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Identification and Characterization of the Duffy Antigen Receptor for Chemokines (*DARC*) Gene in Sichuan Golden Monkey (*Rhinopithecus roxellanae*)

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Abstract: The Duffy Antigen Receptor for Chemokines (*DARC*) gene also called Duffy or FY, *Plasmodium vivax* and *Plasmodium knowlesi* use DARC to trigger internalization into red blood cells and cause malaria, the malaria life cycle in humans and nonhuman primates. In order to investigate *DARC* gene in golden snub-nosed monkey (*Rhinopithecus roxellanae*), two pairs of primers were designed based on *DARC* gene sequence of the *Macaca mulatta* (HQ285849.1) and used to amplify approximately 1.0 and 1.1 kb DNA fragments respectively by PCR technique from genomic DNA sample of golden monkey. The DNA, sequencing and combing results showed that the *DARC* gene of the golden monkey was 1593 bp in length and contained a 47 bp 5 flanking region, two extrons (21 and 990 bp), one complete intron (478 bp) and a 57 bp 3 flanking region. The Open Reading Fragment (ORF) was 1011 bp and encoded 336 amino acid residues. The DARC was a hydrophobic protein with less hydrophilic components. The prediction of topological structure for the protein indicated that it contained 16 potential function sites: three N-glycosylation sites, one protein kinase C phosphorylation site, two casein kinase II phosphorylation sites and ten N-myristoylation sites. In addition, the protein comprised seven transmembrane helix regions and four extracellular regions and four intracellular regions. Alignment analysis revealed that the homologies of *DARC* gene nucleotide sequence of golden snub-nosed monkey with other primate species and human was 95-99% and the homologies of amino acid sequence was 80-99%. These results would provide the molecular basis for golden monkey against human malarias.

Key words: *Rhinopithecus roxellanae*, *DARC* gene, sequencing analysis, primates, malaria

INTRODUCTION

The genus *Rhinopithecus* is comprised of four distinct allopatric species: *R. brelichi* (the gray snub-nosed monkey), *R. bieti* (the black snub-nosed monkey), *R. roxellana* (the golden snub-nosed monkey) and *R. avunculus* (the Tonkin snub-nosed monkey) (Groves, 2001) all of four species are found only in Asia with the exception of *R. avunculus* which is distributed in temperate areas of China and inhabit six isolated mountainous regions (Boonratana and Le, 1998; Kirkpatrick, 1998). Sichuan golden monkey (*Rhinopithecus roxellanae*), namely the golden snub-nosed monkey which distributes only in Qinling, Sichuan/Gansu and Shennongjia Mountains in China (Li *et al*., 2003). Sichuan golden monkey is enigmatic and threatened primates that belong to the subfamily Colobinae (Kirkpatrick, 1998; Ren *et al*., 1998).

The *DARC* gene also called Duffy or FY (Demogines *et al*., 2012), encodes a membrane-bound chemokine receptor, different human alleles of this gene underlie the designation of different Duffy blood groups (Meny, 2010). But DARC also plays a role in the biology of malaria, in humans, the severe form of malaria is caused by *Plasmodium falciparum*, *P. vivax* and *P. knowlesi*, exploit DARC for internalization into red blood cells, interaction with DARC is mediated by a Plasmodium surface ligand called Duffy-Binding Protein (DBP) (Haynes *et al*., 1988; Wertheimer and Barnwell, 1989). The N-terminal extracellular tail of DARC interacts with the DBP of *P. vivax* and *P. knowlesi* and also contains some of the determinants for chemokine binding (Chitnis *et al*., 1996; Tournamille *et al*., 2003, 2005).

In a fascinating example of convergent evolution, polymorphisms in the cis-regulatory region of DARC in African baboons are also associated with resistance to a

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malaria-like parasite common in baboon populations (Tung *et al*., 2009). Malaria caused the attention of people again according to the World Malaria Report 2008, 3.3 billion people were at risk of acquiring malaria by the end of 2006, 250 million clinical episodes of malaria occur each year (mainly due to *P. falciparum* and *P. vivax* infections) of which more than 1 million people die (WHO, 2008). *R. roxellana* was widely known for its shining golden coat and funny snub nose (Li *et al*., 2003) which was categorized as an endangered species by The World Conservation Union (IUCN, 2007) and was also listed as a Category I species under the Chinese Wild Animal Protection Law (Yang *et al*., 2002). Despite substantial research on the physiological, morphological and behavioral characters of golden monkeys were investigated, little research has examined to against human malarias survival in the species at the molecular level. It is therefore interesting and necessary to investigate the key gene for understand the molecular mechanisms that underlie the prevention and treatment of malaria.

