

RESEARCH ARTICLE

Genetic Diversity, Natural Selection and Haplotype Grouping of *Plasmodium knowlesi* Gamma Protein Region II (Pk γ RII): Comparison with the Duffy Binding Protein (PkDBP α RII)

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Abstract

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Background

Plasmodium knowlesi is a simian malaria parasite that has been reported to cause malaria in humans in Southeast Asia. This parasite invades the erythrocytes of humans and of its natural host, the macaque *Macaca fascicularis*, via interaction between the Duffy binding protein region II (PkDBP α RII) and the Duffy antigen receptor on the host erythrocytes. In contrast, the *P. knowlesi* gamma protein region II (Pk γ RII) is not involved in the invasion of *P. knowlesi* into humans. Pk γ RII, however, mediates the invasion of *P. knowlesi* into the erythrocytes of *M. mulata*, a non-natural host of *P. knowlesi* via a hitherto unknown receptor. The haplotypes of PkDBP α RII in *P. knowlesi* isolates from Peninsular Malaysia and North Borneo have been shown to be genetically distinct and geographically clustered. Also, the PkDBP α RII was observed to be undergoing purifying (negative) selection. The present study aimed to determine whether similar phenomena occur in Pk γ RII.

Methods

Blood samples from 78 *knowlesi* malaria patients were used. Forty-eight of the samples were from Peninsular Malaysia, and 30 were from Malaysia Borneo. The genomic DNA of the samples was extracted and used as template for the PCR amplification of the Pk γ RII. The PCR product was cloned and sequenced. The sequences obtained were analysed for genetic diversity and natural selection using MEGA6 and DnaSP (version 5.10.00) programmes. Genetic differentiation between the Pk γ RII of Peninsular Malaysia and North Borneo isolates was estimated using the Wright's F_{ST} fixation index in DnaSP (version 5.10.00). Haplotype analysis was carried out using the Median-Joining approach in NETWORK (version 4.6.1.3).

Results

A total of 78 Pk γ RII sequences was obtained. Comparative analysis showed that the Pk γ RII have similar range of haplotype (Hd) and nucleotide diversity (π) with that of PkDBP α RII. Other similarities between Pk γ RII and PkDBP α RII include undergoing purifying (negative) selection, geographical clustering of haplotypes, and high inter-population genetic differentiation (F_{ST} index). The main differences between Pk γ RII and PkDBP α RII include length polymorphism and no departure from neutrality (as measured by Tajima's D statistics) in the Pk γ RII.

Conclusion

Despite the biological difference between Pk γ RII and PkDBP α RII, both generally have similar genetic diversity level, natural selection, geographical haplotype clustering and inter-population genetic differentiation index.

Introduction

Malaria is caused by protozoa of the genus *Plasmodium*. Four species are responsible for human malaria: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Human infections by the simian malaria parasite *P. knowlesi* have once been thought to be rare [1]. This was because *P. knowlesi* had been erroneously identified under microscopy examination as *P. malariae*, since both species have similar trophozoite morphology in the infected erythrocytes. This problem was eventually overcome by the use of highly specific polymerase chain reaction (PCR). Hence, a study at a district hospital in Sarawak (Malaysia Borneo) in 2004 reported PCR detection of a large number of knowlesi malaria cases that were initially diagnosed by microscopy examination as *P. malariae* infections [2]. Subsequent PCR tests on archived blood smears have also detected significant number of *P. knowlesi* infections. Human knowlesi malaria has now been documented in almost all countries in Southeast Asia [3].

P. knowlesi invades the erythrocytes of human and of its natural host, the macaque *Macaca fascicularis*, via interaction with the Duffy antigen on the erythrocytes [4–6]. The Duffy binding protein of *P. knowlesi* (PkDBP) is a large protein which is divided into seven regions (I–VII). Region II contains the critical motifs for binding to the erythrocyte Duffy antigen [5]. PkDBP is encoded by the α -gene, and is closely related to two other homologous proteins of *P. knowlesi*—Pk β and Pk γ . However, unlike region II of PkDBP α (PkDBP α RII), the region II of β (Pk β RII) and γ (Pk γ RII) does not bind to the Duffy antigen of human, but binds to rhesus monkey (*M. mulata*) erythrocytes [7]. Hence, Pk β and Pk γ are responsible for the Duffy-independent pathways for invasion of rhesus erythrocytes. Pk β RII binds to a sialic acid receptor on rhesus erythrocytes [8]. Although the receptor for Pk γ RII remains to be identified, it has recently been demonstrated that Pk γ RII is also a sialic acid-dependent invasion ligand [9].

