The *Plasmodium berghei* RC strain is highly diverged and harbors putatively novel drug resistance variants

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Background. The current first line drugs for treating uncomplicated malaria are artemisinin combination therapies. However, Plasmodium falciparum parasites resistant to artemisinin and partner drugs are spreading, which threatens malaria control efforts. Rodent malaria species are useful models for understanding antimalarial resistance, in particular genetic variants responsible for cross resistance to different compounds. Methods. The Plasmodium berghei RC strain (PbRC) is described as resistant to different antimalarials, including chloroguine and artemisinin. In an attempt to identify the genetic basis for the antimalarial resistance trait in *Pb*RC, its genome was sequenced and compared with five other previously sequenced *P. berghei* strains. **Results.** We found that PbRC is eight-fold less sensitive to the artemisinin derivative artesunate than the reference strain PbANKA. The genome of PbRC is markedly different from other strains, and 6974 single nucleotide variants private to PbRC were identified. Among these PbRC private variants, non-synonymous changes were identified in genes known to modulate antimalarial sensitivity in rodent malaria species, including notably the ubiquitin carboxylterminal hydrolase 1 gene. However, no variants were found in some genes with strong evidence of association with artemisinin resistance in *P. falciparum* such as K13 propeller protein. **Discussion.** The variants identified in *Pb*RC provide insight into *P. berghei* genome diversity and genetic factors that could modulate chloroquine and artemisinin resistance in *Plasmodium* spp.



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23 Abstract

24 Background.

The current first line drugs for treating uncomplicated malaria are artemisinin combination therapies. However, *Plasmodium falciparum* parasites resistant to artemisinin and partner drugs are spreading, which threatens malaria control efforts. Rodent malaria species are useful models for understanding antimalarial resistance, in particular genetic variants responsible for cross resistance to different compounds.

30 Methods.

The *Plasmodium berghei* RC strain (*Pb*RC) is described as resistant to different antimalarials, including chloroquine and artemisinin. In an attempt to identify the genetic basis for the antimalarial resistance trait in *Pb*RC, its genome was sequenced and compared with five other previously sequenced *P. berghei* strains.

35 **Results.**

We found that *Pb*RC is eight-fold less sensitive to the artemisinin derivative artesunate 36 37 than the reference strain *Pb*ANKA. The genome of *Pb*RC is markedly different from other strains, and 6974 single nucleotide variants private to PbRC were identified. Among these PbRC 38 private variants, non-synonymous changes were identified in genes known to modulate 39 antimalarial sensitivity in rodent malaria species, including notably the ubiquitin carboxyl-40 terminal hydrolase 1 gene. However, no variants were found in some genes with strong evidence 41 of association with artemisinin resistance in *P. falciparum* such as K13 propeller protein. 42 **Discussion.** 43 The variants identified in *Pb*RC provide insight into *P. berghei* genome diversity and 44

45 genetic factors that could modulate chloroquine and artemisinin resistance in *Plasmodium* spp.

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47 Keywords: *Plasmodium berghei RC*, artemisinin, malaria, genome, chloroquine

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50 Introduction

The incidence of malaria is declining around the world, and efforts are being directed 51 towards elimination of this disease in many endemic areas (Tanner et al., 2015); (WHO Malaria 52 Policy Advisory Committee and Secretariat, 2015). However, malaria parasite resistance to first-53 54 line artemisinin combination therapy is evolving in Southeast Asia (Woodrow & White, 2016), including resistance to partner drugs such as piperaquine (Duru, Witkowski & Ménard, 2016); 55 (Amato et al., 2016); (Imwong et al., 2017). This is alarming given that parasites resistant to 56 chloroquine (CQ) (Wootton et al., 2002) and pyrimethamine (Nair et al., 2003) are highly 57 prevalent in this region. The specter of multi-drug resistant *Plasmodium falciparum* parasites 58 59 could undermine all recent advances in reducing the disease burden. Laboratory models of antimalarial resistance are needed to develop new drugs effective against parasites resistant to 60 61 currently available antimalarials, and to understand the molecular mechanisms of resistance.