MATERIALS AND METHODS

Genomic DNA extraction: The golden monkey was provided by Wildlife and Natural Reserve Laboratory, Sichuan Agricultural University, Ya'an, Sichuan, China. Genomic DNA was extracted from frozen muscle of golden monkey using a standard proteinase K, Phenol/Chloroform Extraction Method (Sambrook *et al*., 1989). Both the quantity and quality of total DNA were assessed by agarose gels and Gel Electrophoresis Imaging Analysis System (CBIO-GelPro, Ultraviolet Technologies, Beijing, China).

Primer design, DNA cloning and sequencing: The PCR primers were designed based on the sequence alignment of *Macaca mulatta* and *Homo sapiens* using Primer 5.0 Software. The primer sequences are listed:

(PF1: FY1-F 5'-CCTCATTAGTCCTTGGCTCTTATCT-3';
FY1-R 5'-ACCATACCAGACACAGTAGCCCA -3'.
PF2: FY2-F 5'-CCTCAACTCAGAACTCAAGTCAGC-3';
FY2-R 5'-TCTCCCCAGACAAAATAAGAAACC-3')

To amplify the *DARC* gene sequences from golden monkey, PCR reactions were carried out in 50 μL total volume and included 5 μL diluted samples. PCR reaction parameters were: initial denaturation 94°C for 5 min 35 cycles of denaturation 94°C for 30 sec, annealing 61°C for 35 sec and extension 72°C for 45 sec followed by final elongation 72°C for 5 min then stored at 4°C. PCR products were subjected to agarose gel electrophoreses in 1×TAE buffer. PCR products were purified from the gel slices using the gel extraction kit (Sangon Biotech Co., Ltd. Shanghai, China). Purified PCR products were eluted into 50 μL ddH$_2$O then sequenced directly using Big Dyeterminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems, Darmstadt, Germany) in an automatic sequencer (ABI-PRISM3730, Genetic analyzer, Applied Biosystems, California, USA).

Data analysis: The sequences of assembled contigs and corresponding CDs sequences were aligned and anslyzed by the SeQman Protean module of the Lasergene 7.1 Software (DNASTAR, Madison, WI, USA). Compared the sequence to *M. mulatta*'s and predicted the ORF of the golden monkey *DARC* gene. The Simple Modular Architecture Research tool (SMART) was employed to predict the hydrophobicity. PredictProtein online tool was used to predict potential function sites. The informations of hydrophobicity analysis were obtained through the DNAMAN. Transmembrane helices were displayed with the help of expasy tools (http://www.enzim.hu/hmmtop/html/submit.html).

Sequences from the *DARC* gene family were collected from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the Blast search program. The alignments of amino acid and nucleotide sequence used for the analysis of the construction of phylogenetic trees were made using Clustal X. *Homo sapiens* (*H. sapiens*, JN251916.1); 3 Great Ape Species: *Gorilla gorilla* (*G. gorilla*, AF311914.1), *Pan troglodyte*s (*P. troglodyte*s, AF311920.1), *Pongo pygmaeus* (*P. pygmaeus*, JN544135.2) that diverged from humans about 5-14 million years ago; 2 New World monkey species: *Ateles geoffroyi* (*A. geoffroyi*, GU219525.1), *Callithrix jacchus* (*C. jacchus*, GU219520.1) that diverged from humans about 25 million years ago and 12 Old World monkey species: *Macaca mulatta* (*M. mulatta*, HQ285849.1), *Macaca thibetana* (*M. thibetana*, HQ285852.1), *Macaca fascicularis* (*M. fascicularis*, HQ285848.1), *Mandrillus sphinx* (*M. sphinx*, HQ285854.1), *Macaca nigra* (*M. nigra*, HQ285851.1), *Mandrillus leucophaeus* (*M. leucophaeus*, HQ285853.1), *Lophocebus. aterrimus* (*L. aterrimus*, HQ285847.1), *Papio anubis* (*P. anubis*, GU219518.1), *Theropithecus gelada* (*T. gelada*, GU219519.1), *Cercocebus agilis* (*G. agilis*, GU219528.1), *Cercopithecus mona* (*C. mona*, GU219517.1), *Trachypithecus francoisi* (*T. francoisi*, JN544126.2) that diverged from humans about 40 million years ago (Goodman *et al*., 1998) and the sequence of *Mus musculus* (*M. musculus*, NM_010045.2) was loaded down from ensemble.

