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

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# Abstract

The human malaria parasite *Plasmodium falciparum* continues to be a major focus for public health research, as elimination efforts confront the spread of artemisinin resistance in South-East Asia. These efforts stand to gain from a deeper knowledge of genetic diversity and recombination in parasite populations, yet *P. falciparum* remains a difficult genome to study. To provide a foundation for ongoing research in these and other areas of *P. falciparum* genome biology, we describe a data resource comprising whole genome deep sequencing of haploid parents and progeny from three genetic crosses. We use the sequence data to construct a map of genome variation within each cross, spanning both coding and non-coding regions, and integrating SNP, INDEL and complex polymorphisms. We confirm that INDELs are exceptionally abundant, being more common than SNPs and thus the dominant mode of polymorphism within the core genome. This map of variation can serve as a robust reference point for studies of variation in natural populations, as well as a tool for fine-grained genotype-phenotype studies within the crosses themselves. We further illustrate the value of this resource by addressing open questions regarding meiotic recombination, including the relative contribution of crossover and non-crossover events, and the factors influencing variation in recombination rate over the genome. Finally, we study a peculiar phenomenon within these crosses whereby several progeny have undergone meiotic recombination within regions of copy number amplification associated with drug resistance, and discuss the implications for the potential of this species to undergo bursts of evolution within genome regions under strong selection.

# Introduction

Genome variation in the eukaryotic pathogen *Plasmodium falciparum* underpins both fundamental biology, such as the ability of the parasite to evade the human immune response, and clinical outcomes, through the evolution of antimalarial drug resistance. This is of particular concern with the recent spread of resistance to front-line therapies in South-East Asia (Ashley et al. 2014). High throughput sequencing is a proven technology for the study of genome variation in *P. falciparum*, and has yielded insights into natural patterns of variation and population structure (Manske, Miotto et al., 2012; Miotto et al., 2013), the generation of antigenic diversity (Claessens et al. 2014) and the genetic basis for artemisinin resistance (Ariey et al. 2014). However, knowledge of genome variation is still incomplete, in part because several factors make the *P. falciparum* genome difficult to study in its entirety. The highly compact 23Mb genome has an extremely biased nucleotide composition, with 80.6% (A+T) content overall and ~90% (A+T) in non-coding regions (Gardner et al. 2002). Despite being free of transposable elements, the genome is highly repetitive, with short tandem repeats and other low complexity sequences unusually abundant in both coding and non-coding regions (Gardner et al. 2002; Zilversmit et al. 2010; Muralidharan & Goldberg 2013; DePristo et al. 2006). Several multi-copy gene families encoding erythrocyte surface antigens are hypervariable, diversifying within the course of a single infection via ectopic recombination (Bopp et al. 2013; Freitas-Junior et al. 2000; Claessens et al. 2014). Other genes associated with the blood-stage exhibit deep and possibly ancient allelic dimorphisms under balancing selection, where one or more allele families are highly diverged from the reference sequence (Roy et al. 2008). These factors challenge both the sequencing technology itself and methods for variant discovery and genotyping from high throughput sequence data. Data on insertion/deletion (INDEL) variation are limited, although there is evidence that INDELs may be unusually abundant (Jeffares et al. 2007; Haerty & Golding 2011; Tan et al. 2010). Little is known about variation in non-coding regions, which could have a significant impact on phenotype by regulating gene expression (Mok et al. 2014; Gonzales et al. 2008). Knowledge of complex variation, where haplotypes are highly diverged from the reference genome, is constrained to a few well-studied loci.

There are many potential sources of error in the process of high throughput sequencing and variant calling (Robasky et al. 2014). Sequencing of replicates provides one strategy for mitigating errors (ibid.). Also, by sequencing related individuals, genotypes that are in violation of Mendelian inheritance can be identified. While a small number of de novo mutations are expected in progeny of a cross, the vast majority of Mendelian inconsistencies will reflect errors in variant discovery and/or genotyping, and thus can be used to calibrate methods and filter data (Saunders et al. 2007). Unfortunately, although pedigrees can be readily obtained for many species, this is currently not the case for *P. falciparum*. The parasite is sexually reproducing but remains haploid for the majority of its life cycle, existing only briefly as a diploid when taken up into the mosquito mid-gut where it undergoes meiosis (Walliker et al. 1987). Crossing *P. falciparum* parasites currently involves a laborious and difficult process of mixing clones that are fed to mosquitoes, which are then used to generate blood stage parasites by biting non-human primates. As a result, only three experimental crosses have been published to date, where they have led to key discoveries regarding the causes of drug resistance (Wellems et al. 1991) and host specificity (Hayton et al. 2008). However, although only a limited number of crosses are currently available, typically more than 30 genetically distinct progeny clones can be obtained from a single cross (Ranford-Cartwright & Mwangi 2012). The large number of progeny provides a higher power to observe Mendelian errors than smaller pedigrees or trios, and thus to identify variants which are spurious or where genotyping is unreliable. These three crosses are therefore a precious resource for studying genome variation, because biological replicates can be readily obtained by *in vitro* culture methods, and because they represent the only *P. falciparum* experimental system within which Mendelian errors can be observed and used to guide variant discovery.

Here we describe a data resource comprising whole genome deep sequencing of parents and progeny of three experimental *P. falciparum* genetic crosses, involving the parental clones 3D7, HB3, Dd2, 7G8 and GB4, representing a broad range of genetic and phenotypic diversity (Ranford-Cartwright & Mwangi 2012). We use a combination of methods for variant discovery, leveraging both alignment of sequence reads to the 3D7 reference genome (DePristo et al. 2011; Li & Durbin 2009; McKenna et al. 2010) and reference-free sequence assembly (Iqbal et al. 2012) to build a map of genome variation within each cross incorporating SNP, INDEL and complex polymorphisms. All of the variants included in the final call sets are highly consistent with Mendelian inheritance and show almost perfect genotype concordance between biological replicates, and hence could serve as a positive training set to underpin variant discovery in other studies, particularly those surveying variation in natural populations. These data also reveal some interesting features of genome variation between the parental clones, including an exceptionally high abundance of INDELs relative to SNPs. Because these data are a rich resource and valuable for further research and education, we describe a novel web application providing a means for exploring and interacting with the data in an intuitive way.

