the fact that there are nearly three times as many rifin genes as var genes in the genome. The rate of var gene recombination differed depending on var gene UAA type and parasite strain. We found a lower rate of recombination in HB3 than in 3D7, Dd2 or W2 parasites. The HB3 genome does not appear to be unable to recombine var genes per sec, as we did identify a recombinant exon 1 var gene in the ‘parent’ strain used for one of our two HB3 clone trees and all clonal descendants from that parent inherited the recombinant var gene. We cannot use this example to measure the recombination rate because we do not know the time in culture prior to performing our first clonal dilution, but the observation indicates that mitotic var exon 1 recombination in HB3 does occur, although perhaps at a rate that is too slow to be detected over the course of our clone trees. We have also observed instances of var recombinations in genetically modified HB3 lines (Hamilton *et al.*, manuscript in preparation). A longer time in culture between clonal dilutions, or more clones per dilution, or both, would be necessary to capture enough var recombination events in this strain to determine its rate accurately. What factors influence var gene recombinogenicity, and whether there is a connection with drug resistance, remains to be determined. It is possible that HB3 is inherently less recombinogenic than other strains, or that the particular HB3 isolate we used in our clone experiments had, for unknown reasons, lost or reduced its capacity to undergo var recombination. Moreover, although var gene recombination in recently adapted *P. falciparum* field isolates does occur (Hamilton *et al.*, manuscript in preparation), the frequency of such events in the wild and in vivo is currently unknown.

The fact that recombination was more common in group B and C var genes is consistent with the observation that group A var genes from geographically diverse strains are more conserved than those of groups B and C [26]. Var recombination group-specificity also has implications for the underlying mechanism, raising the possibility that the upstream sequence or some other group-specific sequence feature plays a role in co-localising var genes prior to recombination. Var genes can also be classified into six groups based on their DBLα sequences [27]. However there was no association between var recombination and that specific subgrouping (Chi-square, p>0.40). Whether some specific var genes are inherently more recombinogenic than others, as the *Dd2var34/var45* example could suggest, requires an even larger dataset, perhaps involving several years of clone tree evolution, to properly assess. Further studies such as these will also be required to test the hypothesis that specific var genes being actively expressed in a given strain are more likely to recombine. If the latter hypothesis is true, it would partly explain the lack of recombination in group A genes that we observed, as cultured lab strains typically express very few, if any, of that subgroup [28]. It would also fit with the hypothesis that longer and more conserved var genes show a lower activation rate and lower sequence diversity [29]. Further investigation into DNA repair and recombination in *P. falciparum* will be essential to identify the precise molecular mediators of var recombination and elucidate the mechanism in full.

To date, var gene diversity has typically been studied using short DBLα tag sequences from field isolates [30,31], or from 7 sequenced lab strains [8,26,32]. In either case, these var sequences represent snapshots of diversity at a particular time and place. They reflect the polymorphism found in the wild, under the intense pressure of the human immune system. Our study uses a novel approach to analyse var gene sequences as they are being generated, i.e. without any immunological selection. However, it has to be noted that lethal recombinations, for example between internal and subtelomeric var genes, would never be observed due to the resulting loss of genetic material. But only selection-free experiments such as this one can accurately measure the var recombination rate and the diversity of chimeric sequences being generated. As var gene assemblies from worldwide sampling will become available (Otto *et al.*, manuscript in preparation), it will be of major interest to compare the sequence diversity under selection versus our dataset, for example to differentiate true recombination hotspots from selection hotspots. This will also help modelling studies to define and test recombinational constraints [32].