MEGA 4.0 (Kumar *et al*., 2008) was used to construct the phylogenetic tree: the corresponding nucleotide sequences were then imported into MEGA 4.0 for multiple sequence alignment by ClustalX and phylogenetic analysis by using the Neighbor-Joining and Bootstrap Methods. In the NJ analysis, the Kimura 2-parameter nucleotide model with a pairwise deletion option for gaps was used and the reliability of the tree topologies was evaluated using bootstrap support (Felsenstein, 1985) with 1000 replicates.

RESULTS AND DISCUSSION

Gene sequencing and characteristics: The DNA sequencing and combining result showed that a 1593 bp *DARC* gene sequence of the golden monkey was gained, full-length DNA sequences contained a 47 bp 5'-flanking region, two extrons (21 and 990 bp), one complete intron (478 bp) and a 57 bp 3'-flanking regionan, an open reading frame of 1011 bp encoded 336 amino acids, initiator codon and terminator codon were ATG and TAG, respectively, the average content of A, G, T and C are 16.12, 26.71, 26.81 and 30.37%, respectively. In which, A+T was 42.93% and G+C was 57.07%. The DARC protein molecular weight was 35.597 kDa, the isoelectric point was 6.213 and its charge quantity was -3.952 at pH 7.0. The estimated half-life was 30 h (mammalian reticulocytes, *in vitro*), the instability index (II) was 41.02 and the protein was classified as unstable. The protein contained 160 hydrophobic amino acids (Ala, Ile, Leu, Phe, Trp and Val) 83 polar amino acids (Asn, Cys, Gln, Ser, Thr and Tyr), 11 positively charged amino acid residues (Lys, Arg) and 16 negatively charged amino acid residues (Asp, Glu).

PredictProtein Online Software was used to predict the Duffy protein functional sites, it contained 16 potential function sites: three N-glycosylation sites (16NSSQ, 27NSSY, 33NDSF), one Protein kinase C phosphorylation site (122STR), two casein kinase II phosphorylation sites (35SFPD, 327SHLD) and ten N-myristoylation sites (72GILASS, 119GLGSTR, 138GSAFAQ, 148GCHASL, 160GQVPGL, 168GLTVGL, 189GASGGL, 224GLFGAK, 230GLKKAL and 257GVVLGL) (Fig. 1). In addition, the protein comprised seven transmembrane helix regions (64-83, 92-116, 135-154, 163-187, 208-227, 234-253 and 284-307), four extracellular regions (1-63, 117-134, 188-207 and 254-283) and four

