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Original article

Selective pressure on the merozoite surface protein-1 genes of *Plasmodium vivax, P. knowlesi* and *P. cynomolgi*

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Background: The merozoite surface protein 1 (msp-1) of malarial parasites is a promising target for malaria vaccine development. Because antigenic polymorphism of this protein occurs in both *Plasmodium falciparum* and *P. vivax*, it is important to know the extent of sequence variation of this gene in nonhuman primate malarias that can infect humans.

Objective: To determine the complete nucleotide sequences of the msp-1 genes of *Plasmodium knowlesi* (Pkmsp-1) and *P. cynomolgi* (Pcymsp-1) and their evolutionary history comparing with an orthologous gene of *P. vivax* (Pvmsp-1).

Method: The msp-1 genes of *P. knowlesi* and *P. cynomolgi* were amplified by the polymerase chain reaction and their nucleotide sequences were determined directly from the amplified products. Sequences were aligned using the Clustal X program. The extent of polymorphism is expressed in terms of nucleotide diversity (π) and the average number of nucleotide substitutions per site between populations (Dxy) in pair-wise comparison. The ratio of nucleotide substitutions at synonymous and nonsynonymous sites was computed by the McDonald and Kreitman test to detect evidence of departure from neutrality. The phylogenetic tree was constructed by the neighbor-joining method using the maximum composite likelihood with 1,000 bootstrap iterations.

Results: The deduced amino acid sequences of the msp-1 of *P. knowlesi, P. cynomolgi* and *P. vivax* exhibit sequence similarity, ranging from 56.2 to 60.8 %. Variable blocks detected in Pkmsp-1 and Pcymsp-1 are located in regions corresponding to variable blocks of Pvmsp-1. Sliding window plots of Dxy along the entire coding regions of the msp-1 genes gave similar profiles for each pair of comparison. Pvmsp-1 is more closely related to Pcymsp-1 than that of Pkmsp-1 based on a phylogenetic analysis. However, the Tajima's relative rate test suggests that the merozoite surface protein 1 (msp-1) of each species has not evolved at the same rate. The McDonald-Kreitman test detects positive selection on regions equivalent to blocks 1 and 9 of Pvmsp-1.

Conclusion: The msp-1 genes of *P. knowlesi, P. cynomolgi* and *P. vivax* exhibit a similar pattern of sequence variation. Regions equivalent to conserved blocks 1 and 9 of Pvmsp-1 have evolved under positive selective pressure, suggestive of functional importance probably as targets for host immune responses.

Keywords: Malaria, merozoite surface protein 1, natural selection, *Plasmodium vivax, Plasmodium knowlesi, Plasmodium cynomolgi,* selective pressure.

Despite enormous efforts based on various strategies to control malaria, it remains one of the leading causes of morbidity and mortality in the tropics. Each year 300-500 million people are infected, and between one and two million victims die of severe complications caused by malaria [1]. Malaria is caused by a hemoprotozoan parasite of the genus *Plasmodium* and four species, i.e. *Plasmodium falciparum, P. vivax, P. malariae* and *P. ovale*, are known to be implicated in human infections. Of these, *Plasmodium falciparum* is the most prevalent and most virulent species. Although death caused by *P. vivax* infection has rarely been reported, infection with this species is estimated to be around 70 to 80

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million people annually and the majority of infections occur in Asia and South America [2].

