

The genome of the simian and human malaria parasite *Plasmodium knowlesi*

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Plasmodium knowlesi is an intracellular malaria parasite whose natural vertebrate host is *Macaca fascicularis* (the 'kra' monkey); however, it is now increasingly recognized as a significant cause of human malaria, particularly in southeast Asia^{1,2}. *Plasmodium knowlesi* was the first malaria parasite species in which antigenic variation was demonstrated³, and it has a close phylogenetic relationship to *Plasmodium vivax*⁴, the second most important species of human malaria parasite (reviewed in ref. 4). Despite their relatedness, there are important phenotypic differences between them, such as host blood cell preference, absence of a dormant liver stage or 'hypnozoite' in *P. knowlesi*, and length of the asexual cycle (reviewed in ref. 4). Here we present an analysis of the *P. knowlesi* (H strain, Pk1(A+) clone⁵) nuclear genome sequence. This is the first monkey malaria parasite genome to be described, and it provides an opportunity for comparison with the recently completed *P. vivax* genome⁴ and other sequenced *Plasmodium* genomes^{6–8}. In contrast to other *Plasmodium* genomes, putative variant antigen families are dispersed throughout the genome and are associated with intrachromosomal telomere repeats. One of these families, the KIRs⁹, contains sequences that collectively match over one-half of the host CD99 extracellular domain, which may represent an unusual form of molecular mimicry.

The *P. knowlesi* genome sequence was produced by whole-genome shotgun sequencing to eightfold coverage, with targeted gap closure and finishing (Supplementary Table 1). The 23.5-megabase (Mb) nuclear genome is composed of 14 chromosomes and contains the expected complement of non-coding RNA (ncRNA) genes with known function (Supplementary Table 2) and a large number of novel structured ncRNA candidate genes (Supplementary Figs 1–5 and Supplementary Tables 3 and 4). The presumed centromeres are similar to those found in other *Plasmodium* species^{4,6}, and are positionally conserved within regions sharing synteny with *P. vivax* (see Fig. 1 of ref. 4). The overall G+C base composition is 37.5%. A total of 5,188 protein-encoding genes were identified, which is

slightly lower than the predicted proteome size of *P. falciparum* and *P. vivax*^{4,6}.

Unusually for *Plasmodium* species, (G+C)-rich repeat regions containing intrachromosomal telomeric sequences (ITSs, containing the heptad sequence GGGTT[T/C]A) are found at multiple internal sites in the *P. knowlesi* chromosomes, arrayed tandemly or as components of larger repeat units (Fig. 1). These sequences appear infrequently in *P. vivax* and *P. falciparum* at internal chromosome sites (Supplementary Figs 6 and 7). In the protozoan parasite *Trypanosoma brucei*¹⁰, ITSs may be the templates for recombination events that result in gene conversion among variant antigen VSG genes¹¹. In mammalian genomes¹², ITSs are common and may represent the 'scars' of double-stranded DNA break repair¹². Alternatively, ITSs may have a role in transcriptional control.

For approximately 80% (4,156 out of 5,185) of predicted genes in *P. knowlesi*, orthologues could be identified in both *P. falciparum* and *P. vivax* (for details, see ref. 4). The *P. knowlesi*-specific variant antigen gene families, *SICAvar* genes¹³ and *kir* genes⁹, form the largest groups of *P. knowlesi*-specific expansions (Supplementary Tables 5 and 6). Five distinct gene families of unknown function, with 4–15 paralogous members, are unique to *P. knowlesi* (referred to as Pk-fam-a to Pk-fam-e in Supplementary Table 7). Pk-fam-a and Pk-fam-b each have more than nine paralogous members (Supplementary Fig. 8), which have a two-exon gene structure with a signal peptide, a carboxy-terminal transmembrane region, but lack typical export motifs^{14,15}. Members of the protein family Pk-fam-c and Pk-fam-e represent two new families with putative protein export signals (Supplementary Fig. 8 and Supplementary Table 8).

A comparison of Pfam domains¹⁶ between the predicted proteomes of *P. knowlesi*, *P. vivax* and *P. falciparum* (Supplementary Table 9, Supplementary Information) revealed major differences in domains that distinguish species-specific protein families involved in antigenic variation. The remainder of the proteome was relatively conserved albeit with some interesting copy number variations of a

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few key housekeeping enzymes (Supplementary Fig. 9 and Supplementary Table 9).