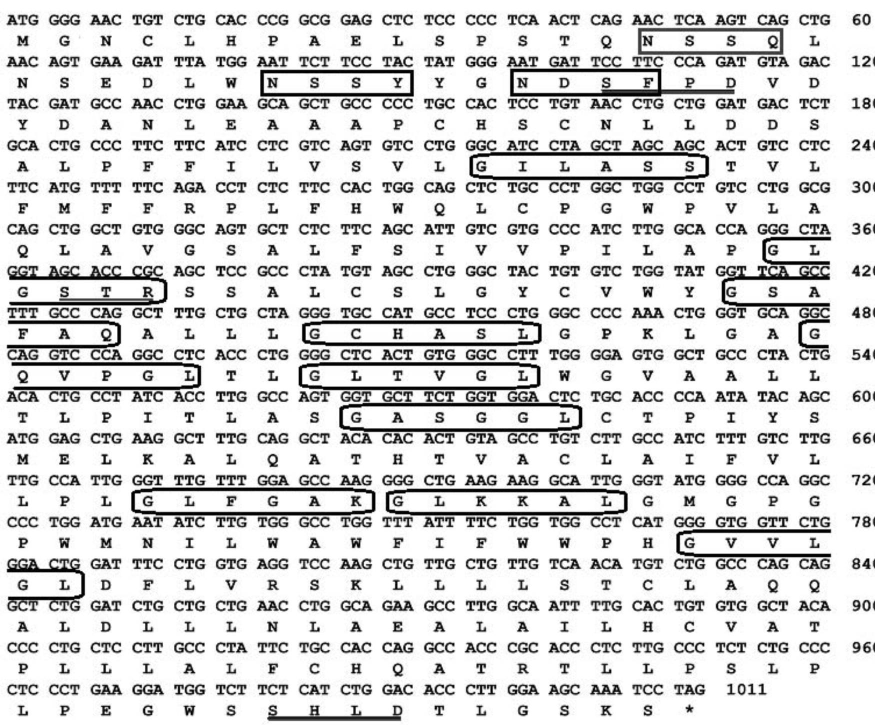

Fig. 1: The coding nucleotide sequence and deduced amino acid sequence of *R. roxellanae DARC* gene. Different signs are used to mark the following function sites: “ ” shows N-glycosylation sites (16NSSQ, 27NSSY and 33NDSF), “ ” shows Protein kinase C phosphorylation site (122STR), “___” shows Casein kinase II phosphorylation sites (35SFPD, 327SHLD), “ ” shows N-myristoylation sites (72GILASS, 119GLGSTR, 138GSAFAQ, 148GCHASL, 160GQVPGL, 168GLTVGL, 189GASGGL, 224GLFGAK, 230GLKKAL, 257GVVLGL); in addition, N-glycosylation sites (33NDSF) and Casein kinase II phosphorylation sites (35SFPD) are overlaped partly

intracellular regions (84-91, 155-162, 228-233 and 308-336) (Fig. 2). In order to distinguish regions having conserved properties, namely hydrophobicity researchers got the informations using DNAMAN Software, there were 238 hydrophobic amino acids in the whole protein, the Grand Average of hydropathicity (GRAVY) was 0.7083, aliphatic index was 119.11, it was obvious that the DARC protein was a hydrophobic protein with less hydrophilic components from the diagram (Fig. 3).

Phylogenetic and homologies analysis: A single *DARC* gene tree was inferred from the coding region (Fig. 4), the tree was congruent with phylogenetic species tree, there was a faster evolution of DARC in Old World monkey than apes and humans. The tree indicated a closer relationship of the *R. roxlanae DARC* gene with *H. sapiens* researchers obtained the information that all the New World monkeys were formed a clade, all the Old World monkeys were fomed a clade and all the apes were fomed a clade with *H. sapiens*. Besides, homologies of the *DARC* gene, nucleotide sequence with that of other 18 species was 95-99%, the amino acid level was 80-99%, the sequence of mouse is the least identical one either at the nucleotide (73%) or at the amino acid (71%) which suggested that the DARC region was highly conserved. There was a aspartic acid (D) at position 42 in the golden monkey of Duffy protein which defines as isoform FY*A Duffy blood.

DARC, it is considered as silent chemokine receptor because of the important role in immunity system, various

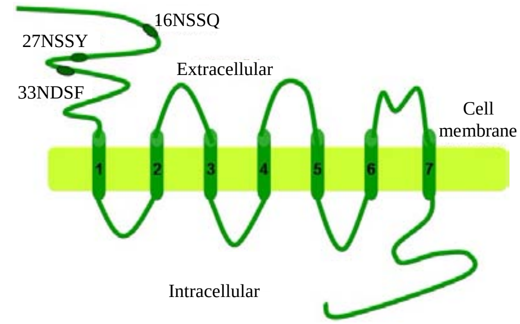

Fig. 2: The structural model of Duffy protein with seven transmembrane domains. Numbers associated with circles are the N-glycosylation sites in the golden monkey Duffy protein sequence, interaction with Duffy-binding protein of Plasmodium

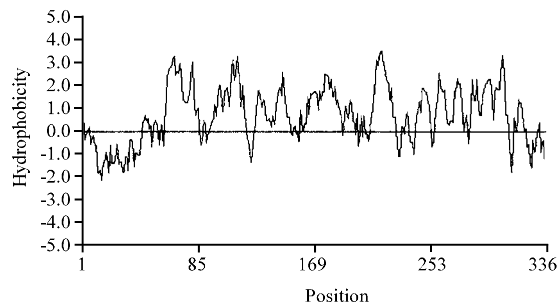

Fig. 3: Hydrophobicity plot of the deduced amino acid sequence of Duffy protein. Hydrophobic segments were characterized by positive values