In Thailand, *P. falciparum* and *P. vivax* account for more than 99 % of malaria cases based on conventional microscopy detection while *P. malariae* and *P. ovale* are infrequently encountered. However, recent reports that *P. knowlesi* may be causing symptomatic malaria in humans in Thailand and Malaysian Borneo have raised concern about the potential for spreading of this zoonotic malaria species [3, 4]. It is already known that *P. knowlesi* that can cross transmit from macaques to humans by natural transmission cycle, *P. brasilianum* from African chimpanzees and *P. simium* from howler monkeys in South America causing diseases in humans via infective mosquitoes [5, 6]. Furthermore, other simian malaria, *P. cynomolgi* and *P. inui* whose main natural hosts are Southeast Asian macaques, can accidentally or experimentally establish infections in humans and are responsible for febrile paroxysm [7, 8]. Although *P. cynomolgi*, *P. inui* and *P. knowlesi* are structurally and biologically different from one another, these simian malarias are placed within the same cluster as *P. vivax* on a phylogenetic tree inferred from small subunit ribosomal RNA (SSU rRNA), mitochondrial cytochrome oxidase I, and the elongation factor Tu of the apicoplastid genome [9-11].

It is likely that proteins on the merozoites of these simian malarias involved in erythrocyte invasion by the merozoites are structurally related to human proteins. One of these molecules is a high molecular weight glycoprotein of 195-200 kDa, designated merozoite surface protein-1 (msp-1). Msp-1 is synthesized at the time of schizogony and is a major protein component on the merozoite surface [12]. Thereafter, msp-1 undergoes two steps of proteolytic cleavage, resulting in several processed fragments. One of these is the 19 kDa fragment located at the C-terminus of the protein that is carried into the newly invaded erythrocyte and appears until the ring stage [13]. Immunization studies in monkeys have revealed that msp-1 of *P. falciparum* (Pfmsp-1) confers protection upon parasite challenges [14]. Field studies have shown that parasitemia and symptomatic malaria inversely correlate with antibodies against Pfmsp-1 among individuals living in malaria endemic areas, suggesting a strong promising role of Pfmsp-1 as a malaria vaccine candidate [15]. It is, however, important to note that Pfmsp-1 exhibits extensive antigenic diversity among isolates, an issue that may compromise malaria vaccine incorporation because antibodies against certain forms of Pfmsp-1 may not cross react and confer cross protection against other variants [16]. On the contrary, the gene encoding Pfmsp-1 displays allelic dimorphism and can be divided into conserved, semi-conserved and variable blocks [17-19]. Further analysis reveals that polymorphism in Pfmsp-1 results from intragenic recombination between distinct parental alleles along with repeat length polymorphism generated by slipped strand mispairing or related mechanisms [17-20].

Meanwhile, sequence comparison of the entire Pvmsp-1 genes of a number of *P. vivax* clinical isolates and two monkey-adapted strains, i.e. the Belem and the Salvador 1 strains, has shown that Pvmsp-1 can be divided into 13 blocks, consisting of seven conserved and six variable blocks. Pvmsp-1 has mosaic organization with heterogeneity in frequency of allelic recombination and the recombination rate is effectively high [21-23]. However, Pvmsp-1 clearly differs from Pfmsp-1 because numerous recombination sites occur throughout Pvmsp-1 in both conserved blocks and variable blocks, while the recombination sites of Pfmsp-1 are confined to the 5' and 3' regions with no recombination events in the central region [17, 18]. Therefore, Pvmsp-1 is far more polymorphic than Pfmsp-1 except that the C-terminal 19 kDa-encoding region of Pvmsp-1 shows a very low level of nucleotide diversity, characterized by a single dimorphic exchange.

To gain insights into the structural variation of the msp-1 genes of the *P. vivax*-related malaria species and their evolutionary aspects, we cloned and determined the complete nucleotide sequences of the msp-1 genes of *P. knowlesi* (Pkmsp-1) and *P. cynomolgi* (Pcymsp-1). Sequence comparison has shown a high similarity of sequences of these malarias and Pvmsp-1. We have also identified two conserved regions of these genes that have evolved under positive selection, suggesting functional significance probably as targets for host immune pressures.

Materials and methods

Source of parasite DNA

Genomic DNA of *P. knowlesi* strain PK57 and *P. cynomolgi* strains DCM320 and MR4 was extracted from infected monkeys' blood spotted onto

filter paper. Malarial DNA was extracted from 0.2 ml of EDTA blood samples by using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA purification procedure was essentially as described in the manufacturer's instruction manual with minor modification as described by Sakihama et al [24]. The purified DNA was dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and stored at – 20 °C until used.