Comparison with our clone tree data suggests that much of the var gene recombination observed in the progeny of experimental genetic crosses likely arose during mitosis prior to the cross itself (Fig. 7). The dominance of mitotic recombination is inherent to the fact that the life cycle of *P. falciparum* consists of a single meiotic event for dozens or hundreds of mitoses. We propose a model whereby var gene sequence polymorphism is mainly generated during the asexual part of the life cycle, while meiosis has the crucial function of creating a new repertoire of ~60 var genes from two different parents. There is also a clear biological advantage in having extensive recombination in antigen-coding
genes take place during mitosis: it allows the continuous generation of antigenic diversity inside a human host over the course of a single infection. An adult with malaria fever typically carries on the order of $10^7$ to $10^{10}$ infected erythrocytes (parasitaemia 5 to 5,000 parasites/µl), so this implies that on the order of $10^{15}$ to $10^{18}$ new mosaic var gene sequences could be generated through recombination every two days in a single infected individual. Var gene expression is a mechanism of immune evasion, enabling parasitized erythrocytes to sequester in small blood vessels and thus avoid circulation through the spleen. But var genes are themselves the targets of host immunity as they elicit a strong antibody response, which the parasite evades by switching expression between different var genes. Ectopic recombination of var genes may be of limited value in the initial phase of infection, as each new var sequence is present in only a tiny fraction of parasites. However it could be of great importance in chronic asymptomatic infection, which is increasingly recognized a crucial problem for malaria elimination [33]. As infection progresses, the parasite population carried by a single individual has the potential to accumulate an almost limitless repertoire of antigenic variants, thus allowing the evolutionary selection of variants which are best equipped to evade the immune response in that particular individual over a protracted period of time.

**Materials and Methods**

**P. falciparum culture and clone tree generation**

Our 3D7 'parent' clone was provided by Bob Pinches. It was one of several 3D7 reference stablilates produced in 1989 following the isolation of 3D7 from NF-54. Thus, its genome should be very close to the canonical 3D7 reference genome. Dd2, W2 and HB3 parasites were obtained originally from the Malaria Research and Reference Reagent Resource Center (MR4). All *P. falciparum* strains were cultured in human O+ erythrocytes with heat-inactivated 10% pooled human serum as in [34]. For the clonal dilution step, parasites were ultra-diluted into a 96-well plate, to reach a theoretical concentration of 0.2 to 0.5 parasites per well, supplemented with 100 µl of complete medium at 2% haematocrit. The chamber was gassed every 2–4 days with medium changes on day 4, 12 and 20 and 50% dilution with fresh 2% hct medium on day 8, 16 and 24. Positive wells were identified by changes on day 4, 12 and 20 and 50% dilution with fresh 2% hct. The chamber was gassed every 2–4 days with medium changes on day 4, 12 and 20 and 50% dilution with fresh 2% hct medium on day 8, 16 and 24. Positive wells were identified by medium colour change confirmed microscopically after day 16–24 post clonal dilution. The clones were further cultured in either 6-well plates or 10 ml flasks at 4–5% hct. Cultures were pelleted, mixed with RPMI to a total volume of 2 ml and frozen at ~80°C for DNA extraction (see below), with most clones also being frozen in glycerolyte for reculture. After 1–2 months growth, one clone was arbitrarily selected for the next round of clonal dilution.

**DNA extraction, quantification and whole-genome sequencing**

DNA extraction was performed with Qiagen whole blood midi kits [12145] following the manufacturer’s instructions. DNA was quantified using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions, with 1 µl from the extraction step. An average of 2 µg DNA (range 1–5 µg) per sample was submitted in 100 µl volume for PCR-free library preparation for next generation sequencing on the Illumina HiSeq, as described in [33], except without the initial PCR amplification step.

**Mapping to reference genome**

All whole-genome sequencing output samples (100 bp paired-end reads) were processed through the MalariaGEN pipeline, as described in [33]. Briefly, FASTQ files were mapped to the *P. falciparum* 3D7 reference genome (PlasmoDB version 5.3) using BWA with default parameters (See S1 Table for coverage of each sample). For HB3, Dd2 and W2, the resulting BAM files were also mapped to the HB3 and Dd2 genomes from BROAD (assembled in 1187 and 2837 supercontigs, respectively) and to a Dd2 assembly made following the PAGIT protocol [36] (14 chromosomes +636 contigs, by T. Otto, unpublished data). When an in silico control was needed, these reference genomes were modified according to the predicted chromosomal rearrangements. For the discovery of translocations within var genes, samples were also mapped to each var sequence from their respective strain (sequences from [8]). This allows the identification of potential translocations between var genes that are located on the same chromosome.