Although PkDBP α and Pk γ are closely related erythrocyte binding proteins (EBP), each uses a different pathway to mediate invasion of *P. knowlesi* merozoite into the host erythrocyte. The PkDBP α RII binds with the Duffy protein receptor for chemokines (DARC) on the surface of erythrocytes, whereas Pk γ RII binds to N-glycolylneuraminic acid-sialylated receptors [9]. Previous studies [10, 11] have revealed high genetic diversity of PkDBP α RII in *P. knowlesi* isolates from Peninsular Malaysia and North Borneo. Also, the PkDBP α RII was observed to be undergoing purifying (negative) selection. Furthermore, phylogenetic analysis found that

PkDBP α RII haplotypes of North Borneo isolates were genetically distinct from those of Peninsular Malaysia.

The aim of this study therefore is to determine whether Pk γ RII is subjected to the same selection forces as the PkDBP α RII, by measuring its genetic diversity level, selection trend (positive or negative) and geographical clustering of its haplotypes.

Materials and Methods

Ethics statement

Ethical clearance for this study was obtained from University of Malaya Medical Ethics Committee (Ref No. 817.18) and the Medical Research Ethic Committee (MREC), Ministry of Health, Malaysia (National Medical Research Register ID No.13079). Informed verbal consent was obtained from patients for use of their blood samples for diagnosis and research. Written consent was found to be unnecessary as verbal consent would be sufficient for the purpose of this study and patient details were noted down solely for personal recordkeeping. This consent procedure was approved by the ethics committees.

Blood sample collection

Seventy-eight blood samples were used in this study. The samples (0.5 ml) were collected by trained medical personnel from knowlesi malaria patients in the University of Malaya Medical Centre (UMMC), Kuala Lumpur, Peninsular Malaysia (n = 48) and several public hospitals in two Malaysian states (Sabah and Sarawak) in North Borneo (n = 30). The samples were collected between 2010 and 2013.

Extraction of DNA

Total DNA of *P. knowlesi* was extracted from each blood sample using the QIAGEN Blood DNA Extraction kit (QIAGEN, Hilden, Germany). In each extraction, 100 μ l of blood was used. The extracted DNA was suspended in water to a final volume of 50 μ l.

PCR, cloning and sequencing of the Pk γ RII

The Pk γ RII was amplified by nested PCR using oligonucleotide primers F1: 5' -CGCATTTTG AAGGAATCCAC-3' and R1: 5' -TGCTAGACTTACCTTCACCT-3' for nest 1. The primers for the nest 2 reaction were F: 5' -TCCTCAAAGGCGGTGACCATCC-3' and R: 5' -ACTGGC TGCCTTAGATTCAACACCA-3'. Cycling conditions for nest 1 were as follows: 95°C for 4 mins, 30 cycles at 95°C for 30 secs, 48°C for 30 secs, and 72°C for 90 secs, followed by a 10 min extension at 72°C. The amplification for nest 2 was performed using the following cycling profile: 95°C for 4 mins, 30 cycles at 95°C for 30 secs, 56°C for 30 secs, and 72°C for 90 secs, followed by a 10 min extension at 72°C. The PCR products were purified with QIAquick Gel Extraction Kit and ligated into pGEM[®]-T plasmid vector (Promega Corp., USA). Each ligation mixture was transformed into *Escherichia coli* TOP 10 competent cells. Plasmid DNA from clones having the desired DNA fragment was extracted using the QIAprep Spin Miniprep Kit. The plasmids were sent to a commercial laboratory (MyTACG Bioscience Enterprise, Malaysia) for DNA sequencing. To verify the sequences, the recombinant plasmids of three clones from each isolate were sequenced. In addition, the sequencing was performed in both strands of DNA.