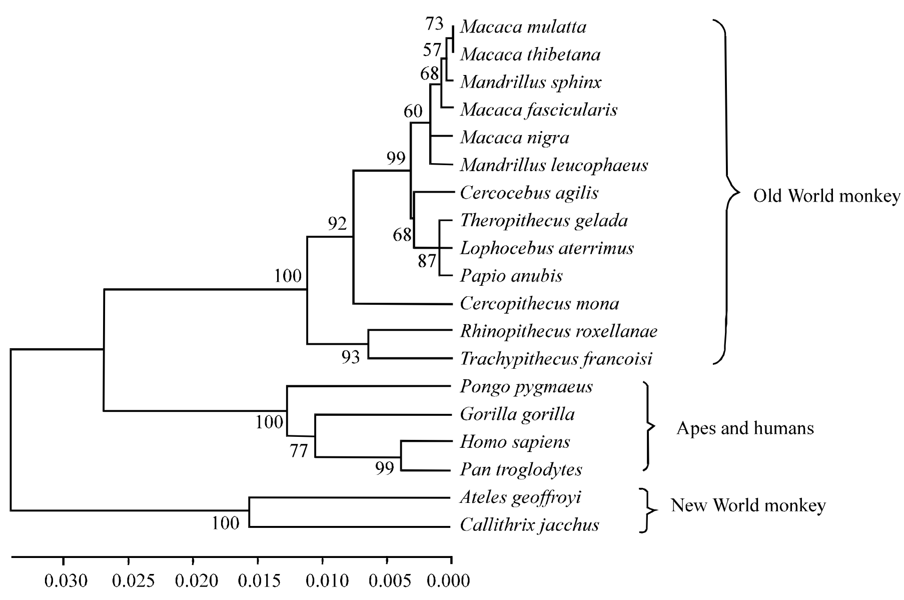

Fig. 4: Phylogenetic tree derived from the multiple sequence alignment for the coding region nucleotide sequence of DARC using ClustalX. And repertoire based on Neighbor-Joining Method with 1000 bootstrap values of MEGA 4.0 programme. Bootstrap support values for the main branches defining different species were shown in the tree

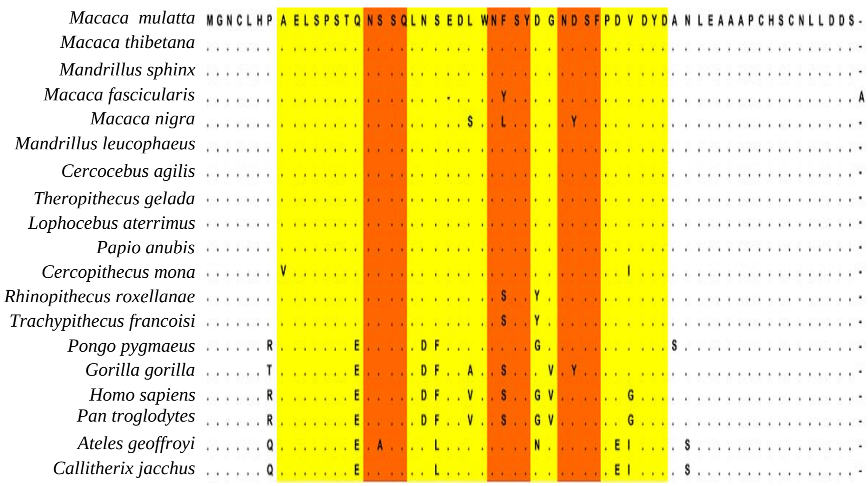

Fig. 5: Multiple sequence alignment of the N-terminal tail sequence of DARC using ClustalX, the *M. mulatta* sequence was used as reference with a dot for amino acids identity, a dash for a deletion. The N-terminal tail sequence which the first 60 codons contains the interaction domain with *P. vivax* and *P. knowlesi* (Chitnis *et al*., 1996), DARC N-terminal tail (aa 1-60) with Plasmodium interaction domain (aa 8-42), N-glycosylation site (aa 16-19, aa27-30, aa33-36)

Table 1: Potential function sites of Duffy protein of *H. sapiens*, *R. roxellana*, *T. francoisi* and *M. mulatta*