Rodent malaria parasites are widely used laboratory models for human malaria as they 62 can be studied *in vivo* in animal and mosquito hosts. Antimalarial-resistant parasites can be 63 selected by repeated dosing of infected animals, and stably resistant parasite clones can be 64 isolated after serial passage in animal hosts. Moreover, parasites cross-resistant to different drugs 65 have been obtained by this approach. CQ and artemisinin (ART) resistant *Plasmodium chabaudi* 66 rodent malaria parasites from laboratory selection were isolated in (Afonso et al., 2006). 67 Laboratory selected CQ and ART-resistant *Plasmodium voelii* rodent malaria parasites display 68 69 impaired hemozoin production and elevated level of glutathione (GSH), suggestive of a common mechanism of resistance against the two drugs (Witkowski et al., 2012). Laboratory selection of 70 antimalarial resistance has also been performed for the most virulent rodent malaria species 71 *Plasmodium berghei*. The *P. berghei* RC strain (*Pb*RC) was obtained by laboratory selection 72 73 with CQ, and is defective for production of hemozoin (Peters, 1964); (Peters, Fletcher & Staeubli, 1965). This strain is also reported as resistant to other drugs, including ART (Pérez-74 Rosado et al., 2002). Given that *Pb*RC also shows an elevated level of GSH (Vega-Rodríguez et 75 76 al., 2015), similar to cross-resistant *P. voelii* (Witkowski et al., 2012), it may harbor resistance mutations in the same genes as other drug-resistant rodent malaria parasites. 77

In this study, we found that *Pb*RC is resistant to artesunate, a water-soluble derivative of
ART. We sought the genetic factors responsible for the cross-drug resistant phenotype of *Pb*RC

80 by performing whole genome sequencing. The *Pb*RC genome is markedly different from other

characterized *P. berghei* strains, and we identified several variants unique to *Pb*RC in genes

82 which may modulate antimalarial resistance.

83

84 Materials & Methods

Six to ten-week old female BALB/c mice were purchased from the National Laboratory
Animal Center, Mahidol University, Thailand. The following parasites were obtained from the
Malaria Research and Reference Reagent Resource Center (MR4; http://www.beiresources.org),
a part of BEI Resources, NIAID, NIH: *Plasmodium berghei* RC, MRA-404, deposited by W
Peters and BL Robinson; *Plasmodium berghei* ANKA 507m6cl1, MRA-867 deposited by CJ
Janse and AP Waters. Artesunate was a gift from Dafra Pharma, Belgium. CF11 was purchased
from Whatman. Other reagents, unless otherwise noted, were purchased from Sigma Aldrich.

92

93 Four-day suppressive test for *in vivo* drug sensitivity

BALB/c mice were injected intravenously with 1 x 10⁷ *P. berghei* infected red blood
cells. For *Pb*RC, five to six mice were used per group. For *Pb*ANKA, four to eight mice were
used per group. Oral doses of artesunate were given at 4, 24, 48 and 72 h post infection. Four
days post infection, parasitemia was determined by manually counting infected and uninfected
red blood cells in Giemsa-stained thin blood smears. Percent inhibition was calculated using the
following formula:

100 Percent inhibition = 100 - ((100 x parasitemia of each dose)/Parasitemia of untreated control)

101 Data of percent inhibition at different doses of artesunate were fitted to the two-parameter

sigmoidal dose response equation using the drc package in R (Ritz & Streibig, 2005). This study

103 was carried out in accordance with the guidelines in the Guide for the Care and Use of

104 Laboratory Animals of the National Research Council, Thailand. All animal experiments were

105 performed with the approval of BIOTEC's Institutional Animal Care and Use Committee (Permit

106 number BT-Animal 02/2557). At the end of the experiments, mice were euthanized by CO_2

107 asphyxiation. All efforts were made to alleviate pain and suffering.