Sequence diversity, natural selection and haplotype analyses

Multiple alignment of Pk γ RII sequences was performed using CLUSTAL-Omega programme [12]. Both nucleotide and the deduced amino acid sequences were aligned and analysed. DnaSP ver. 5.10.00 [13] was used in the polymorphism analysis of the Pk γ RII sequences. Information such as haplotype diversity (Hd) and nucleotide diversity (π) was generated. The rates of synonymous (dS) and non-synonymous (dN) mutations were estimated and compared by the Z-test ($P < 0.05$) in MEGA6 using the Nei and Gojobori's method with Jukes and Cantor correction [14]. In purifying (negative) selection, mutations are usually not advantageous, so that dN will be less than dS ($dN/dS < 1$). However, in positive selection, non-synonymous mutations can be advantageous and dN will exceed dS ($dN/dS > 1$).

The Tajima's D statistics [15] was used to test departures from the neutral theory of evolution, with the assumption that the population size was constant. The Wright's F_{ST} fixation index [16] was used to measure genetic differentiation between the Pk γ RII of Peninsular Malaysia and North Borneo isolates. Both the Tajima's D statistics and Wright's F_{ST} index were determined using DnaSP 5.10.00. The Median-Joining method [17] in NETWORK v4.6.1.3 programme [18] was used to establish genetic relationship among the Pk γ RII haplotypes.

Results and Discussion

The Pk γ RII of *P. knowlesi* clinical isolates from patients in Peninsular Malaysia and North Borneo were successfully amplified, cloned, and sequenced. Three clones from each isolate were used for sequencing. All clones from each isolate showed identical Pk γ RII sequences. A total of 78 Pk γ RII nucleotide sequences was obtained (GenBank Accession No. KR053974-KR054021, KU216673-KU216702). For analysis, the Pk γ RII sequence of the reference *P. knowlesi* strain H GenBank (GenBank Accession No. M90695) was also included. Each sequence was trimmed, using the codons for amino acids at the N- and C-terminals of PkDBP α RII as reference points.

Previous studies have found no length polymorphism in PkDBP α RII [10, 11]. All PkDBP α RII sequences of *P. knowlesi* isolates from Peninsular Malaysia and North Borneo were 307 amino acids in length. In the present study, multiple alignment of the nucleotide sequences revealed length polymorphism in the Pk γ RII gene, ranging between 915 and 921 base pairs, hence encoding amino acid sequences of 305–307 in length. Full alignment of these amino acid sequences (S1 Fig) showed 54 Pk γ RII sequences with 307 amino acids, and 24 sequences with 306 amino acids. The Pk γ RII of *P. knowlesi* strain H was the only sequence with 305 amino acids. Close inspection of the alignment showed two deletions in the Pk γ RII of *P. knowlesi* strain H, N at position 55 and K at position 166. For sequences with 306 amino acids, the deletion was K at position 166. Uniquely, no length polymorphism was seen in the North Borneo Pk γ RII sequences. All the North Borneo sequences were 307 amino acid in length.

The PkDBP α RII contains 12 conserved C residues (positions 16, 29, 36, 45, 99, 176, 214, 226, 231, 235, 304, 306) [11, 19]. These C residues form six disulphide bridges in the folding of PkDBP α RII for interaction with the erythrocyte Duffy antigen chemokine receptor (DARC). Surprisingly, despite having length polymorphism and no interaction with DARC, Pk γ RII still retained the 12 conserved C residues at same positions (S1 Fig) as in PkDBP α RII. Apart from the conserved C residues, the conserved residues Y94, N95, K96, R103, L168, and I175 are required for the interaction of PkDBP α RII with DARC [19]. In this regard, however, Pk γ RII showed no similarity with PkDBP α RII. Except for Y94, the remaining residues in Pk γ RII (S95, E96, K103, K168, N175) were different from those of PkDBP α RII. The amino acid changes at

Table 1. Genetic diversity and selection pressure of PkγRII of *P. knowlesi* isolates in Peninsular Malaysia and North Borneo.