We also address open questions regarding meiotic recombination in *P. falciparum*. Previous studies using lower-resolution genotyping methods have estimated crossover (CO) recombination rates (Su et al. 1999; Jiang et al. 2011; Walker-Jonah et al. 1992) and provided evidence for non-crossover (NCO) recombination (Su et al. 1999). One study used high-throughput sequencing to resolve recombination events in two progeny of the HB3xDd2 cross, finding evidence that rates of CO and NCO recombination may be similar (Samarakoon, Regier, et al. 2011). Previous studies have also demonstrated that at least some recombination events occur within coding regions (Kerr et al. 1994) and suggested that recombination events are not uniformly distributed over the genome (Jiang et al. 2011). Here we combine SNP and INDEL markers to obtain a resolution of ~300bp within each cross, which is sufficient to differentiate between crossovers and non-crossovers, and estimate rates for both types of recombination event. This resolution is also sufficient to resolve the location of most recombination events relative to gene and exon boundaries and study spatial variation in recombination rates. Finally we investigate recombination in the context of two large regions of copy number amplification, both of which segregate in the crosses and are associated with drug resistance (Wellems et al. 1990; Samarakoon, Gonzales, et al. 2011). We find evidence for crossover recombination within these amplifications leading to regions of pseudo-heterozygosity within progeny clones. This has implications for evolutionary biology, because it demonstrates a mechanism whereby fitness costs associated with a drug resistance mutation could be compensated, and different copies of an amplified gene could diverge more rapidly than by point mutation alone.

# Results

## Whole genome sequencing and genome accessibility

Whole genomes of parent and progeny clones from the crosses 3D7×HB3 (Walliker et al. 1987), HB3×Dd2 (Wellems et al. 1990; Wellems et al. 1991) and 7G8×GB4 (Hayton et al. 2008) were sequenced using Illumina high throughput technology, with the majority of samples obtaining an average depth above 100X (Table S1). All DNA libraries were derived from haploid parasite clones in culture, and sufficient DNA was available to use PCR-free library preparation throughout, which has been shown to reduce some of the biases associated with the AT-rich *P. falciparum* genome and hence improve the evenness of coverage across both coding and non-coding regions (Kozarewa et al. 2009). PCR can also induce false INDELs, providing an additional motivation for using PCR-free libraries (Fang et al. 2014). The clone HB3 is a parent in two crosses, however because DNA samples were obtained from different sources and had different culturing histories the two HB3 clones were sequenced and genotyped separately, and are here labelled HB3(1) and HB3(2) for crosses 3D7×HB3 and HB3×Dd2 respectively. For several progeny clones biological replicates were obtained, where libraries were created from DNA extracted from different cultures of the same parasite clone. These were also sequenced and genotyped separately to enable analysis of concordance between replicates. All sequence data has been deposited at the European Nucleotide Archive and a mapping from clone identifiers to ENA accessions can be obtained via the web application at <http://www.malariagen.net/apps/pf-crosses> where sequence alignments can also be browsed interactively.

Sequence reads from all samples were aligned to the 3D7 reference genome and various metrics were calculated per genome position including depth of coverage and average mapping quality. Visual examination of these data revealed a clear, qualitative difference between acore genome (20.8Mb) comprising regions of near-complete coverage and unambiguous alignments in all samples; hypervariable regions(1.9Mb) where accessibility is severely affected by both extensive paralogy and extreme divergence from the reference genome; and subtelomeric repeat regions (0.6Mb) where accessibility is limited by repetitive sequence (Supplementary Information; Figures S1-S3; a BED file defining the boundaries of these regions can be downloaded from the FTP site[[11]](#footnote-11)). Hypervariable regions contained all genes in the *var* family, which are known to undergo frequent non-allelic recombination during mitosis (Bopp et al. 2013; Claessens et al. 2014), and almost all genes in the associated *rif* and *stevor* families. Hypervariable regions also corresponded closely with regions of heterochromatin (Flueck et al. 2009) confirming a strong association between chromatin state and qualitative differences in genome variability. All samples exhibited some degree of bias such that coverage was lower where (A+T) content was above 80%, however the high depth of sequencing meant that more than 99.6% of the core genome was covered in all parental clones. Because of the poor accessibility of hypervariable and subtelomeric repeat regions we excluded them from further study and limit ourselves to the core genome for the remainder of this paper. However, raw variant calls and other data for hypervariable regions can be obtained via the accompanying data and web application, and may be of use to others studying these immunologically important regions.

## SNP, INDEL and complex variation within the core genome

SNPs, small INDELs and regions of complex polymorphism were discovered and genotyped within each cross by two independent methods, one based on alignment of sequence reads to the 3D7 reference genome (DePristo et al. 2011; Li & Durbin 2009), the other based on partial assembly of sequence reads and comparison of assembled contigs (Iqbal et al. 2012). Variants where genotype calls in one or more progeny clones were inconsistent with Mendelian segregation (Mendelian errors) were used to calibrate the outputs of both calling methods and decide variant filtering strategies (Methods and Supplementary Information; Figures S4-S7). After variants were filtered, both methods achieved near-perfect concordance between biological replicates for both SNPs and INDELs (Table S2) demonstrating that the process from DNA extraction through sequencing and variant calling was highly reproducible. The patterns of inheritance of parental alleles within the progeny of each cross were also highly consistent when comparing SNPs with INDELs (Figure S8) or comparing results of the two variant calling methods (Figures S6 and S7).