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109 Sanger dideoxy sequencing of PCR products from selected regions

Selected genomic regions with candidate variants in the *P. berghei* RC strain were PCRamplified using high-fidelity Phusion DNA polymerase (New England Biolabs) using primers
listed in Supplemental Table S1. The resulting PCR products were sent for Sanger dideoxy
sequencing (1st BASE, Malaysia).

114

115 Whole genome DNA sequencing

116 Parasitized blood obtained from a single mouse infected with the *Pb*RC parasite was passed through a CF11 (Whatman) column to remove white blood cells. Cells were harvested by 117 centrifugation and parasites were liberated from red cells by lysis of the red blood cell membrane 118 with 0.2% saponin. Parasites were washed twice with phosphate buffered saline (137 mM NaCl, 119 120 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4), resuspended in lysis buffer (8.5 mM Tris-HCl, 342 mM NaCl, 2 mM Na₂-EDTA, 0.14 mg/ml proteinase K, 0.14% SDS, pH 8.2) 121 and incubated at 37°C overnight. Proteins were precipitated by the addition of NaCl to 1.5 M, 122 centrifuged, and discarded. Isopropanol was added to the supernatant to precipitate nucleic 123 acids. The nucleic acid pellet was dried and resuspended in TE buffer (10 mM Tris, 1 mM 124 125 EDTA pH 8.0). RNA was removed by digestion with RNAseA. Phenol chloroform extraction and ethanol precipitation of DNA were performed. The integrity of genomic DNA was checked 126 by agarose gel electrophoresis. Genomic DNA was submitted to the Chulalongkorn Medical 127 Research Center, Bangkok, Thailand for genome sequencing. Genomic DNA was sheared by 128 129 sonication and DNA fragments < 1 kb were gel-purified. Sequencing libraries were constructed from sheared genomic DNA using a TruSeq kit (Illumina). 2x 150 bp reads were obtained using 130 a MiSeq instrument (Illumina). The raw data are deposited in the NCBI Sequence Read Archive, 131 accession number PRJNA277169. 132

133

134 Sequence Data Analysis

Raw sequencing data were obtained in FASTQ format. Genome sequence data for strains 135 K173, NK65NY, NK65E, SP11 RLL, SP11 A (Otto et al., 2014) were downloaded from the 136 European Nucleotide Archive. Filtering of raw data to remove poor quality reads (average Q-137 score<30) and removal of adapter sequences were performed using FASTQC (Andrews, Simon, 138 2010). Preprocessed read data were aligned to the P. berghei ANKA version 3 reference genome 139 (downloaded from GeneDB; (Logan-Klumpler et al., 2012)) using BOWTIE version 2.2.2.6 140 software (Langmead et al., 2009) under the setting of length of seed substring 22 bases without 141 clipping. GATK software version 3.3.0 (McKenna et al., 2010) was used to identify potential 142 base-substitution single nucleotide variants (SNV) and small insertion/deletions (INDEL) 143 between all strains different from the reference strain by the HaplotypeCaller method combined 144 with the GenotypeGVCFs method, under the settings: --minReadPerAlignmentStart (10), --145 min base quality score (10), --sample ploidy(2), --heterozygosity(0.001). SnpEff software 146 version 4.3g (Cingolani et al., 2012) was used to annotate the variants using the genome 147 annotation file downloaded from GeneDB. The .vcf file containing all raw variants called by 148 GATK is provided in Data S2. The raw variants were filtered to retain only high-confidence 149 150 SNVs for genome analyses. INDEL variants were not included, since many occur within simple sequence repeats that could be prone to PCR and sequencing artifacts. Potential false positive 151 152 SNVs resulting from sequencing artifacts were removed by excluding variants with heterozygous or missing genotypic calls in any strain. Potential false variants resulting from read alignment 153 154 error in low complexity regions were removed using the DustMasker program (Morgulis et al., 2006). Variants present among multigene families listed in Additional File 4 of (Otto et al., 155 2014) were also treated as potential false positives and were removed. After filtering, 8681 SNV 156 markers remained. 157