**SNP calling**

SAMtools mpileup followed by BCTools were used for SNP detection [37]. Parent samples were analysed with the default parameters except for the following changes: Extended BAQ computation, unlimited read depth, use Bayesian inference, "--P" option <0.9 (probability that the site is a variant). Progeny samples were analysed with the following parameters: Extended BAQ computation, unlimited read depth, use Bayesian inference. The strict filter for progeny samples directly removes false positives. As the downstream analysis only keeps *de novo* SNPs called in progeny but not in parent, the looser filter for parent samples set here is also directed to remove false positives. Each progeny VCF file was then parsed using R with the following criteria: variant Quality >120, mapping quality >25, reject bi- or tri-allelic calls, at least 5 non-ref reads, number ref reads less than 20% of all reads. From this output list, each potential SNP was then visualised on LookSeq [38], an online tool in which sequence coverage is represented by a pile-up of blue reads matching the 3D7 reference genome, with mismatches in red. Finally, 95% of all detected SNPs had maximal quality >222 and a mapping quality >54. Note that a "progeny" sample was also analysed as a "parent" if that sample was subcloned.

**Detection of structural variants**

In order to detect translocations involving var genes located on the same chromosome, reads from each sample were mapped with BWA to separate var gene sequences, i.e. each var sequence is considered its own "chromosome". DELLY identifies structural variation (>150 bp) by integrated paired-end and split-read analysis [39]. Paired ends mapping to loci with an increased or reduced spacer distance relative to what is expected based on insert size suggests either a deletion or an insertion, respectively. Face-away reads with increased spacer distance and higher coverage is suggestive of a duplication, and reads mapping to different chromosomes ("trans-locus reads") indicate a translocation (or recombination). DELLY was run on each 3D7-mapped BAM file, as well as all var gene mapped BAM files, with default parameters. For deletions and duplications, outputs were parsed to a minimum of 20 reads and a maximum distance of 50,000. For translocations, outputs were parsed to a minimum of 10 reads. Similar to SNP detection, non-specific hits were discarded by detecting structural variants in progeny that were also identified in parents. All resulting hits were inspected on LookSeq.

Progeny clones from the *P. falciparum* crosses 3D7xHB3 [18] (16 samples +2 parents), 7G8xGB4 [16] (27 samples +2 parents) and HB3xDd2 [17] (30 samples +2 parents) were whole genome sequenced as part of another genetic study (Miles, Pearson et al,
manuscript in preparation). These clones were also searched for var gene translocations.

Validating SNPs and structural variants

SNPs were validated by PCR amplifying a 150–800 bp fragment containing the putative SNP and capillary sequencing the PCR product (S6 Table).

Chimeric var genes identified by DELLY were validated through PCR, by designing primers that bridge the putative translocation site and only amplify product in samples possessing that translocation (S6 Table). The chimeric PCR product was then sub-cloned into a pCR Blunt-end Invitrogen vector backbone and 1–3 positive clones were capillary sequenced.

Quantitative real-time PCR (qPCR) was used to validate copy number variations (CNV) in var genes. Primers were designed flanking the putative translocation site in the PF3D7_0421100-0421300 chimera on chromosome 4. Assay was conducted in triplicate on a 96 well plate using a Roche Light Cycler 480 real-time PCR system. We estimated gene copy number relative to wild type 3D7, and normalized samples against the AMA1 gene (which has one copy in 3D7). Conditions as described in [40,41].

Calculating the SNP mutation and var exon 1 recombination rates

We estimated the SNP mutation rate per life cycle for each generation in the 3D7, Dd2 and HB3 clone trees, based on the number of SNPs identified per clone within that generation, and the number of life cycles since the previous clonal dilution:

\[
M_i = \frac{\sum S}{LG}
\]

Where \(M_i\) is the SNP mutation rate per life cycle for that clonal dilution generation, \(\sum S\) is the total number of SNPs identified for all the clones in that clonal dilution generation, \(\sum G\) is the total number of clones that were analysed in that clonal dilution generation, \(L\) is the number of life cycles that took place between the previous clonal dilution and the current one (see below), and \(G\) is the Genome size, taken as 23.3 × 10^6 bp [42]. The number of life cycles was calculated as follows:

\[
L = \frac{24d}{t}
\]

Where \(d\) is the days in culture between clonal dilutions, 24 to convert to hours, and \(t\) is the estimated life cycle time in hours. \(t\) has been measured in HB3 and Dd2 at 49.7 and 44.1 hours, respectively [43]. 48 hours was used for 3D7.