In other *Plasmodium* genomes sequenced so far, variant gene families involved in antigenic variation (Supplementary Figs 6 and 7) are typically arranged in the subtelomeres, and only a few members of these families have hitherto been found at intrachromosomal sites. Notably, the *P. knowlesi* genome sequence has revealed that the major variant gene families (that is, *SICAvar*¹³ and *kir*⁹) are randomly distributed across all 14 chromosomes (Fig. 1) and often co-localize with ITS-containing repeats (Supplementary Information). Although all of the telomeres were not fully assembled, we know that in the case of chromosome 7, *P. knowlesi* and *P. vivax* have atypical gene content—the subtelomere encodes proteins associated with merozoite invasion (for example, MAEBL and members of the reticulocyte-binding-like (RBL) family) (Supplementary Fig. 10).

Variant SICA (schizont-infected cell agglutination) antigens on the surface of infected red blood cells⁵ are associated with parasite virulence¹⁷ and are encoded by the *SICAvar* gene family¹³—the largest variant antigen gene family in *P. knowlesi*. Switching of variant types underlies the establishment of a chronic infection in the vertebrate host, a process that is essential in all species, to ensure

mosquito transmission and the completion of the life cycle. Full-length *SICAvar* genes have 3–14 exons (Supplementary Table 5 and Supplementary Fig. 11), resulting in a range of sizes for the predicted proteins of 53–247 kDa. Although many of the *SICAvar* genes are present only as fragments, we estimate that there are up to 107 members in the H strain of *P. knowlesi* based on the number of conserved final exons.

Twenty-nine predicted *SICAvar* genes have complete gene structures and were divided into two subtypes (Fig. 2). The type I *SICAvar* genes with 7–14 exons predominate, with a few containing unusually long introns (Fig. 2). The type II subgroup represents small *SICAvar* genes with 3–4 exon structures. Unusually large introns (5.8–13.6 kb) are a unique feature of *SICAvar* genes and have not previously been seen in any other sequenced apicomplexan gene (Fig. 2).

SICA antigens have a modular structure (Fig. 3, Supplementary Fig. 12) comprising a variable number of highly diverged cysteine-rich domains (CRDs) encoded by multiple exons, a transmembrane domain and a cytoplasmic domain. A high level of sequence diversity was observed, with the exception of the 3' terminal exon¹³. We investigated the domain organization of the CRDs using profile hidden Markov models (HMMs; Fig. 3 and Supplementary Fig. 13). The