Principal Components Analysis (PCA) was performed using the ipPCA tool (Limpiti et 158 al., 2011), implemented in MATLAB version R2009b. The 8681 filtered SNV markers were 159 used as input for ipPCA. Tajima's D scores were calculated using PopGenome, a population 160 genomic analysis tool in R (Pfeifer et al., 2014). Scores were calculated in sliding non-161 overlapping genomic windows of five consecutive variants (1730 windows in total), and 162 separately for annotated genes (1784 genes in total, Table S3). Plots of Tajima's D scores were 163 made using the ggplot2 package in R (Wickham, 2009). Gene ontology analysis of genes with 164 Tajima D scores greater than 1 was performed using the Gene ontology web service provided in 165

the PlasmoDB website (Aurrecoechea et al., 2009). Terms were considered significant using aBonferroni-corrected p-value threshold of 0.05.

168

169 **Results**

The *Pb*RC parasite is reported as CQ and ART resistant (Pérez-Rosado et al., 2002). We tested the sensitivity of the *Pb*RC strain to artesunate, a water soluble derivative of ART. *Pb*RC is approximately eight-fold less sensitive to artesunate compared with *Pb*ANKA (effective dose for 50% inhibition of parasite (ED₅₀) of 2.8 (s.e. = 0.4) and 23.1 (s.e. = 7.3) mg/kg in *Pb*ANKA and *Pb*RC, respectively; Fig. 1), which confirms the multi-drug resistant phenotype for this strain.

176

177 *Pb*RC is widely diverged from other *P. berghei* strains

Illumina sequencing of PbRC genomic DNA was performed. Sequence reads were 178 obtained from 17,801,263 clusters, and 80.5% of the preprocessed reads could be mapped to the 179 *Pb*ANKA v3 reference genome. Genomic sequence data of previously sequenced strains K173, 180 NK65NY, NK65E, SP11 RLL and SP11 A (Otto et al., 2014) were aligned to the reference 181 genome using the same analytical procedure. SNV and small INDEL variants were called from 182 the mapped reads for all strains. A total of 27,495 variant markers were identified among the six 183 strains (Data S2), of which 8681 SNV markers remained after applying stringent filtering 184 criteria. The numbers of variants identified in each strain are shown in Table 1. 185

The majority of filtered SNVs are private to each strain, and the PbRC strain shows the highest number of variants, indicating that it is markedly different from the other strains. This is confirmed by Principal Components Analysis, in which PbRC is clearly separated from the other strains in the first Principal Component, which captures most of the genotypic variance among these strains (Fig. 2).

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192 Genetic diversity across the *P. berghei* genome

A previous analysis of *P. berghei* genotypic diversity revealed a low SNV frequency
compared with *P. chabaudi*, another rodent malaria species (Otto et al., 2014). However, the

extreme divergence of *Pb*RC from other strains may provide further insight into *P. berghei* 195 intraspecific genetic diversity. The Tajima's D score was calculated for sliding genomic 196 windows along all chromosomes, and also for annotated genes. Tajima's D is a measure of the 197 observed versus expected genetic diversity, calculated as the difference between the mean 198 number of pairwise differences and the number segregating sites (Tajima, 1989). The mean 199 Tajima's D is negative for each chromosome, indicating a genome-wide pattern of negative 200 Tajima's D (Fig. 3A). However, there are several regions with positive Tajima's D values that 201 are spread throughout the genome (Fig. 3B). At the gene level, 125 genes were identified with a 202 Tajima's D score greater than one (Table S3). No specific gene ontology terms or biological 203 processes are significantly enriched among these genes with high Tajima's D scores. However, a 204 few genes homologous to Plasmodium genes with known functions in host cell invasion showed 205 high Tajima's D scores, including PBANKA 132170 (berghepain 1), PBANKA 041290 206 (circumsporozoite- and TRAP-related protein, PbCTRP), PBANKA 1115300 (glideosome-207 associated protein 40, PbGAP40), PBANKA 1137800 (glideosome-associated connector, 208 PbGAC) and PBANKA 1400091 (reticulocyte binding protein, Pb235). 209