We took into account the number of clones in each clonal dilution generation to produce a weighted average mutation rate (S5 Table) of 4.07 × 10^{-10}, 3.63 × 10^{-10} and 3.78 × 10^{-10} SNPs per erythrocytic life cycle per nucleotide for 3D7, Dd2 and HB3 respectively. We did not calculate a SNP mutation rate in W2 as the clone tree was not as extensive and we did not feel there was enough data for an accurate calculation. We observed one instance of two SNPs occurring ‘back to back’, i.e. two consecutive nucleotides were mutated from the reference, and one occasion where a single nucleotide separated two SNPs. These were all counted as separate SNP mutations for the rate calculation, though it may be that they represent single ‘mutation events’ at a molecular level.

The Bopp et al [9] published mutation rates for 3D7 and Dd2 in the absence of drug selection was 1.7×10^{-9} and 3.2×10^{-9}, respectively. However, they adjusted their raw data to account for deleterious non-synonymous SNPs that may have been eliminated by purifying selection before being identified. Using their methods, our raw mutation rates become 4.28×10^{-9}, 3.82×10^{-9} and 3.98×10^{-9} SNPs per erythrocytic life cycle per nucleotide for 3D7, Dd2 and HB3 respectively. Thus, there is high consistency both between ours and Bopp et al’s SNP mutation rate estimates and between different P. falciparum lab strains.

Our calculation of exon 1 var gene recombination in the Dd2 clone tree was very similar to our SNP mutation method described above (S5 Table). Because we often observed multiple crossovers per recombining var gene pair, we calculated two recombination rates: First, we calculated the rate at which pairs of var genes recombine in exon 1 in Dd2 as 2.52 × 10^{-3} per life cycle, i.e. a pair of var genes will recombine in ∼0.2% of infected erythrocytes per life cycle. We did not calculate the rate per life cycle per nucleotide because our analysis was focused on var exon 1, so dividing by the total nuclear genome was not appropriate. We therefore also calculated ‘per life cycle’ rates for SNP mutations to enable comparison between var recombination and SNPs. Second, we calculated the rate of var exon 1 crossover events at 3.45 × 10^{-3} per life cycle. This is higher than the rate of recombining var pairs per life cycle because some pairs have >1 crossover.

Supporting Information

S1 Fig Generating clone trees. (A) The 3D7 clone tree. Each box indicates a whole-genome sequenced clone. Sample 3D7_1o was thawed and clonally diluted following whole genome sequence analysis indicated the presence of var gene recombination, to confirm that the mutation was inherited by clonally-derived progeny. (B) The Dd2 clone tree: samples Dd2_{(A)1a} and Dd2_{(B)1b} were further clonally diluted for three generations forming two ‘branches’, referred to as the A and B branches. (C) and (D) show two HB3 clone trees started independently. Unlike all other clone trees, HB3 (c) was initiated from a subclone of HB3. (E) The W2 clone tree. Asterisks on the x-axes indicate when clonal dilutions were performed.

(PDF)