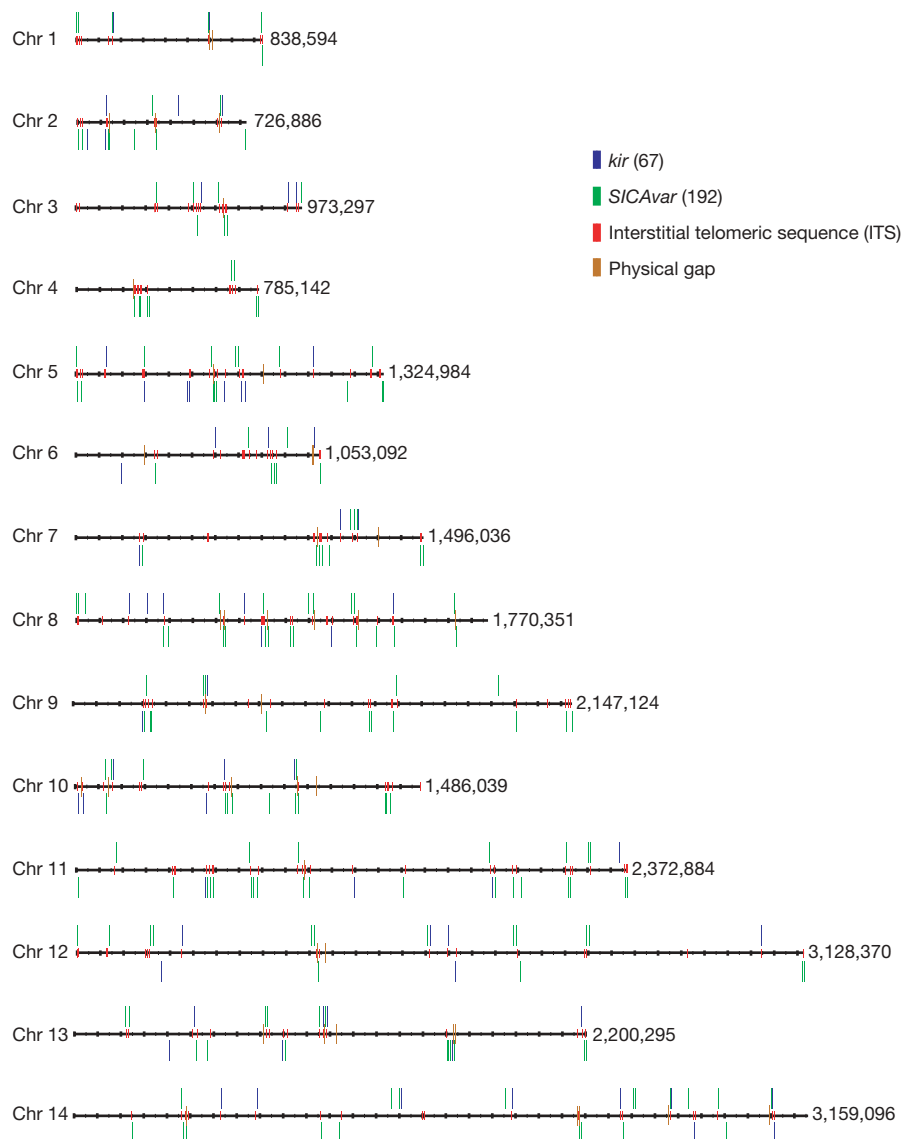


Figure 1 | Distribution of *SICAvar* genes, *kir* genes and telomere-like repeats on chromosomes 1 to 14 of *P. knowlesi* (H strain). The positions of *kir* (shown in blue) and *SICAvar* (green) genes and gene fragments are shown on all 14 chromosomes. Interstitial telomeric sequences

(GGGTT[T/C]A) are found surrounding *kir* and *SICAvar* genes (shown in red). The values along the right of each chromosome indicate the total sequence length in base pairs.

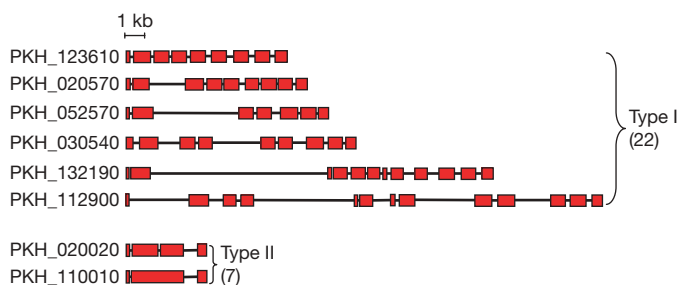


Figure 2 | Structural organization of complete (full length) *SICAvAr* genes in *P. knowlesi* (H strain). Schematic view of the exon structure of type I and type II *SICAvAr* genes. Exons are shown as red boxes with introns as joining lines.

full-length SICA proteins contain a distinct five-cysteine CRD (termed SICA- α) at the amino terminus, which occurs once or twice and may have a stabilizing role analogous to the cysteine-rich N-terminal capping motifs of extracellular leucine-rich repeat proteins¹⁸. There are 1–8 CRDs (referred to as SICA- β) with 7–10 conserved cysteine residues. The transmembrane domain and a conserved domain follow at the C terminus (termed SICA_C in Supplementary Figs 12 and 13).

Although *P. knowlesi* and *P. falciparum* are phylogenetically distant, the SICA and *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) variant antigens share many fundamental biological characteristics (reviewed in ref. 19). Common regulatory mechanisms involving post-transcriptional gene silencing have been proposed between the *var* gene family in *P. falciparum* and the *SICAvAr* family in *P. knowlesi*¹⁹. We have identified conserved sequence motifs between the single *var* intron and *SICAvAr* introns (Supplementary Figs 14–18) in the region thought to be the origin of a ncRNA transcript involved in the silencing of *var* genes²⁰, indicating possible commonality in regulatory mechanisms.