Origin	N	Hd ± SD	π ± SD	dN	dS	dN/dS	Z-test			Tajima's D
							dN = dS	dN > dS	dN < dS	
Malaysia	79	0.991 ± 0.005	0.021 ± 0.001	0.019	0.030	0.633	P = 0.171 [#]	P = 1.000 [#]	P = 0.082 [#]	-1.538 [#]
Peninsular Malaysia	49	0.979 ± 0.013	0.009 ± 0.002	0.009	0.010	0.900	P = 0.706 [#]	P = 1.000 [#]	P = 0.375 [#]	-2.352 [*]
North Borneo	30	0.991 ± 0.011	0.013 ± 0.001	0.009	0.029	0.310	P = 0.004 ^{&}	P = 1.000	P = 0.002 [*]	-1.275 [#]

N: number of sequences; Hd: haplotype diversity; π: nucleotide diversity; dN: rate of non-synonymous mutations; dS: rate of synonymous mutations

[#] not significant (P > 0.10)

^{*} significant (P < 0.01)

[&] reject null hypothesis of strict neutrality, i.e., dN = dS

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these key positions may possibly explain the inability of PkγRII to bind to human and *M. falciparum* erythrocytes.

DNA sequence analyses were conducted to determine nucleotide diversity and genetic differentiation of PkγRII (Table 1). By taking the total 79 sequences as a single population, the overall haplotype diversity (Hd) and nucleotide diversity (π) were 0.991 ± 0.005 and 0.021 ± 0.001, respectively. To determine whether natural selection contributed to the diversity, the rate of non-synonymous (dN) to synonymous mutations (dS) was determined. dN (0.019) found to be lower than dS (0.030). The dN/dS ratio was 0.633, which suggested purifying (negative) selection of PkγRII. Although the overall Z-test did not show significant natural selection, there was indication towards purifying selection (dN < dS, P = 0.082). Tajima's D statistics (-1.538, P > 0.10) revealed no significant departure from neutrality.

However, when the PkγRII sequences of Peninsular Malaysia and North Borneo were analysed as two separate populations, some differences were observed (Table 1). The North Borneo PkγRII was noted to have slightly higher diversity (Hd = 0.991 ± 0.011; π = 0.013 ± 0.001) than the PkγRII of Peninsular Malaysia (Hd = 0.979 ± 0.013; π = 0.009 ± 0.002). Tajima's D statistics revealed significant departure of neutrality (-2.352, P < 0.01) only in the Peninsular Malaysia PkγRII. Although the dN/dS ratios showed purifying selection of PkγRII on both populations, the Z-test indicated strong purifying selection of the North Borneo PkγRII (dN < dS, P = 0.002). Comparative analysis showed that the PkDBPαRII of Peninsular Malaysia [9] and North Borneo [10] have almost similar range of haplotype (Hd = 0.986, 0.999) and nucleotide diversity (π = 0.013, 0.012) with that of PkγRII. Purifying selection on the PkDBPαRII was equal in both population (dN < dS, P < 0.05). Unlike PkγRII, the PkDBPαRII of both Peninsular Malaysia and North Borneo showed significant departure from neutrality by the Tajima's D statistics (-2.085, P < 0.05; -2.459, P < 0.01 respectively) [9, 10].

It is interesting that higher haplotype diversity was seen in PkDBPαRII as compared to PkγRII despite the lesser number of samples used in the PkDBPαRII studies [10, 11]. The previous and present studies used only human/clinical *P. knowlesi* isolates. Therefore, the PkDBPαRII of the isolates is likely to be under selection pressure to generate high polymorphism (i.e., high haplotype diversity) for the parasite to escape the host's immune defenses. PkγRII, on the other hand, does not mediate invasion of *P. knowlesi* into human erythrocyte and therefore is likely under less selection pressure to display high polymorphism for immune evasion.

Phylogenetic analysis from a previous study reported two haplotype groups of PkDBPαRII in *P. knowlesi* from Peninsular Malaysia [10]. The distribution of the haplotypes though was uneven, because majority (83.3%) of the haplotypes clustered in a large or major group. A subsequent study reported distinct phylogenetic grouping of PkDBPαRII haplotypes from North