210 Next, variants private to *Pb*RC located within genes were examined to identify putative causal variants of the drug resistant trait in this strain. To our knowledge, the only prior evidence 211 of PbRC genetic variants compared with the PbANKA reference is an Ile to Lys substitution at 212 residue 413 (I413K) of the gamma glutamylcysteine synthetase (*Pby-gcs*) gene (Pérez-Rosado et 213 al., 2002). We confirmed this mutation in *Pb*RC from whole genome and Sanger sequencing. 214 215 Comparison of *P. berghei* strain genomes identified this variant as private to *Pb*RC (Table 2). A previous study of PbRC reported a possible chromosomal translocation in the vicinity of the 216 217 MRP gene (Gonzalez-Pons et al., 2009). However, visualization of PbRC mapped reads in this region showed no evidence of translocation (data not shown). 218

We searched for candidate drug resistance variants in genes known to modulate CQ
and/or ART drug resistance in other *Plasmodium* spp. The variants in these genes private to *Pb*RC are shown in Table 2. Mutations in the *P. chabaudi* ubiquitin carboxyl-terminal hydrolase
1 (*ubp1*) gene modulate CQ and ART resistance in laboratory-selected drug resistant parasites
(Hunt et al., 2007); (Henriques et al., 2013). Five non-synonymous variants private to *Pb*RC
were found in the homologous *Pbubp1* gene. Mutations in the chloroquine resistance transporter

(crt) gene modulate CQ resistance in *P. falciparum* (Martin & Kirk, 2004); (Ecker et al., 2012). 225 A non-synonymous V42F variant private to PbRC was found in the P. berghei homologue 226 (Pbcrt). Mutation of the P. falciparum multidrug resistance associated protein 1 (mdr1) gene 227 modulates sensitivity to several antimalarials, including CQ and ART (Sanchez et al., 2010). A 228 non-synonymous V54A variant private to PbRC was found in the homologous Pbmdr1 gene. 229 Among other proteins implicated as modulators of ART sensitivity in *P. falciparum*, a non-230 synonymous F320C variant private to PbRC was found in the Pbpi3k gene homologous to 231 phosphatidylinositol-3-kinase (PfPI3K) (Mbengue et al., 2015). PbRC mutations were notably 232 absent from some genes strongly implicated as modulators of ART sensitivity, including the 233 homologues of µ subunit of adaptor protein 2 complex (AP2-µ; PBANKA 1433900), a gene 234 mutated in the P. chabaudi CQ- and ART-resistant AS-ART strain (Henriques et al., 2013) and 235 K13 Kelch propeller (PbK13; PBANKA 1356700), mutations of which confer reduced ART 236 sensitivity in *P. falciparum* (Ariey et al., 2013); (Ghorbal et al., 2014). 237

238 Catabolism of hemoglobin is tied to the mechanisms of action of both CQ and ART. CQ prevents crystallization of heme, a product of hemoglobin catabolism (Martin & Kirk, 2004), 239 whereas the antimalarial effect of ART is strongly dependent on catabolism of hemoglobin 240 (Klonis et al., 2011); (Xie et al., 2015). Given that the CQ and ART-resistant PbRC is defective 241 for hemoglobin catabolism and hemozoin formation (Peters, 1964); (Peters, Fletcher & Staeubli, 242 1965), we investigated whether PbRC harbored private variants in genes encoding proteins 243 known to function in catabolism of hemoglobin (Ponsuwanna et al., 2016); (Lin et al., 2015). 244 Among these genes, a non-synonymous variant private to *Pb*RC was found only in the dipeptidyl 245 aminopeptidase 3 gene (Pbdpap3) (Table 2). 246