Polymerase chain reaction

The DNA fragment spanning the entire coding region of the msp-1 genes of *P. knowlesi* and *P. cynomolgi* was amplified by PCR using primers whose sequences were derived from the 5' and 3' portions of the Pvmsp-1 gene of the Salvador 1 strain (GenBank Accession No. AF435593). Sequences of the PCR primers were PMSP1F0, 5'-ATGAAGGCG CTACTCTTTTG-3' and PMSP1R0, 5'-GTGTTAT TTTTAAAGCTCCATG-3'. The thermal cycling profiles for PCR contained denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 seconds and extension at 72°C for 5 minutes for 38 cycles of amplification. DNA amplification was performed by using a GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA). To minimize the error introduced in the sequences during PCR amplification, we used ExTaq DNA polymerase (Takara, Shiga, Japan) that has efficient 5' → 3' exonuclease activity to increase fidelity and shows no strand displacement. The size of the PCR product was examined by electrophoresis in a 1 % agarose gel and visualized with an ultraviolet transilluminator (Mupid Scope WD, Japan).

PCR purification

PCR products were inserted into DNA purification column as supplied in the PCR Purification kit (Qiagen). PCR purification procedure followed that described in the instruction protocol of the manufacturer. After elution of PCR product from the column, an aliquot of the solution was used for semi-quantitative determination of the DNA concentration.

DNA sequencing

DNA sequences were determined directly from the purified PCR products. Sequencing analysis was performed from both directions for each template using the BigDye Terminator version 3.1 Cycle Sequencing Kit on an ABI3100 Genetic Analyzer (Applied Biosystems). Overlapping sequences were obtained by using sequencing primers (available upon request). Whenever a singleton occurred, the sequence was re-determined using DNA templates from two independent amplifications from the same DNA samples.

Data analysis

Sequences were aligned by the Clustal X program with minor manual adjustment made by visual inspection [25]. The average number of nucleotide differences per site between two sequences (nucleotide diversity, π) and/or the average number of nucleotide substitutions per site between populations (Dxy) were computed by the methods described [26]. Tajima's relative rate test was used to estimate the mutation rate between species calibrating with an outgroup [27].

Evidence of departure from neutral evolution was computed by the McDonald-Kreitman test and the statistical significance was considered at $P < 0.05$ [28]. Phylogenetic construction was performed by the neighbor-joining method with 1,000 bootstrap iterations [29]. The Pvmsp-1 sequences of the Salvador 1 and the Belem strains and Pkmsp-1 strain A1 were retrieved from the GenBank database under the accession numbers AF435593, AF435594 and DQ220743, and used for comparative analysis.

Results

Sequence of Pkmsp-1

A comparison of the alignment of the Pkmsp-1 sequence of strain PK57 that was originally isolated from an infected macaque with that of isolate A1 obtained from a patient with naturally acquired malaria in Thailand revealed that both sequences exhibited 96.7 % amino acid identity (**Fig. 1**). It is of note that two regions corresponding to blocks 8 and 10 of Pvmsp-1 displayed more sequence divergence than the remaining parts of the gene. A closer look into the nucleotide sequences has shown that these regions possessed repeats with sequence and size difference (**Fig. 2**). In total, we observed 144 nucleotide substitutions, resulting in 72 amino acid exchanges between two strains of *P. knowlesi* used in this study.