S2 Fig Chimeric var gene created by recombination. This is the same example as simplified in Fig. 3A in the main manuscript. The translocations in clone 3D7_1o involved var genes PF3D7_0426000 (‘var4’, red) on chromosome 4, PF3D7_0937800 (‘var9’, green) on chromosome 9, and PF3D7_0300100 (‘var3’, blue) on chromosome 3, all located in subtelomeres. There is a 2x increase in coverage for the first part of var4 exon 1 in sample 3D7_1o, with the ‘parent’ strain (3D7_P) shown for reference (A). The trans-locus read peak located where the coverage drops is mate-paired with reads mapping to var9 (B). Var9 has zero coverage for the first part of exon 1. The boundary where the coverage starts is identical to the trans-locus reads peak, with mate-paired reads from var4. The chimeric gene therefore had a duplicated first section of var4 exon 1, joined with the remainder of var9. We validated this model by PCR amplifying and capillary sequencing a product bridging the var4/9 translocation site, with no other tested samples producing a band. The translocation was also inherited by all four progeny of 3D7_1o, with the ‘parent’ strain (3D7_P) shown for reference (A). In addition, a second trans-locus read peak in var9 was found in exon 2 (B), with mate pairs mapping to var3 exon 2 (C). Var9 lacks any coverage in exon 2, whilst var3 has 2x exon 2 coverage. We thus suspect three translocation events in clone 3D7_1o, involving chromosomes 4, 9 and 3. The final arrangement left 3D7_1o with intact var4 and var9.
and a chimeric var4-9-3 composed of var4+9 for exon 1 with the exon 2 from var3 (D). TLR = Trans-locus reads.

(PDF)

S3 Fig Example of 7 recombination events within var gene exon2 in 3D7_1b. An example of multiple cross-overs in between the same two var gene is found in clone 3D7_1b, in which most of PF3D7_0300100 was deleted and replaced by a duplicated copy of PF3D7_1373500, as shown by TLR peaks, coverage and validated by PCR amplification. Capillary sequencing the PCR amplicon of the chimeric var3/13 exon 2 revealed that the new exon 2 sequence switched back and forth between var3 and var13 seven times within a ~700 bp distance. The shortest distance observed between recombination break points was only 54 bp, indicating an incredibly active cross-over recombination process. (A) The two upper panels show reads mapped to the exon 2 of PF3D7_0300100 in 3D7_P and 3D7_1b. All other progeny showed the same mapping as the 3D7_P parent. (B) Primers that bridge the var3/13 translocation site (i.e. one annealing to var3, the other to var13) amplified a product in 3D7_1b but not in any other sub-clones tested. Note the fainter band in the 3D7 parent (3D7_P), meaning that the frequency of the var3/13 mutation in 3D7_P was high enough prior to sub-cloning for it to be detectable by PCR. The band from 3D7_1b was gel extracted, sub-cloned into the pCR Blunt-end Invitrogen vector backbone and 3 positive clones were capillary sequenced. This was repeated with an independent primer pair bridging the translocation site. (C) The capillary sequence of this entire exon 2 in 3D7_1b was used to reconstruct the model. The chimeric sequence swaps 7 times from PF3D7_0300100 (red) to PF3D7_1373500 (blue). Blocks where red and blue colours are merged (limits indicated by nucleotide coordinates) correspond to the Identity Blocks. Finally, the var exon 2 of PF3D7_0300100 was replaced by the capillary sequence in the reference genome. All reads from 3D7_135 were mapped to this updated reference genome (lower panel of Fig. A).

(PDF)

S4 Fig Triple recombination event within less than 50 bp. DELLY identified one translocation event between PF3D7_1041300 and PF3D7_0500100 in sample 3D7_1o. (B) is a simplified model of (A). Trans-locus reads on LookSeq are blue when they match the reference sequence and red when there is a mismatch. All mismatches near the DELLY-predicted recombination breakpoint actually correspond to the sequence from the other var that has been translocated. Mismatches and perfect matches indicate the following chimeric sequence from upstream to downstream: PF3D7_1041300 sequence (blue), Identity Block of 11 bp, PF3D7_0500100 sequence (1 bp), second identity block (11 bp), PF3D7_1041300 sequence (1 bp), third identity block (27 bp), PF3D7_0500100 sequence (red). The capillary sequence from sample 3D7_1o confirms this model (although the capillary sequence does not cover the full alignment shown here, it does confirm the triple recombination events).

(PDF)

S5 Fig Duplications in internal var gene clusters can also generate chimeras. Var genes within the body of a chromosome are found in clusters of >4 on chromosomes 4, 8 and 12 in 3D7, and all face the same direction. 'Duplication-chimeras' in our clone tree were formed by duplicating the second half of one var gene and the first half of the var gene immediately adjacent, splicing the two halves together as a chimera. In this example (A), there is 2-fold increase coverage for the second part of PF3D7_1240400 (red) and the first part of the adjacent gene, PF3D7_1240600 (green), on chromosome 12 of all 3D7 samples. This generated a chimeric var gene, validated by PCR, capillary sequencing and transgenerational inheritance of the chimeric product (B). The chimeric gene was deleted in clone 3D7_2b, as shown by PCR and the loss of 2x coverage (C).