The near-perfect reproducibility, concordance between calling methods and highly parsimonious patterns of inheritance and recombination indicate that variants discovered here correspond to genuine genetic differences segregating within the crosses. However, some variants may be false discoveries, in the sense that the variant allele is not correctly ascertained. To estimate rates of false discovery and sensitivity we compared variant alleles called in each of the HB3 replicates with the HB3 draft assembly (Birren et al. 2006) and publicly available HB3 gene sequences (Supplementary Information). We estimated a SNP FDR between 0.6-2.7% for the alignment-based calling method and between 0.0-1.1% for the assembly-based calling method (Table S4). INDEL FDR estimates were higher for both calling methods, in the range 8.3-12.5%, however we noted a number of discordances between previously published sequences, making accurate FDR estimation difficult (Supplementary Information). For sample HB3(1) SNP sensitivity was above 84% and INDEL sensitivity was above 70% for both calling methods, however sensitivity was lower for HB3(2) particularly for the assembly-based calling method (Table S4). This lower sensitivity was partly due to the fact that the assembly-based calling method was only capable of genotyping biallelic variants. All segregating variants in the 3D7xHB3 cross are expected to be biallelic because 3D7 is the reference clone, however segregating variation in the HB3xDd2 and 7G8xGB4 crosses could be multiallelic if each parent has a different non-reference allele at a variant site. This technical limitation accounts at least in part for the lower number of segregating INDELs discovered in the HB3xDd2 and 7G8xGB4 crosses compared with 3D7xHB3 (Table 1).

To provide the greatest possible resolution for the present study, filtered variants called by each method were combined into a single call set for each cross (Methods and Supplementary Information). All variant calls can be downloaded from a public FTP site[[12]](#footnote-12) or browsed via the web application at <http://www.malariagen.net/apps/pf-crosses>.

### INDELs are the most abundant form of polymorphism

Analysis of the combined variant call sets revealed that, within the core genome, segregating INDELs were more abundant than SNPs in all three crosses (Table 1). Overall 83% of INDELs were found in non-coding regions, where INDELs were 3 times more abundant than SNPs. INDELs were also relatively abundant in coding regions, with the ratio of SNPs to INDELs being approximately 2:1. This relative abundance of INDELs is exceptionally high when compared with other species, for example a comparative study found a SNP to INDEL ratio of approximately 10:1 in primates and 20:1 in bacteria (Chen et al. 2009). The vast majority of INDELs found in the crosses were expansions or contractions of short tandem repeats (STRs), i.e., microsatellites (Figure 1A). In non-coding regions 83% of INDELs were STR length variations, of which 71% were variations within poly(AT) repeats. In coding regions 77% of INDELs were STR variations, of which the majority were within poly(asparagine) tracts (Figure 1B). Tandem repeat sequences are prone to slipped strand mis-pairing during DNA replication (Li et al. 2002; Lovett 2004) and are known to be associated with high rates of INDEL mutation (Montgomery et al. 2013). Poly(AT) repeats are very common in the non-coding regions of the *P. falciparum* genome (Gardner et al. 2002) and poly(asparagine) repeats are unusually abundant within the exome (Muralidharan & Goldberg 2013; Tan et al. 2010), hence the high INDEL diversity overall may be accounted for by the abundance of STRs in the genome, coupled with the high mutability of tandem repeats due to replication slippage.

Regarding the phenotypic consequences of INDEL variation, frame shift mutations within coding regions are expected to have severe consequences and hence be negatively selected. Here, 94% of coding INDELs were found to be size multiples of 3 and hence preserved the reading frame (Figure 1A). Within non-coding regions, the potential consequences of INDEL mutations are harder to predict. Relatively little is known about the transcription machinery in *P. falciparum*, however, Brick et al. (2008) predicted the location of core promoters upstream of genes based on a training set of known transcription start sites. Intergenic INDEL diversity in the crosses was found to display a specific architecture relative to the central positions of these predicted promoters, with an excess of non-STR INDELs within the first 50bp upstream of the promoter centre, and a deficit of STR INDELs 100-200bp upstream and 0-100bp downstream of the promoter centre (Figure 1C). Further experimental work will be required to determine whether these INDELs have any impact on promoter activity.

### Low nucleotide diversity is punctuated by complex variation in merozoite-stage genes

Average nucleotide diversity across the core genome was low (5x10-4) in all three crosses (Table 1). However, this low diversity was punctuated by 19 loci with highly diverged alleles, where local diversity over a region up to 2kb was up to 3 orders of magnitude greater (Figure 2). These divergent loci were found almost exclusively within coding regions of genes associated with the merozoite life cycle stage, where the parasite is briefly exposed to the host immune system before invading another erythrocyte, and include several well-studied merozoite surface antigens. The most extreme example was MSP1, a highly expressed protein located at the merozoite surface, where several regions of the gene are known to exhibit deep allelic dimorphism (Ferreira et al. 2003). The complex variation at these loci could not be accessed by the alignment method, because sequences were too diverged from the reference genome, and hence coverage was locally patchy or non-existent (Figure S9). However, the assembly method was able to construct complete and correct sequences for all parents and progeny in the two main divergent regions of msp1 (blocks 4-11 and blocks 13-16 (Ferreira et al. 2003)) as verified by comparison with publicly available capillary sequence data for clone HB3. Other genes where peaks of diversity were found and alleles could be assembled include four members of the *msp3* family (*msp3*, *msp6*, *dblmsp*, *dblmsp2*), members of the *surf* family (*surf1.2*, *surf4.1*, *surf4.2*, *surf8.2*, *surf13.1*, *surf14.1*) and PF3D7\_0113800 (encoding a DBL-containing protein with unknown function on chromosome 1). A notable exception to the pattern of merozoite expression is PF3D7\_0104100 which is transcribed by the sporozoite specifically within the mosquito salivary gland, suggesting involvement in the early stages of infection (Lasonder et al. 2008). Several of these genes are blood-stage vaccine candidates and/or are being actively studied for their role in erythrocyte invasion, and comprehensive knowledge of variation at these loci is essential for the design of effective vaccines and invasion assays. These results show that complex variation in a number of key *P. falciparum* genes can be ascertained in a high throughput manner via assembly of short sequence reads.