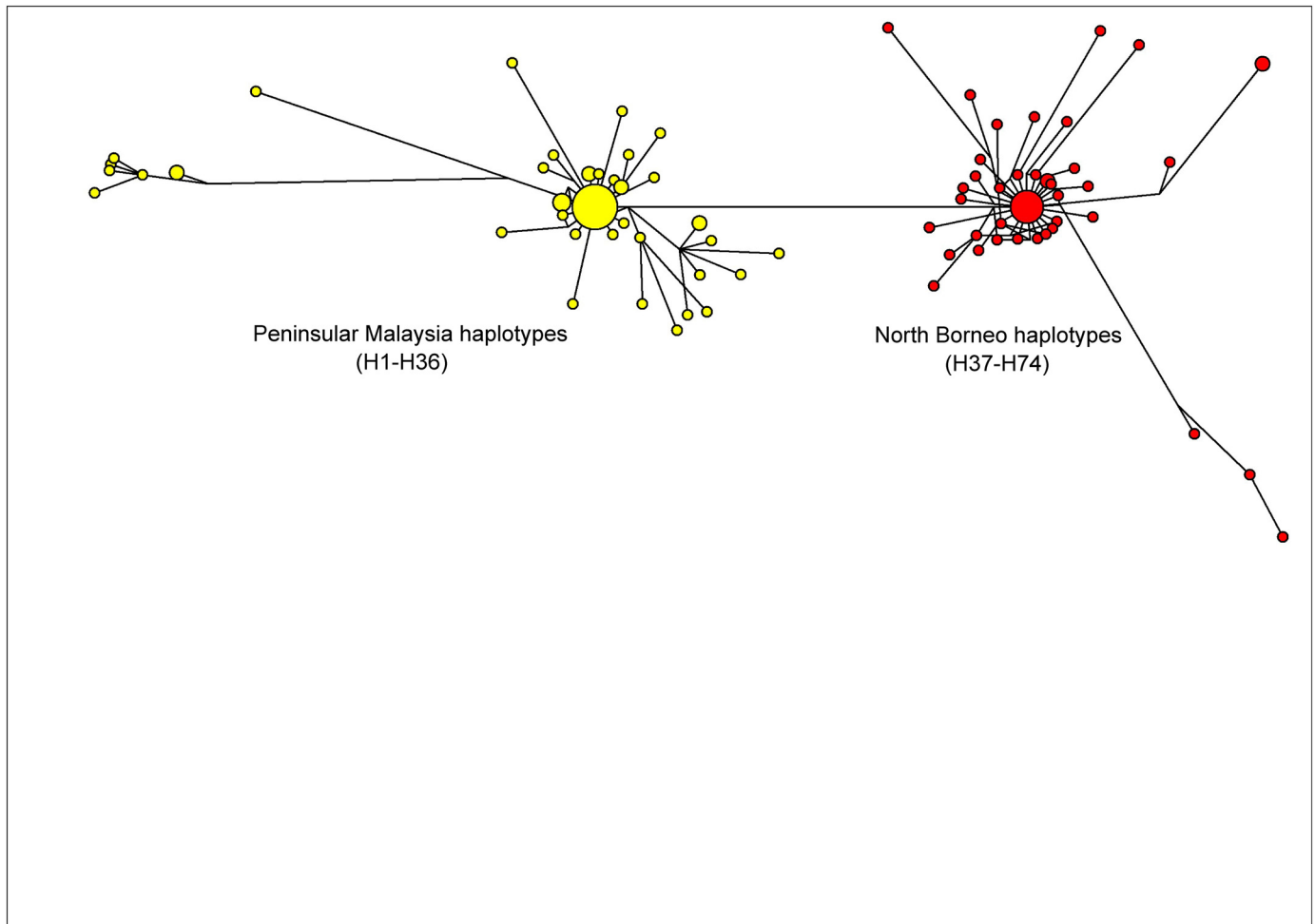


Fig 2. Median Joining network of PkDBP α RII haplotypes. The network shows geographical clustering of PkDBP α RII haplotypes from Peninsular Malaysia (yellow) and North Borneo (red). Amino acid sequences used for the construction of this network were from a previous study [9].

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the short link connecting the small subgroups (Peninsular Malaysia's H31-H36, and North Borneo's H42, H46, H48, H53, H54) suggests close relationship among the haplotypes. This was attributed to the sharing of some common amino acid residues in these haplotypes, namely, E107, D138, E139, K140, K142, E143 and E147. In contrast to PkDBP α RII and Pk γ RII, the highly diverse *P. knowlesi* circumsporozoite Th2R/Th3R epitope region displays no geographical clustering of haplotypes, and many shared haplotypes from Peninsular Malaysia and Malaysian Borneo were observed in the Median Joining Network [20].