247

248 Discussion

Our analysis of *P. berghei* genomes revealed that the majority of SNVs are private to each strain, in which *Pb*RC is most diverged strain of all. Moreover, the genome-wide pattern of negative Tajima's D score (Fig. 3) can be interpreted as evidence of an excess of rare variants because of a recent population bottleneck. Other *Plasmodium* spp. also show overall negative Tajima's D score (Parobek et al., 2016). The overall negative Tajima's D score (and other measures (Rutledge et al., 2017); (Otto et al., 2016)), are consistent with recent population

bottlenecks in *Plasmodium* spp. Although Tajima's D score is negative overall for *P. berghei*, 255 we identified some genes with high Tajima's D scores. Some of these genes are known to 256 function in host cell invasion, including PbCTRP, berghepain 1, PbGAP40, PbGAC and Pb235. 257 The *P. berghei* CTRP protein is important for mosquito midgut invasion by the ookinete 258 (Dessens et al., 1999). The berghepain 1 gene may also function in midgut invasion, as mutants 259 of the P. falciparum homologous gene (falcipain 1) are defective for oocyst production (Eksi et 260 al., 2004). The GAP40 and GAC genes may function as part of the glideosome complex, an 261 actin- and myosin-based machine conserved across Apicomplexa that powers parasite motility, 262 migration, host cell invasion and egress (Frénal et al., 2010); (Jacot et al., 2016). Pb235 belongs 263 to a rodent malaria family of proteins which are thought to interact with host cells during the 264 invasion process (Gruner et al., 2004). The high Tajima's D scores for these genes suggests 265 266 balancing selection could operate to favour alleles that produce antigenically diverse proteins allowing invasive parasites to evade host immune systems. 267

The large number of variants private to PbRC makes it difficult to pinpoint causal 268 variants of its drug-resistant phenotype. Moreover, the N strain progenitor from which PbRC 269 270 was derived by chloroquine selection was not available to us, and forward genetic mapping is not possible since *Pb*RC does not produce gametocytes (Peters, Fletcher & Staeubli, 1965). 271 272 Therefore, we manually curated variants private to *Pb*RC to identify those in candidate genes known to modulate drug sensitivity in other *Plasmodium* spp. The level of GSH modulates 273 chloroquine sensitivity in *P. falciparum*, which is controlled by the level of expression of γ -274 glutamyl cysteine synthetase enzyme (Ginsburg & Golenser, 2003). PbRC carries a I413K 275 variant in the Pby-gcs gene encoding this enzyme (Table 2). However, P. berghei parasites with 276 knockout of *Pby-gcs* do not show altered sensitivity to CQ or ART (Vega-Rodríguez et al., 277 278 2015); (Songsungthong et al., 2016). Furthermore, the *Pbγ-gcs* I413K variant may have preexisted in the CQ-sensitive N strain progenitor (Pérez-Rosado et al., 2002), and so is not likely to 279 modulate drug sensitivity. 280

*Pb*RC harbors five non-synonymous variants in the *Pbubp1* gene. The homologous gene
is mutated in drug-resistant *P. chabaudi* (Hunt et al., 2007); (Henriques et al., 2013). The *ubp1*mutated residues in drug-resistant *P. chabaudi* are located in a conserved putative ubiquitin
binding region of the protein, which may disrupt its function and lead to increased proteasomal