## Meiotic crossover and non-crossover recombination

These crosses are currently the only available experimental system for *P. falciparum* where meiotic recombination can be directly observed. No previous study has had comparable data for all three crosses, or had the resolution available from deep whole genome sequencing, making this study uniquely able to provide definitive answers regarding the extent and features of meiotic recombination inthis species. For each cross, SNP and INDEL variants combined from both calling methods were used as a set of segregating markers for analyses of meiotic recombination (Table 1). The average distance between markers was ~300bp in all three crosses, at least an order of magnitude greater resolution than available previously (Jiang et al. 2011). In eukaryotes, programmed double strand breaks (DSBs) during meiosis are resolved by either crossover (CO) or non-crossover (NCO) between homologous chromosomes (Hastings 1992; Youds & Boulton 2011; Mancera et al. 2008; Baudat & de Massy 2007). A CO is a reciprocal exchange accompanied by a conversion tract, whereas an NCO is a conversion tract without reciprocal exchange (also known in the literature as a gene conversion, although NCO events can occur in either coding or non-coding regions; see also Figure 1 in Youds & Boulton (2011)). An algorithm was used to infer CO and NCO events from the size and arrangement of parental haplotype blocks transmitted to the progeny, and to identify both simple conversion tracts (all alleles converted to the same parent) and complex conversion tracts (containing switches between parental alleles) (see Methods and Supplementary Information). Because occasional genotyping errors could also manifest as short inheritance blocks, all putative conversion tracts supported by only a single marker or with a minimal length less than 100bp were excluded. This yielded a total of 1194 COs, 230 NCOs and 331 conversion tracts for further analyses (Figure S11).

### Gene coding regions are warm-spots and centromeres are cold-spots of CO recombination

Combining CO events from all three crosses, the total map length of the core genome was 15.7 Morgan (95% confidence interval: 14.8-16.6). The total marker span of the physical chromosomes was 21.16Mb giving an average CO recombination rate of 13.5 kb/cM (95% confidence interval: 12.7-14.3). The CO recombination rate varied between crosses, with 3D7 x HB3 highest (17.7 Morgan), HB3xDd2 intermediate (16.0 Morgan) and 7G8 x GB4 lowest (14.3 Morgan), although this difference was marginally significant (P=0.06, Kruskal-Wallis H-test) (Figure 3A). There was a strong linear correlation between chromosome size and CO recombination rate, with a rate of 0.55 Morgan predicted for the smallest chromosome (Figure 3B) consistent with ~0.5 Morgan expected if crossovers play an essential role in chromosome segregation and thus the recombination rate is calibrated to produce at least one CO per bivalent (Baudat & de Massy 2007; Martinez-Perez & Colaiácovo 2009; Mancera et al. 2008).

The centromeres were cold-spots of CO recombination, as expected from studies in other eukaryotes and confirming previous data from the 7G8xGB4 cross (Jiang et al. 2011), although the effect was highly localised (Figure 3C). Within ~30kb of the centromere the CO rate was significantly lower, however between ~80-120kb from the centromere the rate was slightly higher than average. Due to the high marker density, in many cases it was possible to resolve the location of CO events relative to individual gene and exon boundaries. Of the 1194 CO events, 396 (33%) were observed within a gene (intragenic COs), 162 (13%) were within an intergenic region, and 636 (53%) were ambiguous (flanking markers spanned a gene boundary). The number of intragenic CO events was significantly higher than expected if CO events were distributed uniformly over the genome (P=0.001 by Monte Carlo simulation). Of the 396 intragenic COs observed, 298 (75%) occurred within an exon, 3 (1%) were within an intron, and 95 (24%) spanned an exon boundary. The number of COs observed within an exon was also significantly higher than expected if COs occurred uniformly within genes (P<0.001 by Monte Carlo simulation). Thus a substantial fraction of all CO events were intragenic and occurred within coding regions, in contrast with humans where the majority of recombination occurs within hotspots that preferentially occur near genes but outside of the transcribed domain (Myers et al. 2005).

### Estimation of conversion tract length and NCO recombination rate

Of the 331 conversion tracts observed, an outlying group of 7 very long (>18kb) complex tracts was found (described further below). Of the remaining 324 tracts, 94 were associated with a CO and 230 were assumed to be NCO conversion tracts. 50% of observed NCO conversion tracts had a minimal size less than 1kb and 73% were smaller than 2kb (Figure 3D). The relatively small size of conversion tracts and the available marker density means that some NCO events will not have been observed, because we required tracts to span at least two markers and more than 100bp. To estimate the NCO recombination rate and true tract length distribution, the incomplete discovery of NCO events and bias towards discovery of longer tracts has to be taken into account. In *Drosophila* the distribution of conversion tract lengths was found to fit a geometric model, with parameter *phi* determining the per-base-pair probability of extending a tract (Hilliker et al. 1994). We found that a geometric model also provided a good fit for the observed distribution of tract lengths in the present study with *phi* = 0.9993, although there was a small excess of tracts observed with minimal length greater than 3kb (Figure 3D, Figure 3E). Assuming this model for the tract length distribution, simulations predicted an NCO discovery rate of 40% for HB3 x Dd2, 39% for 7G8 x GB4, and 45% for 3D7 x HB3 where the marker density was slightly higher.