The Wright's F_{ST} fixation index measures population differentiation due to genetic structure [16]. The index is also a measure of gene flow between populations. Populations with F_{ST} values of more than > 0.25 are considered to be highly differentiated. A high Wright's F_{ST} index (0.61) was reported between the PkDBP α II of Peninsular Malaysia and North Borneo, indicating significant genetic difference between the haplotypes groups [11]. The F_{ST} index obtained in this study was equally high (0.62), thus showing high genetic difference between the Pk γ RII of Peninsular Malaysia and North Borneo. The amino acid substitutions in the Pk γ RII which most likely contribute to this genetic difference were at positions 69, 107, 138, 139, 140, 142, 143, 170, 216, 221, 222, 227, 261, 262 and 266 (Fig 1). Similarly, a recent study based on

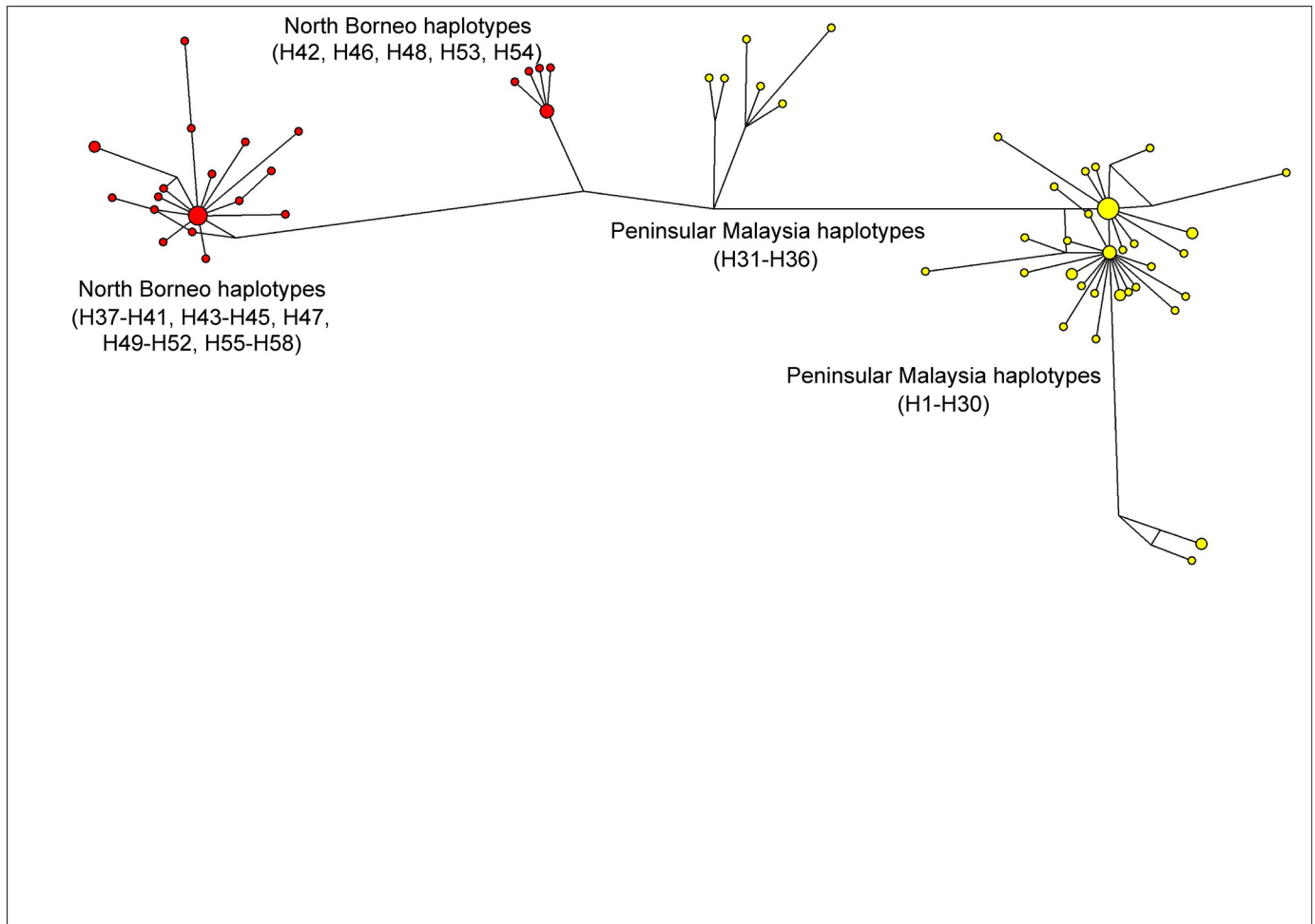


Fig 3. Median Joining network of PkyRII haplotypes. The network shows geographical clustering of PkyRII haplotypes from Peninsular Malaysia (yellow) and North Borneo (red). Note that haplotypes from each region are divided into two subgroups.

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microsatellite DNA data F_{ST} found high level of genetic differentiation between *P. knowlesi* human isolates from Peninsular Malaysia and Borneo [21]. The observation of high F_{ST} values between *P. knowlesi* populations from Peninsular Malaysia and Malaysian Borneo is likely due to the separation of these two land masses since the last ice age 65,000 years ago. The *P. knowlesi*, its macaque hosts and mosquito vectors in Borneo became isolated, and subsequently diverged from their respective species on mainland Asia to form distinct subpopulations. Molecular phylogeny and evolutionary studies have revealed unique history of macaque group formation in Borneo Island [22, 23]. The *Anopheles* mosquitoes have formed species which are uniquely found in the island, such as *An. latens* and *An. balabacensis*, both of which are vectors of *P. knowlesi* [24, 25]. Therefore, the geographical divergence of *P. knowlesi* is most likely the result of host immune selection and adaptation in the macaque and vector hosts respectively.