degradation of UBP-1 substrates (Hunt et al., 2007). However, none of the Pbubp1 variants in 285 *Pb*RC correspond to the *ubp1* mutations in *P. chabaudi*, and except for N2279D, the *Pbubp1* 286 variant residues are not conserved among *Plasmodium* spp. Therefore, it is not known if the 287 *Pbubp1* variants in *Pb*RC affect the function of the protein. Disruption of ubiquitination 288 pathways has been implicated in CQ and ART resistance mechanisms, although such disruption 289 could occur by mutations in different genes. 290 Genome sequencing of the CQ- and ART-resistant clone AS-ART isolated in (Afonso et 291 al., 2006) revealed a non-synonymous mutation in the AP2-µ gene, which was not present in the 292 AS-15CQ progenitor strain (Henriques et al., 2013). Cross resistance to CQ and ART was thus 293 attributed to the presence of *ubp1* and AP2-µ mutations in AS-ART (Henriques et al., 2013). 294 AP2-µ gene mutation in the AS-ART parasite was proposed to change the balance of 295 endocytosis toward a clathrin-independent pathway (Henriques et al., 2013). In this scenario, 296 endocytosis of hemoglobin may be reduced with subsequent lower production of heme catabolite 297 and reduced CQ and ART efficacy. However, no variants in the P. berghei AP2-µ homologue 298 were found in *Pb*RC, suggesting that resistance pathways differ between AS-ART and *Pb*RC. 299 300 Mutations in the *P. falciparum* K13 gene are thought to lead to important changes in ubiquitination patterns that affect sensitivity to ART (Dogovski et al., 2015); however, no PbRC 301 302 variants were found in the homologous gene *Pb*K13. The activity of *Pf*PI3K modulates ART sensitivity in *P. falciparum*, which is controlled by K13-mediated ubiquitination (Mbengue et al., 303 304 2015). PbRC harbors a F320C variant in the homologous Pbpi3k gene. The F320C variant is located in an N terminal domain not present in homologous PI3K proteins with reported 305 structures (i.e. human and *Drosophila*); hence, it is difficult to predict if this mutation affects 306 PbPI3K protein function. 307 308 Mutations in the Pfcrt gene modulate CQ sensitivity in P. falciparum, and a V42F variant

was found in the homologous gene (*Pbcrt*) of *Pb*RC (Table 2). The *Pbcrt* residue 42 is predicted to be in the N-terminal cytosolic part of the protein before the first transmembrane domain; however, the equivalent residue in *Pfcrt* is not reported as mutated among field isolates of CQresistant *P. falciparum* (Ecker et al., 2012). It should be noted that other *crt* mutations, such as the C101F variant in piperaquine-resistant *P. falciparum* parasites (Dhingra et al., 2017) can cause defects in food vacuole morphology similar to *Pb*RC. It is difficult to predict the effect of the V42F variant on *Pbcrt* function by comparison with *Pfcrt*, since allelic replacement of *Pbcrt*

with *Pfcrt* from CQ-resistant *P. falciparum* modulated CQ sensitivity only in sexual stages of *P.*

berghei, pointing to divergence of *crt* gene function between the two species (Ecker et al., 2011).

318 The degree of CQ sensitivity in *P. falciparum* with *Pfcrt* mutation is modulated by variants in

other genes such as *Pfmdr1*, as shown recently by genome editing studies (Veiga et al., 2016).

320 The *Pb*RC *Pbmdr1* V54A variant residue is not equivalent to any *Pfmdr1* residue shown to

modulate CQ or ART sensitivity (Veiga et al., 2016); hence, it is difficult to predict the effect of

the V54A variant on *Pbmdr1* function.

323 The *Pb*RC strain produces less hemozoin than other CQ-sensitive strains (Peters, 1964);

324 (Peters, Fletcher & Staeubli, 1965), which may be due to defective hemoglobin digestive

enzymes. A protein complex of hemoglobin digestive enzymes has been described in *P*.

falciparum (Chugh et al., 2013). This complex may have a simpler composition in *P. berghei*,

since this species possesses only one digestive falcipain-like enzyme (berghepain-2), one

328 plasmepsin (plasmepsin IV) and one hemoglobin digestive protein (HDP) (Ponsuwanna et al.,

2016). No variants were found among these genes in PbRC, and of the downstream digestive

enzymes, only one variant in the *Pbdpap3* gene was found. This gene is non-essential in *P*.

berghei (Lin et al., 2015), and so mutation of this gene may play a minor role in modulating CQ
and ART sensitivity.

333

334 Conclusions

Analysis of genomic variants across six strains of *P. berghei* revealed an excess of rare variants, consistent with a population bottleneck as reported for other *Plasmodium* spp. Several variants were identified as private to *Pb*RC which could modulate drug sensitivity, although direct testing of these variants using approaches such as genome editing is necessary to test causality.

340

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Table 1(on next page)

Summary of *Plasmodium berghei* genomic variants identified from whole genome sequencing.