Adjusting for incomplete discovery, the average rate of NCO recombination over all three crosses was estimated at 7.5 NCO/meiosis (0.36 NCO/meiosis/Mb), thus COs are roughly twice as common as NCOs events. The 95% confidence interval for the NCO recombination rate based on sampling error is 6.8-8.1 NCO/meiosis, however this does not account for additional uncertainty in the estimation of NCO discovery rates for each cross. There was a linear correlation between chromosome size and NCO rate (Figure 3F) however the correlation was weaker than for the CO recombination rate, presumably due to the fewer number of observed NCO events and thus greater sampling error. As with CO events there was a significant enrichment of NCO events within genes (P=0.002 by Monte Carlo simulation) with 37 (16%) NCO conversion tracts falling entirely within a gene, 110 (48%) spanning a gene boundary, 35 (15%) entirely spanning a gene, and 14 (6%) intergenic.

As mentioned above, 7 apparently long (>18kb) complex conversion tracts were found. Two of these tracts occurred in clone JF6 (7G8 x GB4) within a 60kb region on chromosome 11, and thus appear to be part of a single complex long-range recombination event involving a total of 20 switches in inheritance (Figure S12). Two biological replicates of clone JF6 were sequenced and genotyped in this study, and the pattern of recombination was identical in both replicates. Similar observations were made for clone C04 (3D7 x HB3) where a 70kb region on chromosome 14 accounted for 12 switches in inheritance, and clone 3BD5 (HB3 x Dd2) where an 80kb region on chromosome 10 contained 13 switches (Figure S12). At all of these loci there was no evidence of copy number variation or other artefacts that could manifest as an apparent excess of recombination. These observations do not fit well with conventional DSB repair pathways leading to normal CO and NCO events, suggesting other repair pathways may also be utilised during meiosis (Mancera et al. 2008) that have more radical results in terms of generating novel haplotypes.

## Recombination within copy number variations spanning drug resistance genes

Clone Dd2 is known to have a three-fold amplification spanning the multi-drug resistance homologue *mdr1* which confers mefloquine resistance (Cowman et al. 1994). This amplification is also known to segregate in the progeny of HB3xDd2 (Wellems et al. 1990) with evidence for meiotic recombination within the amplified region in two progeny clones (Samarakoon, Gonzales, et al. 2011). Amplifications have also been found spanning *gch1*, conferring resistance to anti-folate drugs, in both HB3 and Dd2, although the amplifications are different in size and extent (Kidgell et al. 2006; Heinberg et al. 2013). The *gch1* amplifications have been shown to segregate in the progeny of HB3xDd2, although one progeny clone (CH3\_61) appeared to inherit both parental amplifications superposed (Samarakoon, Gonzales, et al. 2011). Some form of recombination within the amplified region could explain this phenomenon, although the exact nature of the recombination remains uncertain. All 5 parental clones have been shown to carry some form of amplification spanning *gch1* (Kidgell et al. 2006; Sepúlveda et al. 2013) thus the sequence data generated in this study provide an opportunity to elaborate on previous results for HB3xDd2 and extend the analysis of CNV transmission and recombination at drug resistance loci to 3D7xHB3 and 7G8xGB4.

We combined data on depth of sequence coverage and the orientation of aligned read pairs to study CNV alleles in all three crosses. The sequence data confirmed a three-fold amplification in Dd2 spanning *mdr1* and transmission as either 2 or 3 copies to 14 progeny of HB3xDd2 (Figure S13). Evidence for amplifications spanning *ghc1* was also clear in all parental clones (Figure 4). The 3D7 reference genome has only a single copy of *gch1*, however all studies including ours have found the 3D7 clone to carry multiple copies of *gch1*, suggesting an error in the reference sequence. Parental amplifications spanning *gch1* all differed in extent and copy number, confirming previous findings (@@REFs). Alignment of read pairs indicated that the Dd2 amplification was arranged as a tandem inversion, whereas 3D7, HB3 and 7G8 carried tandem duplications, adding further evidence for the independent origin of these CNV alleles (@@REFs). The HB3(2) sample appeared to be a mixture with approximately 20% of parasites retaining the duplication found in HB3(1) and 80% having no amplification, which is not unexpected given that amplifications can be lost in culture in the absence of drug pressure (Cowman et al. 1994). Transmission of *gch1* CNV alleles was consistent with Mendelian segregation in the progeny of all three crosses except for two progeny of 3D7xHB3 (C05, C06) and one progeny of HB3xDd2 (CH3\_61) where both parental alleles appeared to be inherited together (Figure 4).

#### Recombination within amplified regions leads to pseudo-heterozygosity

To further explore the apparent non-Mendelian inheritance of CNV alleles, we considered possible recombination events that could explain the observed patterns of inheritance. Depending how homologous chromosomes align during meiosis, a crossover within a region that is duplicated in one parent could result in a daughter that maintains the same duplication but inherits one copy from either parent for some portion of the amplified region. Within such a segment a haploid individual would become effectively diploid heterozygous for any SNP, INDEL or smaller CNV variants within the segment that segregated between the two parents. We used the alignment of sequence reads from each progeny clone to the 3D7 reference genome to look for evidence of pseudo-heterozygosity and thus recombination within amplified regions. Within a region of pseudo-heterozygosity, reads supporting each parental allele should appear in a roughly 1:1 ratio, whereas elsewhere one or the other parental allele should be found almost exclusively.