We searched for evidence of gene conversion within the *SICAvAr* family, using the predicted sequences of 20 type I full-length *SICAvAr* genes (Supplementary Information). It is clear that exon shuffling has an important role in *SICAvAr* evolution¹³. The low-complexity repeat regions found within introns might facilitate recombination

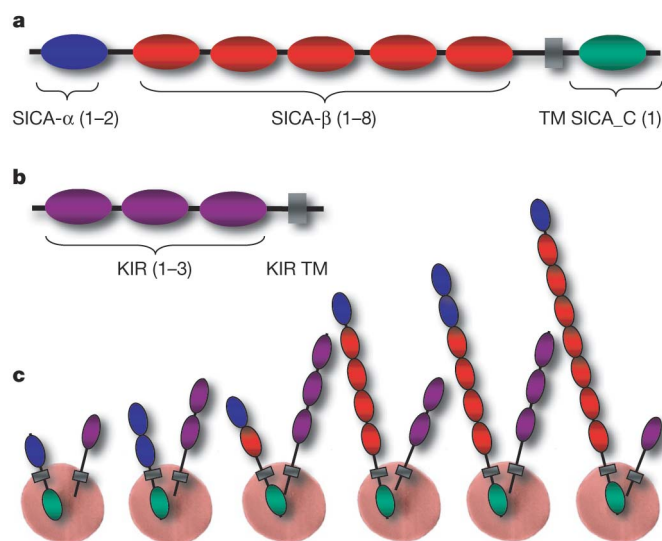


Figure 3 | Domain organization of complete (full-length) SICA and KIR proteins in *P. knowlesi* (H strain). **a**, Domain organization of full-length SICA proteins. The number of different domains (SICA- α , SICA- β and SICA_C) is shown in parentheses. TM, transmembrane. **b**, Domain organization of full-length KIR proteins. **c**, Examples of an infected erythrocyte showing SICA and KIR proteins anchored to the surface in different combinations.

through misalignment during mitosis; this could explain the presence of *SICAvAr* fragments found throughout the genome and/or *SICAvAr* gene models with partial intron/exon structures. These comprise whole, and apparently intact, exons that might provide a reservoir for diversification analogous to that seen with *VSG* genes in *Trypanosoma brucei*¹¹ (Supplementary Information).

Kirs represent the second largest variant gene family. They encode predicted proteins of 36–97 kDa that are hypothesized to be expressed at the surface of infected erythrocytes and undergo antigenic variation⁹. There are 68 predicted *kir* genes, 4 of which have incomplete structures (Supplementary Table 6). They were divided into four types depending on the number of exons (Supplementary Fig. 19). Most (58 out of 64) *kir* genes belong to types I and II. The domain organization of all predicted KIR proteins was also determined using profile HMMs (Fig. 3 and Supplementary Fig. 20). They contain 1–3 domains, followed by a transmembrane domain at the C terminus (referred to as KIR TM in Supplementary Fig. 20). A BLAST analysis of KIR proteins revealed stretches of up to 36 amino acids within the predicted extracellular domain that have 100% identity to host proteins, the most striking of which is to CD99. These matches were evident in several KIR proteins. Interestingly, different family members contain matches to different regions of CD99, such that together, they represent over one-half of the CD99 extracellular domain (Fig. 4). Tests were performed to assess the possibility that such matches could occur by chance (Supplementary Table 10). We have compared the sequences to *Macaca mulatta*, African green monkey and human. The matches exclude conserved cysteine regions and the degree of sequence identity decreases noticeably as the evolutionary distance to the natural host increases (Fig. 4 and Supplementary Table 10). CD99 has a critical role as an immunoregulatory molecule in T-cell function (see <http://www.ncbi.nlm.nih.gov/omim/>). These exact matches may interfere with recognition of parasitized erythrocytes by the host immune system or act as CD99 analogues that interfere by competing with T cells for CD99 partner molecules.

We undertook a more systematic search for other such instances of parasite proteins containing extensive stretches of identical host sequences, using the PMATCH algorithm (Supplementary Information). Unsurprisingly, a large number of matches to highly conserved housekeeping genes were observed, but in addition regions of perfect identity to another host protein (known as AHNAK, see <http://www.ncbi.nlm.nih.gov/omim/>) were detected in two KIRs and one SICA-like protein (Supplementary Fig. 21 and Supplementary Table 10). Analogous searches using the predicted exported protein repertoires (exportome) of *P. vivax* and *P. falciparum* found no such matches to host proteins (Supplementary Table 11). The identity to host proteins is maintained at the amino acid sequence rather than DNA sequence level (data not shown).