The numbers of raw variants refer to Single Nucleotide Variants (SNV) and small insertion/deletions (INDEL) called by GATK using default parameters. The numbers of markers with no genotype calls owing to insufficient mapped reads are indicated as missing for each strain. The numbers of markers with variant allele detected only in one strain are indicated as private. The numbers of filtered variants refer to SNV remaining after filtering to remove variants with heterozygous calls, located in repetitive regions, or present in multigene families. The *Pb*RC strain was sequenced in this study; sequence data from other strains were reported in (Otto et al., 2014).

1

	<i>Pb</i> RC	<i>Pb</i> K173	<i>Pb</i> NK65_E	<i>Pb</i> NK65_NY	<i>Pb</i> SP11_A	PbSP11_RLL
Dow	20300	5263	2034	2480	1561	5834
Naw .	1(774	1001	2054	2400	202	1(02
-private	16//4	1991	305	561	292	1693
-missing	1295	1326	241	259	224	654
Filtered	7726	1251	12	33	1	890
-private	6974	504	1	17	1	311

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Table 2(on next page)

List of genes with non-synonymous variants private to *P. berghei* RC occurring in genes previously associated with chloroquine and/or artemisinin resistance in *Plasmodium* spp.

Single nucleotide variants (SNV) private to the *P. berghei* RC strain (*Pb*RC), when comparing with the reference strain *Pb*ANKA v3 and other strains reported in (Otto et al., 2014) were identified by whole-genome sequencing. Non-synonymous SNVs in candidate genes are listed as the missense mutation annotated by the SnpEff software version 3.6b. Variants were confirmed by Sanger dideoxy sequencing of PCR amplicons; N.D., not done. The SNV causing the I413K missense mutation in the PBANKA_0819800 gene was reported as a raw variant by GATK analysis of genome sequence data; however, this variant was removed by filtering as it is located within a region in which read mapping is equivocal.

1

Gene Description	Gene	Gene ID	Variants private to <i>Pb</i> RC	Confirmed by
	Symbol			Sanger dideoxy
				sequencing
γ-glutamylcysteine	Pby-gcs	PBANKA_0819800	I413K	YES
synthetase				
Ubiquitin	Pbubp1	PBANKA_0208800	R1561K	YES
carboxyl-terminal			K1582E	YES
hydrolase 1			K2102E	YES
			N2279D	YES
			A2402V	YES
Chloroquine	Pbcrt	PBANKA_12195000	V42F	YES
resistance				
transporter				
Multidrug	Pbmdrl	PBANKA_1237800	V54A	YES
resistance gene				
Phosphatidyl-	Pbpi3k	PBANKA_1114900	F320C	N.D.
inositol-3-				
phosphate kinase				
Dipeptidyl	Pbdpap3	PBANKA_1002400	L3I	N.D.
aminopeptidase 3				
2				

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Figure 1(on next page)

Artesunate sensitivity of *Plasmodium berghei* RC (*Pb*RC) in infected mice.

BALB/c mice were infected intravenously with 1×10^7 infected red blood cells. Artesunate was given orally and parasitemia was determined four days post infection. Five to six mice per group were used for *Pb*RC and four to eight mice per group were used for *Pb*ANKA. Percent inhibition compared with untreated control (0%) was calculated for each dose of drug. Data were fitted to the two-parameter variable-slope sigmoidal dose-response equation. The average percent inhibition for each dose are plotted, and the gray bars represent confidence regions calculated by the drc package.



Figure 2(on next page)

Principal Components Analysis of P. berghei strains.

The genotypic data from 8681 Single Nucleotide Variants called from whole genome sequences were encoded as a matrix for Principal Components Analysis. The loadings from the first and second Principal Components for each strain are plotted.



Figure 3(on next page)

Genome-wide distribution of Tajima's D scores in *P. berghei*.

Tajima's D score was calculated for sliding, non-overlapping genomic windows of five variants along all 14 chromosomes using the PopGenome tool. Scores were calculated for a total of 1730 windows from six strains compared with the reference strain *Pb*ANKA v3. The distributions of scores in each chromosome are shown as violin plots in part A. The scores in each window are plotted separately for each chromosome in part B.



Chromosome