At the *gch1* locus, both clones C05 and C06 inherited the large 161kb duplication from parent HB3 as well as the smaller 2kb 4-fold amplification from parent 3D7 spanning *gch1* only (Figure 4). C06 had a region of heterozygosity spanning the leftmost 130kb of the region duplicated in HB3, but was apparently homozygous for the remainder of this region (Figure 4B). The most parsimonious explanation is that a single crossover occurred within the region duplicated in HB3. Clone C05 had a region of heterozygosity spanning the entire region duplicated in HB3, with borders that appeared to coincide closely with the breakpoints of the duplication (Figure 4C). This is harder to explain, as it would require two crossover events at or close to the borders of the duplicated region, which would seem improbable unless the CNV breakpoints are also prone to meiotic crossover (Völker et al. 2010). An alternative explanation is that the HB3 CNV is not a tandem duplication and one copy of the region has been translocated to a different chromosome, however read orientation evidence clearly indicated that the region is tandemly arrayed in both HB3 and C05. For both clones C05 and C06 *gch1* itself lay within the region of effective heterozygosity, thus one copy of *gch1* was inherited from HB3 and 4 copies from 3D7. At the same locus clone CH3\_61 inherited the 161kb duplication from HB3 as well as the 5kb 3-fold tandem inversion from Dd2 (Figure 4E). Two separate regions of heterozygosity were visible at either ends of the HB3 duplicated region, which can be explained if two crossover events occurred. Again *gch1* was within the region of heterozygosity, and thus CH3\_61 acquired 1 copy from HB3 and 3 copies from Dd2. We also confirmed previous evidence for recombination within the 82kb amplification spanning *mdr1* in two progeny of HB3xDd2 (Samarakoon, Gonzales, et al. 2011). Clone QC23 had a region of heterozygosity spanning the leftmost 16kb of the segment, and CH3\_61 was heterozygous for the rightmost 40kb spanning *mdr1* itself (data not shown). Both of these are consistent with a single crossover having occurred within the amplified region.

## A web application to facilitate data exploration and re-use

The sequence and variation data generated in this study are a rich resource and could serve many purposes beyond the analyses presented here. To facilitate re-use of these data we developed a web application that provides a number of novel tools for intuitive, interactive data exploration, available at <http://www.malariagen.net/apps/pf-crosses>. The application includes a tool for browsing and querying a table of variants for each call set (Figure 5B); a tool for visualising and browsing the genotype calls at individual samples and patterns of inheritance and recombination within each cross (Figure 5C); a tool for browsing the genome, allowing the location of variants to be viewed in the context of genome features and alignment metrics (Figure 5D); and a browser for visualising the sequence alignments themselves, implemented by embedding the LookSeq software (Manske & Kwiatkowski 2009) (Figure 5E). All tools are highly interactive, for example when browsing genotypes in parents and progeny the user can hover over any variant and view further information about the reference and alternate alleles, effect prediction, etc. Filters applied to variants can also be changed dynamically, allowing users to explore the entire dataset and compare calling and filtering methods. For the genome browser, a multi-resolution filterbank was implemented to enable highly responsive browsing at all scales from base-pair resolution up to entire chromosomes. The underlying technologies for this web application are being developed as a generic framework so that they can be used with other datasets, as part of an open source project[[13]](#footnote-13) that will be described in detail elsewhere.

# Discussion

@@TODO discuss apparent preference for recombination in coding regions, due to higher GC?

@@TODO find a home for CNV discussion text ... Amplifications spanning *mdr1* are found in clinical isolates, particularly from South-East Asia, and both point mutations and amplifications of *mdr1* have been shown to alter sensitivity to a range of drugs (Price et al. 2004; Anderson et al. 2009).

@@TODO compare NCO rates with Samarakoon, Regier et al.

@@TODO discuss recombination in CNVs in relation to Samarakoon, Gonzales. Emphasise what is novel?

We have presented a resource comprising deep sequence data for three *P. falciparum* genetic crosses. These three crosses are a unique asset in the study of *P. falciparum* and remain an important resource for experimental and genome biology. The development of sequencing technology continues apace, and within the next few years it may be possible to re-sequence these crosses with longer reads and ultimately to fully assemble genomes for all parents and progeny. Meanwhile, these data are a valuable foundation for studies that use current sequencing technology to investigate *P. falciparum* in the lab and the field, and provide a useful benchmark against which new sequencing technologies and methods for variant discovery and assembly can be evaluated.

Using a combination of alignment and assembly methods we have constructed a high resolution map of genome variation within each cross, integrating SNP, INDEL and complex polymorphisms, and spanning both coding and non-coding regions of the core genome. These data have been calibrated using Mendelian errors and verified using sequencing of biological replicates, and thus represent a well-validated resource. They demonstrate that INDELs are the most abundant form of polymorphism within the core genome, in stark contrast to other eukaryotes for which genome-wide data are available. This is perhaps not surprising, considering the abundance of tandem repeats and other low complexity sequences in both coding and non-coding regions of the *P. falciparum* genome (Gardner et al. 2002; DePristo et al. 2006; Zilversmit et al. 2010; Muralidharan & Goldberg 2013) and the evidence that replication slippage causes high variability in short tandem repeats (Lovett 2004; Li et al. 2002; Tan et al. 2010). Nevertheless, the comprehensive data on INDEL variation presented here fill an important gap in current knowledge. The data also provide the first genome-wide view of non-coding variation, which is almost completely unexplored in *P. falciparum*, yet could have important phenotypic consequences (Mok et al. 2014). Increased expression of genes through copy number amplification is known to be an important component of resistance to anti-malarial compounds (Price et al. 2004; Heinberg et al. 2013) although comparable increases of expression can also be obtained through variation in promoter and regulatory elements (Li et al. 2002; Anderson et al. 2009). Using the HB3xDd2 cross, Gonzales et al. (2008) showed that both *cis* and *trans* genetic variation influences gene expression in *P. falciparum*, including a major *trans* regulatory hotspot coinciding with the amplification spanning *mdr1.* Also of relevance, short tandem repeat length variations within regulatory elements have been shown to affect gene activity across a number of species (Li et al. 2002). We found that INDELs are exceptionally abundant in non-coding regions within *P. falciparum*, and furthermore that INDEL diversity has a particular architecture in relation to predicted core promoters, which may indicate that these mutations are non-neutral. @@TODO relate to fungus paper. Experimental work is now required to investigate the phenotypic consequences of these mutations.