(PDF)

S6 Fig Another example of internal var gene clusters generating chimeras. The entire region in our 3D7 parent, containing (at least) four var genes, has double coverage (A), suggesting multiple duplications have occurred in our 3D7 parental strain relative to that used for compiling the 3D7 reference genome. On top of this baseline elevated coverage, clone 3D7_1a appeared to have a further duplication spanning the first half of exon 1 from PF3D7_0421100 and the second half of exon 1 plus the intron and exon 2 from PF3D7_0421300. We predicted this would generate a chimeric PF3D7_0421100-0421300 gene with the two halves of exon 1 linked. To our initial surprise, this model was confirmed by PCR (B) and capillary sequencing as being present in every sample checked, not just clone 3D7_1a. We therefore conjectured that this chimeric var gene was part of the baseline duplications causing elevated coverage here in all samples, but that clone 3D7_1a possessed >1 copy of the chimera. We tested this using quantitative real time PCR (qPCR) of genomic DNA, which confirmed that clone 3D7_1a possessed extra copies of the chimeric sequence (C), whereas three randomly selected control clones (3D7_1h, 3D7_1m and 3D7_1o) all had only one copy. The graph depicts the average 2^-Ct value for the amplification product produced by primers flanking the PF3D7_0421100 - PF3D7_0421300 chimera crossover point. All CP values were normalized against amplification of the AMA1 gene, which has only one copy in the 3D7 genome, and then against sample 3D7_1h. The qPCR suggests that sample 3D7_1a has three copies of the PF3D7_0421100-0421300 chimera. All qPCRs done in triplicate. Note that 'CP' is the value given by the Roche Light Cycler 480 real-time PCR system. Other qPCR machines use a 'Ct' value. (See Methods for further details, and Supplementary S6 Table for primer sequences).

(PDF)

S7 Fig Evolution of a var gene. See also Fig 2 in the main manuscript for a summary. (A) Coverage plots for Dd2var34 and Dd2var45 from LookSeq. The lower part shows trans-locus pair-end reads only. Two peaks are found in sample Dd2_(A)1a and all its progeny (not shown here). The coordinates of these trans-locus peaks correspond exactly to the rise in coverage for Dd2var34 and to the fall in coverage in Dd2var45. (B) Model describing this chromosomal rearrangement: a new chimeric var gene has been created in sample Dd2_(A)1a, the sequence being an hybrid between Dd2var34 and Dd2var45. (C) Trans-locus pair-end reads mapped to a reference genome where the Dd2var34 sequence has been replaced by the chimera Dd2var34-35-43. Note that peaks in Dd2_(A)1a have now disappear. In two of its progeny, Dd2_(A)2g and Dd2_(A)4b, 3 and 1 new translocation peaks have appeared, respectively. (D) PCR with primers overlapping the translocated sequence. In Dd2var34, the primers give a 3974 bp product, which is seen in all clones. In the chimeric var, the primers give a smaller product of 2756 bp, seen only in sample Dd2_(A)1a and its progeny. As predicted, clone Dd2_(A)4b has lost Dd2var34 while clone Dd2_(A)4 1 has lost the chimera Dd2var34-35-43. * Unspecific single read peaks found in all samples.

(PDF)

S8 Fig All var exon 1 translocations identified in this study. Recombination breakpoints are marked by a vertical back line, and linked with the recombining var. The data for this figure can be found in S4 Table. For simplicity, only homology blocks 1 to 10
are represented here, with small colour rectangles above each domain. In two instances, the coverage of trans-locus reads did not allow the identification of the recombination breakpoint coordinates (as it is normally done in S4 Fig, for example), hence the lack of black lines between GB4var21-GB4var43, and GB4var120-GB4var21. NA = unknown chromosomal location.