Acquisition of host proteins, and thus the ability to mimic their function, has been observed in many bacterial and viral pathogens²¹. In parasitic protozoa there are known cases where stretches of amino acids present on a parasite-encoded cell surface protein match perfectly to regions of host proteins²². However, in all such cases, the matches correspond to a common amino acid repeat that is shared between them^{22–24}. Malaria parasites are known to have a potential immunomodulatory role either by secreting functional homologues of host molecules or by binding to host antigen-presenting cells^{25,26}. This is the first observation of its kind in a malaria protein that shows acquisition of host peptide sequences that are likely to be on the infected cell surface and thus may interact with the host. The mechanism by which these host sequences have arisen remains to be clarified. Possible explanations include convergent evolution or horizontal transfer followed by gene degeneration events.

During the intraerythrocytic life cycle, malaria parasites significantly remodel the erythrocyte by exporting numerous proteins^{14,15}. This depends on a short motif, termed the plasmodium export element (PEXEL) or vacuolar transport signal (VTS), which is present in over 300 *P. falciparum* proteins and is common to all *Plasmodium* species

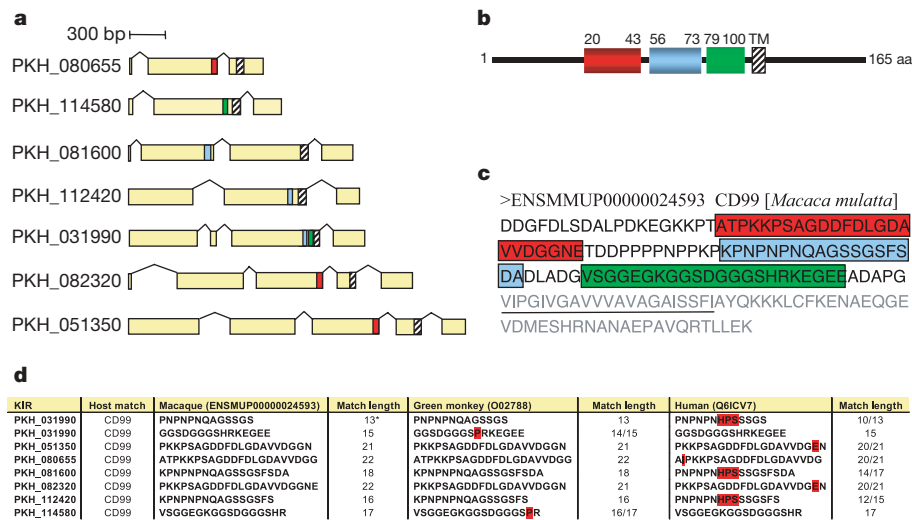


Figure 4 | Matches to CD99 host sequences in *P. knowlesi* (H strain).

a, Seven KIRs show conserved matches to three different regions of CD99 (shown in red, blue and green). **b**, Schematic view of *Macaca mulatta* CD99, showing matches to different KIRs. The numbers represent the amino acid position. TM, transmembrane domain. The highlighted regions represent the summary of perfectly matched amino acid stretches in the CD99 extracellular domain to a subgroup of seven KIR proteins. **c**, Amino acid sequence of *Macaca mulatta* CD99, highlighting the summary of matches to

KIRs. Amino acids corresponding to the transmembrane domain are underlined. The light-grey amino acids represent the transmembrane domain and the intracellular part of CD99. **d**, Comparison of the matches to *Macaca fascicularis*, African green monkey and human. Mismatches are highlighted in red. The asterisk refers to an additional host CD99 match in a KIR protein (PKH_031990) that did not satisfy the minimum length cutoff of 15 amino acids.

sequenced so far²⁷. In addition to the members of the PHIST family²⁷, an additional 100 proteins in *P. knowlesi* have typical PEXEL-like motifs (Supplementary Table 8 and Supplementary Fig. 22).