Species	N-glycosylation site	Protein kinase C phosphorylation site	Casein kinase II phosphorylation site	N-myristoylation site	
Homo sapiens	16NSSQ	122STR	18SQLD	31GVNDSF	72GILASS
	27NSSY		35SFPD	119GLGSTR	138GSAFAQ
	33NDSF		327SHLD	148GCHASL	160GQVPGL
				168GLTVGI	189GASGGL
				224GLFGAK	230GLKKAL
				257GVVLGL	
Rhinopithecus roxellanae	16NSSQ	122STR	35SFPD	72GILASS	119GLGSTR
	27NSSY		327SHLD	138GSAFAQ	148GCHASL
	33NDSF			160GQVPGL	168GLTVGL
				189GASGGL	224GLFGAK
				230GLKKAL	257GVVLGL
Macaca mulatta	16NSSQ		34SFPD	71GILASG	118GLGNTR
	26NFSY		326SHLD	137GSAFAQ	147GCHASL
	32NDSF			159GQVPGL	167GLSVGL
				188GASGGL	223GLFGAK
				219GLKKAL	
				256GVVLGL	

studies about *DARC* gene have been made in different species in the past few years. This was the first time researchers obtained the whole *DARC* gene CDs and a part of 5'- flanking region and 3'-flanking region in golden monkey. The protein domain prediction of the DARC CDs showed the typical DARC structure with seven transmembrane helix regions, four extracellular regions and four intracellular regions. It was the common polymorphism to human at position 42 which defines the FY*A and FY*B Duffy blood group alleles (Meny, 2010). The coding sequence of each ortholog in 19 species was assembled from genomic sequence based on the structure of the human isoform FY*A transcript. The nucleotide mutation rate was very low, phylogenetic relationship was in agreement with accepted primate phylogeny (Perelman *et al*., 2011). Most of 19 species, 990 bases is encoded by a single exon with 21 bases coming from an upstream exon. The alignment of these sequences was straightforward with a single codon deletion, being the only indel present. It is possible that the rapid evolution of these codons may have been driven by a selective pressure of Plasmodium (Demogines *et al*., 2012) old world monkey is faster than apes and humans in *DARC* gene evolution.

The N-terminal tail of DARC was analyzed alone (Fig. 5), a region where high sequence divergence between primate orthologs, several mutations in this domain have been shown. Different primate orthologs of

DARC are all able to bind a relevant human chemokine but could have affected Plasmodium susceptibility by modulating the interaction with DBP (Tournamille *et al*., 2003, 2004, 2005). The zoonotic transmissions of *P. vivax* and *P. knowlesi* from Asian macaques are estimated to have occurred <250,000 years ago (Escalante *et al*., 2005; Mu *et al*., 2005; Lee *et al*., 2011). If so, in the N-terminal tail of DARC, golden monkey was inconformity to *M. mulatta* at aa 28 and aa 31, golden monkey isn't also safe where malaria popular area. Glycosylation sites of DARC are critical region for *P. knowlesi* and *P. vivax* to intrude into erythrocytes, other protein functional sites decided to susceptibility (Demogines *et al*., 2012), the polymorphism 42 isn't in glycosylation site region, thus, the polymorphism of golden monkey DARC is unlikely to play a role in differential susceptibility. N-glycosylation sites of golden monkey were congruent with that of *H. sapiens* and *M. mulatta* (Table 1) so *P. vivax* and *P. knowlesi* mought invade into golden monkey red blood cells and cause malaria. Other protein functional sites (Protein kinase C phosphorylation site, Casein kinase II phosphorylation site, N-myristoylation site and so on) were congruent incompletely, indicated only that the susceptibility was different to golden monkey caused malaria from humans.

CONCLUSION

It is effective that *M. mulatta* DNA vaccine against malaria in human clinical trials (Wang *et al*., 1998). Indeed, evidence exists that African apes are currently under intense pressure, as sampling projects in wild chimpanzees, bonobos and gorillas have uncovered *P. vivax* variants and closely related Plasmodium species beyond those known to infect humans (Kaiser *et al*., 2010; Krief *et al*., 2010; Liu *et al*., 2010). Understanding the host genetics that define species tropism of these pathogens is extremely important both for the protection of wildlife resources of against malaria and for understanding how zoonotic processes give rise to new human diseases.

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