S9 Fig Group B/C var genes do not necessarily recombine with var genes of highest similarity. Var genes were pairwise aligned within each strain. Percentage of nucleotide identity of the recombining var is shown in red. Group A and E var genes, where no recombination had been observed in our dataset, are shown in dark blue. Nucleotide identities between group B/C var genes ranged from 53 to 99% (average = 66.8%). The two recombining exon 1 var genes had an average of 63% Identity (range 42 to 73%). Data from the 7G8 and GB4 strains is not represented here as their var genes are not fully assembled.

S10 Fig Defining identity blocks. Var B/C genes recombine (A) ClustalW alignment of Dd2var34 and Dd2var45 at a crossover. Example sequence reads mapped to Dd2var34 and Dd2var45 are shown above and below the alignment respectively. Sequence reads are coloured blue when they match the reference and red when there is a mismatch. All mismatches near the recombination breakpoint correspond to the sequence from the other var that has been translocated. The sequence between the mismatches is where the crossing-over event must have occurred, and is by definition identical between both var genes. We term these sequences ‘identity blocks’. (B) Cartoon demonstrating formation of ‘Dm2var34/45’ chimera, with recombination breakpoint occurring within the identity block.

S11 Fig Recombination in var genes occurs where sequences are similar. Dot plot matrix with each pair of recombining var genes indicate the length of each BLAST hit (black line). Location of recombination breakpoints are indicated by red crosses. BLAST was run using default parameters.

S12 Fig Var gene exon 1 recombine in short regions of higher homology. Each plot is a ClustalW2 DNA alignment (default parameters) of a pair of recombining var genes. The Y-axis shows the percentage of identities (overlapping 50 bp windows) between the two sequences. The red triangles indicate where the recombination breakpoint was found. Plots without any triangle mean that the precise breakpoint coordinate could not be identified.

S13 Fig Distribution of var exon 1 recombination within each domain class. Blue bars indicate the frequency of each domain, normalized by their length, relative to all domains of group B and C var genes from the 3D7, HB3 and Dd2 strains. Red bars show the frequency of recombination observed within each domain class (data from S4 Table).

S14 Fig No recombination hotspot within DBL domains. Left panel: DBL domains are made of three subdomains (S1, S2 and S3). Homology blocks (HB) 1 to 5 are also shown, with the 100 bp upstream sequence in red. Right panel: The number of DBL recombination breakpoints within HB 1 to 5 (blue) and in the 100 bp upstream of these HB (red) is plotted. Rask et al postulated the existence of a recombination hotspot at the S2-S3 boundary of the DBL domain, i.e. upstream and within HB2, due to high sequence polymorphism found in seven P. falciparum genomes (Rask et al. 2010). Because there is no immune pressure in vitro, our experiment allows us to differentiate underlying recombination rate from the effects of natural selection. We found no evidence of a recombination hotspot in Rask et al’s locus, suggesting that parasites with recombination breakpoints here are being favoured by natural selection, rather than an increased recombination rate per se.

S15 Fig No conserved motifs within Identity Blocks. (A) ClustalW alignment of 71 Identity Blocks. (B) LOGO representation of the most conserved nucleotides. Note that the ‘A’ and ‘T’ at positions 6, 12, 62 and 67 represent only two identity block sequences, i.e. they are not particularly conserved. (C) Top 3 most common motifs identified by MEME, using Identity Blocks ≥ 8 bp.

S1 Table Genome coverage for each subclone.
S2 Table SNPs identified in clone trees.
S3 Table Structural variants identified in the 3D7 clone tree, represented in Fig. 1.
S4 Table All var gene recombinations.
S5 Table Mitotic point mutation rates and var gene recombination rates measured from clone trees.
S6 Table Primers used for validating point mutations and var gene recombination by PCR amplification and capillary sequencing.
S7 Table Crosses progeny culture time and var gene recombinations.

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Author Contributions

Conceived and designed the experiments: AC WLH JCR DK. Performed the experiments: AC WLH MK AF. Analyzed the data: AC WLH JCR DK. Contributed reagents/materials/analysis tools: TDO. Wrote the paper: AC WLH TDO JCR DK.
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