It has previously been difficult to perform high throughput studies of complex polymorphisms observed in genes expressed at the merozoite surface, including *msp1* (Roy et al. 2008; Ferreira et al. 2003) and members of the *msp3* family (Singh et al. 2009). High levels of variability have also been reported in other genes associated with the merozoite stage, such as the *surf* family (Kidgell et al. 2006; Winter et al. 2005), although it is not clear whether these loci exhibit deep allelic dimorphism as seen in *msp1*. The presence of diverged allelic forms and the fact that many of these genes appear to be under balancing selection indicates their clinical importance as potential targets of the immune system and/or components of the erythrocyte invasion machinery. These loci are typically inaccessible to alignment-based methods, because some alleles are too diverged from the reference genome. Thus studies of these genes have so-far relied on more labour-intensive methods, and genome-widediscovery of other loci with similar variation has not been possible. Here we have shown that an assembly-based variant-calling method can access variation at a number of divergent loci, including *msp1* and members of the *msp3* and *surf* families. Some known divergent loci, including *msp2* and *S-antigen* could not be accessed by either calling method due to the presence of longer repeats, and longer sequence reads will be required to access complex variation at all antigenic loci. Also the crosses studied here are clonal samples, and further work will be required to adapt these methods for use on clinical isolates which may comprise a mixture of parasite genotypes. However, these data provide a foundation for high-throughput surveys of complex variation at a number of clinically important loci across natural populations.

*P. falciparum* is a sexually reproducing eukaryotic pathogen, and these crosses originally provided the first demonstration that parasites undergo meiotic recombination whilst in the mosquito (Walliker et al. 1987). These crosses remain the only available experimental system for studying meiotic recombination in *P. falciparum*, due to the practical difficulties associated with generating a cross. Previous work using individual crosses demonstrated a high rate of crossover recombination relative to eukaryotes with larger genomes (Su et al. 1999; Jiang et al. 2011) being approximately 50 times greater than the average recombination rate in humans. Here we have pooled data from all three crosses and used a ~300bp resolution map of SNP and INDEL variation to provide the first estimates for both crossover (CO) and non-crossover (NCO) recombination rates within the core genome and the distribution of conversion tract lengths. @@TODO ensure proper citation of Samarakoon, Regier et al. We confirm a CO recombination rate in the range 12.7-14.3 kb/cM and estimate that CO events are approximately twice as frequent as NCO events. Conversion tract lengths are comparable to yeast (Mancera et al. 2008) but longer than humans (Jeffreys & May 2004) and *Drosophila* (Hilliker et al. 1994) and the observation of long-range complex recombination events spanning >60kb observed in some progeny remains to be explained. In higher eukaryotes the recombination rate is known to be highly variable over the genome, with most recombination concentrated within narrow hotspots (Myers et al. 2005). Previous work on the 7G8xGB4 cross suggested that the rate of recombination may not be uniform over the *P. falciparum* genome (Jiang et al. 2011). Here we confirmed that the CO recombination rate was lower within a highly localised region surrounding the centromeres, and found that CO events occurred with appreciable frequency in both coding and non-coding regions, with a significant excess of events within coding regions relative to a model of uniform recombination. While population data will be required to robustly evaluate the support for different hotspot models, the fact that we observe recombination events frequently in both coding and non-coding regions of the core genome, and that these different regions have very different nucleotide composition and sequence characteristics, suggests that a model of highly punctuate recombination targeted at specific sequence motifs is unlikely, and that the location of recombination events may be less constrained in *P. falciparum*. Note that these findings apply only to the core genome, and entirely different processes may operate within hypervariable regions (Claessens et al. 2014).

There are multiple lines of evidence that gene amplification plays a key role in the evolution of drug resistance in *P. falciparum* (Price et al. 2004; Anderson et al. 2009; Nair et al. 2008; Kidgell et al. 2006; Heinberg et al. 2013; Triglia et al. 1991; Cowman et al. 1994; Thaithong et al. 2001). We have confirmed that amplifications spanning the multi-drug resistance gene *mdr1* segregate in the HB3xDd2 cross, and shown that pleiomorphic amplifications spanning the anti-folate resistance gene *gch1* segregate in all three crosses, thus the data resource described here provides a controlled setting in which to study copy number variation at drug resistance loci and observe it in the context of meiotic recombination. We have extended the previous observation of a recombination event within the *gch1* amplification in the HB3xDd2 cross (Samarakoon, Gonzales, et al. 2011) to illustrate four other cases of recombination within amplified regions, and shown that all of these events are associated with regions of effective heterozygosity within a progeny clone where both parental sequences are inherited and maintained within a single haploid genome. Such events could have several evolutionary consequences. Firstly, drug resistance mutations may confer a fitness cost relative to the wild type allele in the absence of drug pressure (Kondrashov 2012; Anderson et al. 2009; Rosenthal 2013) and can also confer both resistance to one class of drugs and sensitivity to another (Anderson et al. 2009). The process of amplification followed by homologous recombination could provide a mechanism by which both mutant and wild type alleles are acquired, compensating for fitness costs associated with either allele alone. Secondly, the same process could enable copies of an amplified region to diverge in jumps and thus more rapidly than if by the gradual accumulation of mutations alone.