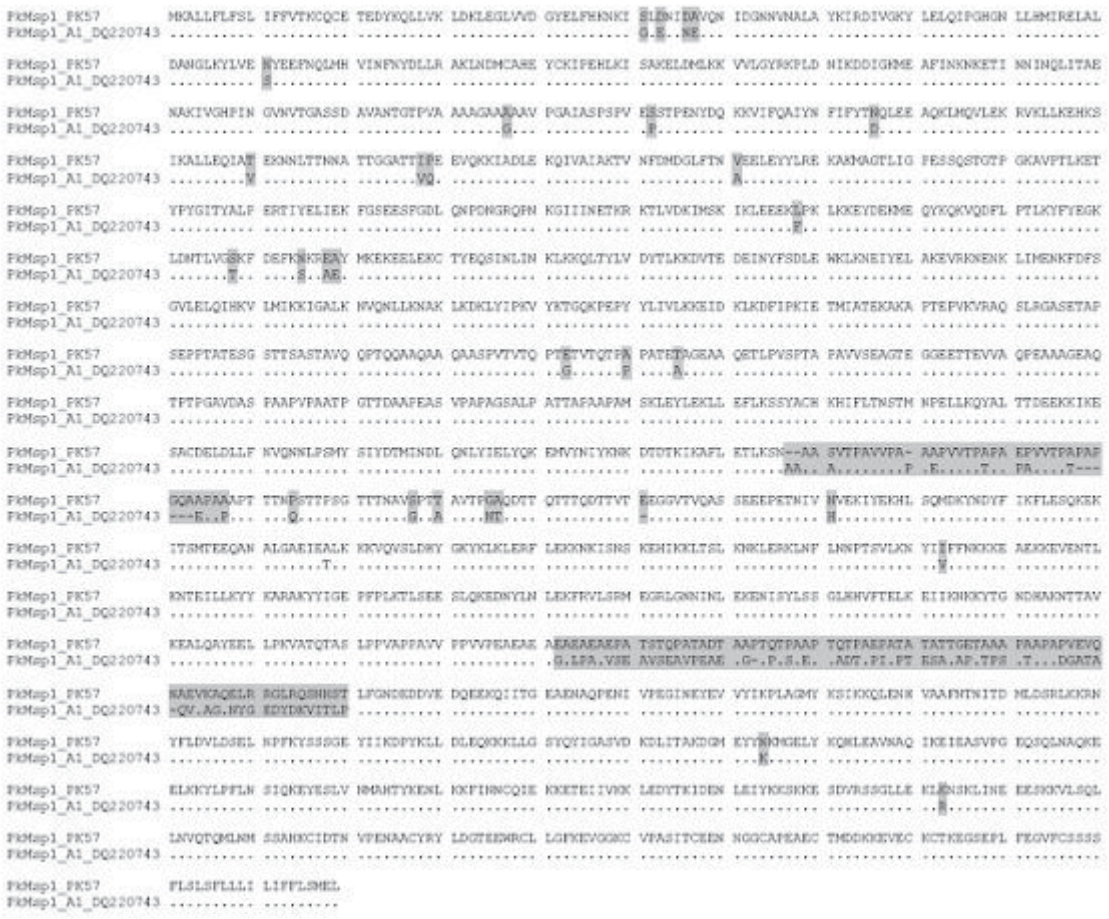

Fig. 1 Amino acid sequence comparison of *Plasmodium knowlesi* merozoite surface protein 1 between strain PK57 and isolate A1. Boundaries of variable blocks and amino acid substitutions are shaded. Dots and dashes denote identical and deleted residues, respectively.

Fig. 2 Alignment of nucleotide sequences of the merozoite surface protein 1 genes of *Plasmodium knowlesi* strain PK57 and isolate A1 showing 2 variable regions. Dots indicate identical residues and dashes represent deletion/insertion.

Sequence comparison of Pcymsp-1

The complete coding sequences of the Pcymsp-1 gene of strains DCM310 and MR4 contained 1797 and 1834 codons, respectively (**Fig. 3**). Sequence comparison of these strains revealed four variable regions corresponding to blocks 2, 6, 8 and 10 of the Pvmsp-1 gene. Like Pkmsp-1, these regions displayed imperfect repeats with sequence and size variation (**Fig. 4**). We observed 204 nucleotide substitutions corresponding to 115 amino acid differences between these strains.