Like the PfEMP1 protein in *P. falciparum*, the SICAs and KIRs lack a signal peptide and a typical PEXEL-motif. We have identified a novel motif in the N-terminal region of SICA- α domains with a positionally conserved tryptophan residue surrounded by hydrophilic residues (Supplementary Fig. 22) that may be the export signal. Similarly, 75% of KIR proteins have a conserved Z-L-P-S motif (where Z denotes a hydrophilic residue) at the beginning of the KIR domain that may also facilitate export (Supplementary Fig. 22). In summary, approximately 280 predicted *P. knowlesi* proteins may be exported to the infected erythrocyte surface via the PEXEL-dependent or PEXEL-independent pathways. By comparison, the exportome of *P. vivax* is considerably larger than that of *P. knowlesi* and seems to be much bigger than previously thought²⁷. About 145 *P. vivax* proteins contain typical PEXEL motifs including the members of the PHIST family and a small subgroup of 12 VIRs.

Genome sequencing of *P. knowlesi* and its comparison with other malaria genomes has highlighted several novel features of this emerging and potentially life-threatening human malaria parasite, and underscores the importance of full genome sequencing of new *Plasmodium* species. Major differences in both content and organization of its genome were revealed that involve the host-parasite interface, reinforcing the notion that malaria species have evolved specific mechanisms for enhancing their survival within their respective hosts. The *P. knowlesi* genome will also greatly enhance the utility of this human-infective species as a model for addressing questions pertinent to all *Plasmodium* species.

METHODS SUMMARY

The random shotgun approach was used to obtain roughly eightfold coverage of the whole nuclear genome sequence from the erythrocyte stage of the Pk1(A+) clone of the H strain of *P. knowlesi*. Sequence reads were assembled (as described in the Supplementary Information) and positional information from sequenced read pairs were used to resolve the orientation and position of the contigs. The assembled *P. knowlesi* contigs were iteratively ordered and oriented by alignment to *P. vivax* assembled sequences (described in ref. 4) and by manual checking. Automated predictions from the gene finding algorithms were manually reviewed by comparison to orthologues in other *Plasmodium* species. Artemis and Artemis

Comparison Tool (ACT) were used (as described previously²⁸) for annotation and curation and viewing the TBLASTX comparisons of regions with conserved synteny between *P. knowlesi*, *P. vivax* and *P. falciparum*. This also allowed us to curate gene models and identify local interruptions of synteny. Functional annotations were based on standard protocols as described previously⁶.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions B.G.B., C.I.N., N.H., A.W.T. and C.M.R.T. initiated the project. M.A.Q., T.C., H.H., S.M., D.O., S.S., N.L., F.S., K.Br., R.S., S.T., S.M., M.Sa., M.Si., B.W. and D.W. constructed DNA libraries and performed sequencing; B.W., M.S. and I.C. finished and assembled sequence data; K.M., D. Harris and C.Ch. managed finishing and sequencing teams; M.A.R. managed the computational and bioinformatics support team; M.A.A., S.B., T.J.C., D. Harper, T.K., A.R.T., E.Z. and N.P. provided computational and bioinformatic support; U.B., A.E.B., E.M.P., S.L. and B.G.B. annotated the genome data. U.B., A.E.B., I.M.M., C.Ca., C.I.N., R.D.F., J.M., T.M., C.M.R.T., T.G.C., K.Bo., M.R.G., C.S.J., T.J.S., M.M., A.F.C., A.P.J., C.H.M.K., M.B. and A.P. contributed specific analysis topics presented in this manuscript or contributed data to characterize the genome and commented on manuscript drafts. U.B. performed data submission in EMBL. A.P., M.B., A.E.B., U.B. and C.I.N. drafted and edited the paper. A.P. and M.B. directed the project and A.P. assembled the manuscript.

Author Information The annotation and sequence data for the 14 chromosomes of the H strain of *P. knowlesi* have been submitted to the EMBL database with the following accession numbers: AM910983–AM910996. The annotation and sequence data are also available at <http://www.genedb.org> and <http://www.plasmodb.org>. Reprints and permissions information is available at www.nature.com/reprints. This paper is distributed under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence, and is freely available to all readers at www.nature.com/nature. Correspondence and requests for materials should be addressed to A.P. (ap2@sanger.ac.uk).

METHODS

Parasite material and isolation of genomic DNA. Genomic DNA was isolated from blood drawn from an infected rhesus monkey at 10% ring stage parasitaemia. Blood was Plasmodipur-filtered five times to remove white blood cells and erythrocytes were lysed in 0.1% saponin. Total parasite DNA was isolated using the PUREGENE DNA isolation kit (Gentra Systems), according to the manufacturer's instructions. All experimental animal work in these studies was carried out under protocols approved by the independent Institutional Animal Care and Use Committee and performed according to Dutch and European laws.