The population substructure may also explain the Tajima's D statistics observed in this study. The absence of a significant Tajima's D < 0 in the total sample (-1.538 , $P > 0.10$) could be linked to the high level of subdivision between the Peninsular and Borneo Malaysia population as revealed by the distribution of the haplotype network (Fig 3) and F_{ST} index. However, since Tajima's D tends to be > 0 in the presence of subdivision, it may be the case that this

tendency is cancelling out the signal of Tajima's *D* negativity observed in the Malaysia Borneo population ($-1.275, P > 0.10$).

Although human knowlesi malaria is generally mild, there has been an increase in the number of severe infections accompanied by high parasitaemias [26]. There is evidence to suggest possible human-to-human transmission [27]. Furthermore, *P. knowlesi* has been observed to expand its preferred host cell niche by invading older red blood cells [28]. All this may suggest increased parasite adaptation to humans. Rapid genetic changes in the invasion ligands including Pk γ RII may play a role in this increased adaptation in humans.

Conclusions

The region II of PkDBP α and Pk γ plays an important role in the invasion of *P. knowlesi* into host erythrocytes. PkDBP α RII binds to the Duffy antigen of human and *M. fascicularis* erythrocytes, but Pk γ RII binds to a hitherto unidentified receptor on *M. mulatta* erythrocytes. Despite this difference, Pk γ RII was found to be almost similar to PkDBP α RII with regards to genetic diversity level, natural selection, geographical haplotype clustering and genetic differentiation index.

Supporting Information

S1 Fig. Full amino acid sequence alignment of *Plasmodium knowlesi* gamma protein region II (Pk γ RII). Amino acid residues identical to those of *P. knowlesi* strain H (GenBank Accession No. M90695) are indicated by dots. Dash indicates amino acid deletion (highlighted blue at positions 55 and 166). The twelve conserved C residues are highlighted in yellow. The crucial amino acid residues in PkDBP α RII required for interaction with DARC are at Y94, N95, K96, R103, L168 and I175. In Pk γ RII, these positions have changed (highlighted orange at S95, E96, K103, K168, N175) except at Y94 (highlighted green). (XLSX)

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Author Contributions

Conceived and designed the experiments: MYF YLL. Performed the experiments: SAAR RY. Analyzed the data: MYF SAAR RY YLL. Contributed reagents/materials/analysis tools: MYF YLL. Wrote the paper: MYF YLL.

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