Taken together, this data resource provides a concrete illustration of how different modes of mutation and recombination could interact to create an evolutionary system that is more than the sum of its parts. Although we have not attempted to precisely map CNV breakpoints here, previous studies have found that CNV breakpoints almost invariably occur at sites with some degree of local homology, due to the presence of tandem repeat sequences found commonly throughout the genome, indicating that amplifications arise due to improper pairing of homologous chromosomes followed by unequal crossover (Nair et al. 2007). The abundance of tandem repeats in the *P. falciparum* core genome creates a rich network of non-allelic sequence homology and thus potential for amplifications to arise. Previous work has also shown that CNV breakpoints are found in repeat regions that are slightly longer than the genome-wide average (ibid.) thus variations in the length of tandem repeat regions could alter the homology network and shift the amplification potential to a different set of loci. We have shown here that INDEL variation within tandem repeat regions is abundant throughout the core genome and thus the homology network is likely to be highly dynamic and variable within populations. Once an amplification has occurred and persisted long enough for parasites to be taken up by a mosquito, meiotic recombination provides an opportunity for copies of the amplified region from different lineages to be acquired within the same genome, creating a region of pseudo-heterozygosity. Both the initial amplification and the acquisition of pseudo-heterozygosity may have fitness benefits which enable short-term adaptation to strong and/or variable selection pressures, for example, due to introduction of new anti-malarial drugs or other changes in drug policy. Over multiple generations, meiotic recombination could enable amplified genes to diverge far more rapidly than by the accumulation of mutations alone, which in turn could accelerate the evolution of modified or novel gene functions. The core genome of *P. falciparum* thus appears stable yet poised to undergo bursts of evolution within any region that may come under selection pressure. It remains to be seen whether meiotic recombination in concert with INDEL mutation and gene amplification is playing a major role in adaptation to drug pressure in natural populations. However, these data indicate that all known modes of mutation and recombination should be integrated when modelling the emergence and spread of drug resistance.

# Methods

## DNA samples

DNA samples from the parents and progeny of the 3D7xHB3 cross were obtained from cultures maintained at Glasgow University. DNA samples from the HB3xDd2 cross were obtained from @@TODO. DNA samples for the 7G8xGB4 cross were obtained from @@TODO, including the provenance of replicate clones that got cultured locally@@. @@TODO say something about DNA extraction methods?

## Whole genome sequencing

All sequencing was carried out using Illumina high throughput technology as described in (Manske et al. 2012) except that the PCR-free method of library preparation as described in (Kozarewa et al. 2009) was used.

## Variant calling

Variants were called by two independent methods. The alignment method used BWA (Li & Durbin 2009) to align reads to the 3D7 version 3 reference genome, then applied GATK (McKenna et al. 2010) base quality score recalibration, indel realignment, duplicate removal, and performed SNP and INDEL discovery and genotyping across samples within each cross simultaneously, then used variant quality score recalibration to filter variants, following GATK best practice recommendations (DePristo et al. 2011; Van der Auwera et al. 2002) with some adaptations for *P. falciparum* described in supplementary information. The assembly method used Cortex (Iqbal et al. 2012) following the independent workflow. Filtered variants from both calling methods were then combined into a single set of segregating variation for each cross.

## Inference of CO and NCO recombination events and conversion tracts

The combined variant call sets was used to infer CO and NCO events and identify conversion tracts within each cross. For each progeny clone, genotype calls were used to identify contiguous regions of the genome where all alleles were inherited from the same parent, by iterating through variants within a chromosome and recording switches in inheritance between adjacent variants. Simulations using previous estimates for the CO recombination rate predicted that two crossovers were highly unlikely to occur in the same meiosis within 10kb of each other under a simple model of random CO recombination (supplementary information). Thus we assumed that any contiguous inheritance block with minimal length shorter than 10kb was not due to a double crossover but represented either the whole or part of a conversion tract. Any such blocks occurring in isolation were assumed to be simple conversion tracts. Any such blocks occurring directly adjacent to each other were merged into a single complex conversion tract. To identify CO events, all genotype calls within conversion tracts were first masked, and remaining switches in parental inheritance were called as CO events. Conversion tracts occurring directly adjacent to a CO were then identified, and the remaining conversion tracts were assumed to be NCO events. Putative conversion tracts supported by a single marker or with a minimal length less than 100bp were excluded from further analyses. This algorithm is similar in motivation to that used by Samarakoon, Regier, et al. (2011) but does not depend on a windowed analysis and thus has greater flexibility for detecting tracts spanning windows.

## Recombination analyses

To calculate the CO recombination rate for each cross the unadjusted recombination fraction was summed across all markers. No map function was used because the marker density was so high and thus we assume no crossovers were missed. To estimate the true conversion tract length distribution, conversion tracts were simulated at different values for the parameter *phi*, and the distribution of tract lengths that would be observed given the available markers within each cross was computed. The parameter *phi* was fitted by examining quantile-quantile plots comparing simulated and actual distributions of observed tract lengths. These simulations also predicted the fraction of conversion tracts that would be discovered given the markers available in each cross and the requirement that tracts must span at least 100bp. The rate of NCO recombination was then estimated by adjusting the number of observed NCO events by the discovery rate predicted by simulations.

## Analyses of copy number variation

The genome was divided into 300bp non-overlapping bins and the number of reads whose alignment started within each bin was calculated for each sample. These binned read counts were then normalised by dividing by the median read count found within the core regions of chromosome 14. Bins where the GC content was lower than 20% were excluded from coverage analyses due to coverage bias in most samples. The fraction of aligned reads with face-away orientation and same-strand orientation was calculated per position for each sample using pysamstats (@@REF). Copy number state was predicted in all samples by fitting a Gaussian hidden Markov model to the normalised coverage data.

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@@TODO

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13. <http://github.com/cggh/panoptes> [↑](#footnote-ref-13)