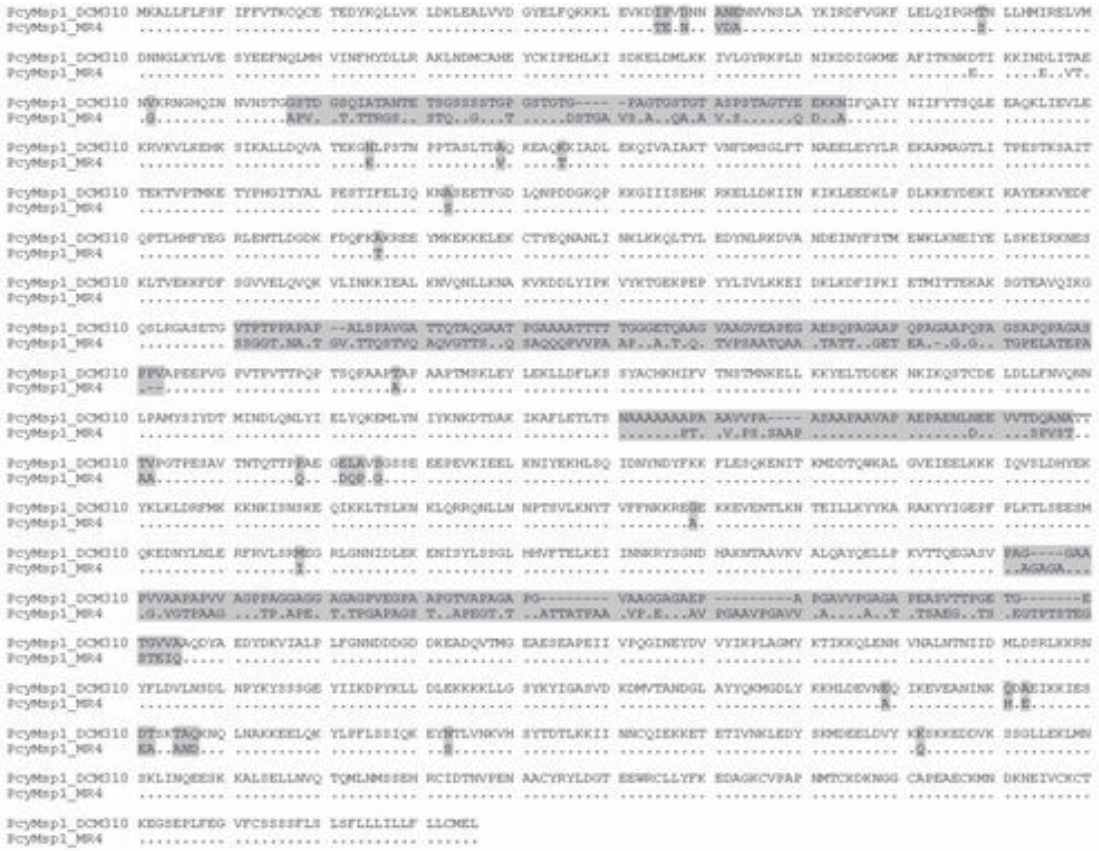

Fig. 3 Amino acid sequence comparison of *Plasmodium cynomolgi* merozoite surface protein 1 between strains DCM310 and MR4. Boundaries of variable blocks and amino acid substitutions are shaded. Dots and dashes denote identical and deleted residues, respectively.