Sequencing. We sequenced the *P. knowlesi* genome from plasmid clones containing small fragments of up to 4 kb inserted into pUC19 vector. Problems associated with high G+C sequence were addressed by optimizing the sequence mixture. The quality of reads for the project was as follows: 97.6% of *P. knowlesi* reads had a quality score of (derived from the PHRED score generated by GAP4²⁹) >70 ($P = 1 \times 10^{-7}$). Regions containing repeat sequences or an unexpected read depth were manually inspected. In addition, a *P. knowlesi* fosmid library was constructed in pCC1FOS vector and end sequences were produced (10.5-fold clone coverage) to obtain paired-end information from 40-kb inserts. In particular, we re-examined regions with apparent breaks in synteny for potential misassembly errors and location of several intrachromosomal telomeric-repeat (GGGTT[T/C]A) sequences associated with *SICAvar* and *kir* genes. Sequence reads were assembled with PHRED/PHRAP on the basis of overlapping sequence and were manually edited in GAP4 database²⁹. Information from oriented read pairs, together with additional sequencing from selected large-insert clones and synteny with *P. vivax* chromosomes, were used to resolve potential misassemblies. Using long-range sequence information from the fosmid end sequences, we were able to bridge 142 out of 190 total gaps (Supplementary Table 1).

Gene finding and genome annotation. Annotation (PK4 version of assembly) was performed using the Artemis³⁰ and ACT software³¹. Genes were identified by manual curation of the output of the gene finding software SNAP³² and Annotaid (an extension of the comparative gene prediction program Projector³³; I. M. Meyer, unpublished). A set of 100 manually curated *Plasmodium knowlesi* genes was used as the training set for SNAP predictions. Annotaid was optimized for genome-wide analysis by training its parameters with a manually curated training set of 180 orthologous gene pairs from *P. knowlesi* and *P. falciparum*.

Functional assignments were based on assessment of BLAST and FASTA similarity searches against public databases and searches in protein domain databases such as InterPro³⁴. In addition, TMHMMv2.0³⁵, SignalPv3.0³⁶ and t-RNA scan³⁷ were used to identify transmembrane domains, signal peptides and t-RNA genes.

To define the orthologous and paralogous relationships between the predicted proteomes of three *Plasmodium* species (*P. falciparum*, *P. knowlesi*, *P. vivax*), the OrthoMCL protein clustering algorithm³⁸ was used with an inflation value of 1.5.

To search for parasite proteins containing stretches of perfectly matched host sequences, the PMATCH algorithm (R. Durbin, unpublished) was used to report

exact matches of 15 amino acids or greater after screening out low complexity sequences (details are provided in Supplementary Information).

Building profile HMMs of SICA and KIR protein domains. Sequence alignments and dotter³⁹ analysis of SICA proteins revealed the presence of a distinct N-terminal cysteine-rich domain (termed SICA- α : in some cases there are two copies of this domain), multiple central cysteine-rich domains (SICA- β) and a C-terminal cytoplasmic encoding domain (SICA_C). For each domain, a profile HMM (using HMMer, <http://hmmer.janelia.org/>) was constructed and searched against the *P. knowlesi* genome to find all examples of the domain (significant matches had *E*-values <0.001). The HMMs were rebuilt, using alignments constructed using all significant hits, and re-searched until no additional examples of the domain were found.

The program Phobius⁴⁰ was used to identify the putative transmembrane region located between the end of the last SICA- β domain and the SICA_C domain in all cases. An identical procedure was used to identify the domains in the KIR proteins. In this case, a single domain type was found on all KIR proteins, repeated between one and three times. Putative transmembrane proteins were identified as before, but only ~50% of KIR proteins had a predicted transmembrane region. Visual inspection of the corresponding C-terminal regions from sequences, both with and without predictions, showed the presence of a common hydrophobic patch. To investigate whether the Phobius⁴⁰ software was insufficiently sensitive to identify all of the KIR transmembrane regions, the predicted transmembrane regions were aligned and used to build a HMM of the transmembrane region. This was then used to iteratively search the whole genome as before.

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