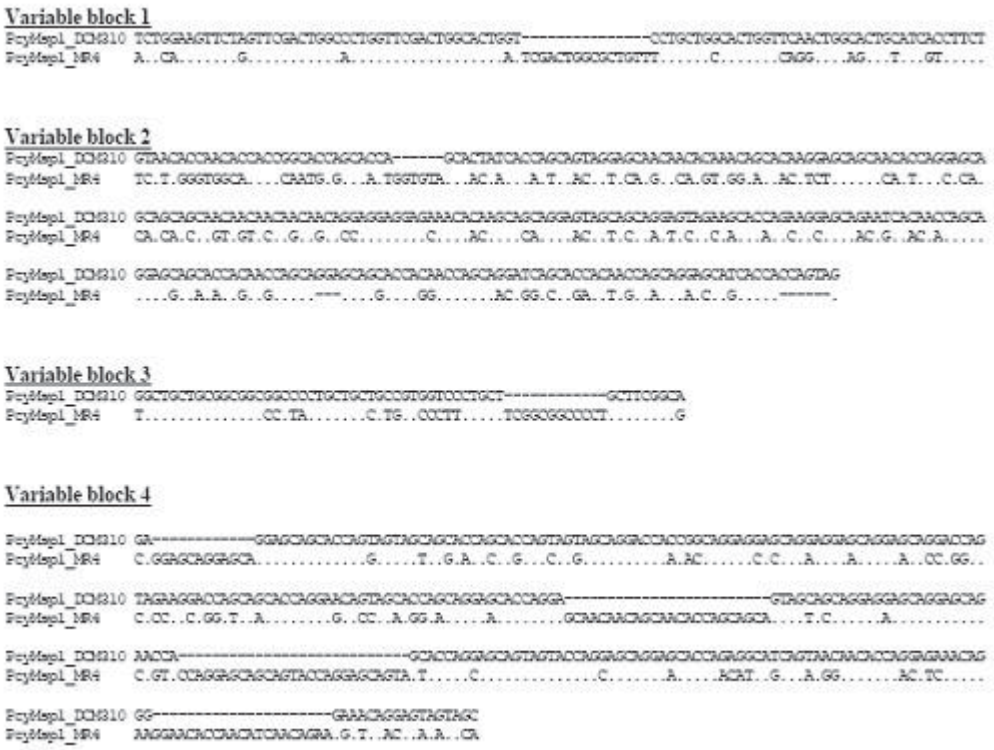

Fig. 4 Alignment of nucleotide sequences of the merozoite surface protein 1 genes of *Plasmodium cynomolgi* strains DCM310 and MR4 showing 4 variable regions. Dots indicate identical residues and dashes represent deletion/ insertion.

Sequence comparison of Pkmsp-1, Pcymsp-1 and Pvmsp-1

Previous analysis of the msp-1 genes of *P. vivax* derived from diverse geographic origins and monkey-adapted strains has shown that the most distantly related sequences were those of the Salvador 1 and the Belem strains [23]. The amino acid sequence alignment of Pvmsp-1 of these two strains, compared with the Pkmsp-1 and the Pcymsp-1 sequences, is shown in **Fig. 5**. It is noteworthy that a number of substituted codons were shared between two species; 93 amino acid residues shared between Pvmsp-1 and Pcymsp-1, 86 residues shared between Pkmsp-1 and Pcymsp-1, and 53 shared between Pkmsp-1 and Pvmsp-1. These substituted residues that were shared between species did not cluster into a particular region but were rather dispersed throughout the proteins. Sliding window plots of nucleotide diversity (π) and the average number of nucleotide substitutions per site between populations (Dxy) in pairwise comparisons are shown in **Fig. 6**. Interestingly, despite differences in the pattern of nucleotide diversity in the msp-1 gene of each species, the patterns of Dxy along the entire coding regions gave similar profiles for each pair of comparisons. Taken together, it is likely that the divergence of these malaria parasites occurred almost contemporarily and these msp-1 genes could have evolved from a common ancestor.

Phylogenetic relationship

We applied the neighbor-joining algorithm using a modified Nei and Gojobori method with Jukes and Cantor correction to construct the phylogenetic relationship based on six taxa of msp-1 of *P. vivax*, *P. knowlesi* and *P. cynomolgi*. It is of note that Pvmsp-1 is more closely related to Pcymsp-1 than Pkmsp-1 and bootstrap support is convincing (**Fig. 7**). However, it is likely that these genes have evolved at different rates because the Tajima's relative rate test in pair-wise comparison has rejected the null hypotheses of equal rates between lineages: Pkmsp-1 vs Pcymsp-1 using Pvmsp-1 as an outgroup, $\chi^2 = 62.08$, $P < 0.00001$, Pvmsp-1 vs Pcymsp-1 using Pkmsp-1 as an outgroup, $\chi^2 = 33.44$, $P < 0.00001$ and Pkmsp-1 vs Pvmsp-1 (Belem strain) using Pcymsp-1 as an outgroup, $\chi^2 = 4.48$, $P = 0.03428$. However, it seems that the msp-1 of the Salvador 1 strain of *P. vivax* has evolved at a relatively similar rate to that of Pkmsp-1, $\chi^2 = 2.43$, $P = 0.11936$.

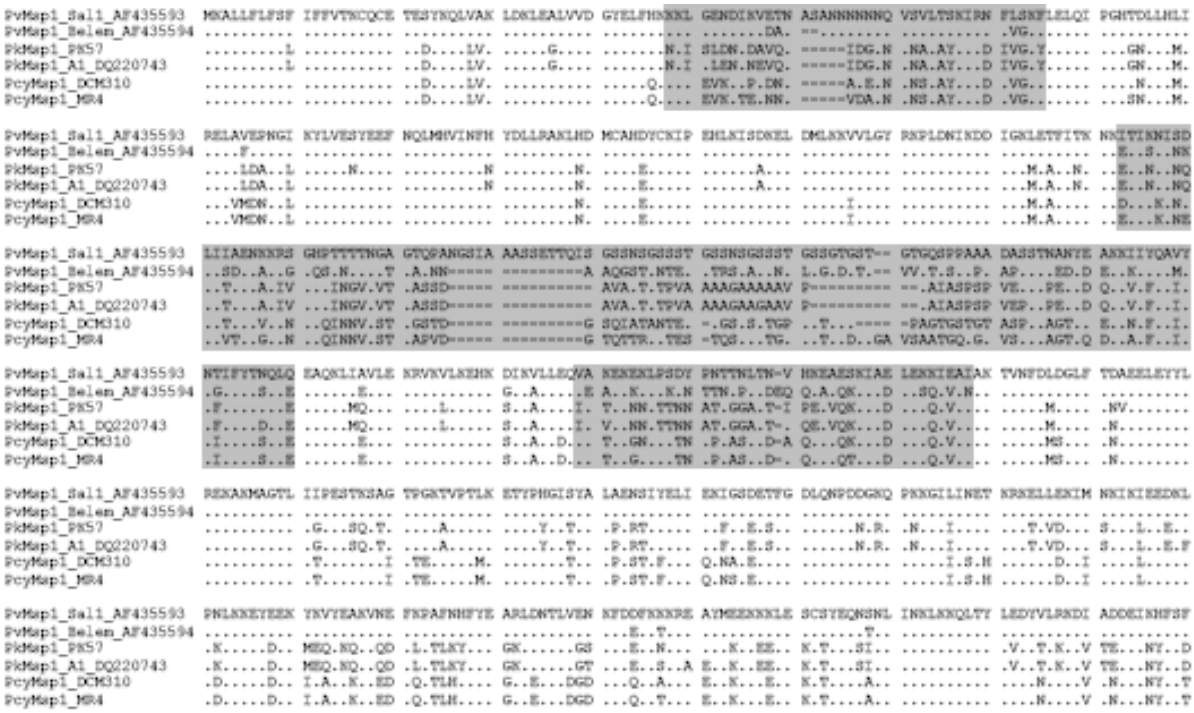

Fig. 5 Amino acid sequence comparison of the merozoite surface protein 1 of *Plasmodium vivax, Plasmodium cynomolgi,* and *Plasmodium knowlesi*. Boundaries of variable blocks are shaded. Dots and dashes denote identical and deleted residues, respectively.

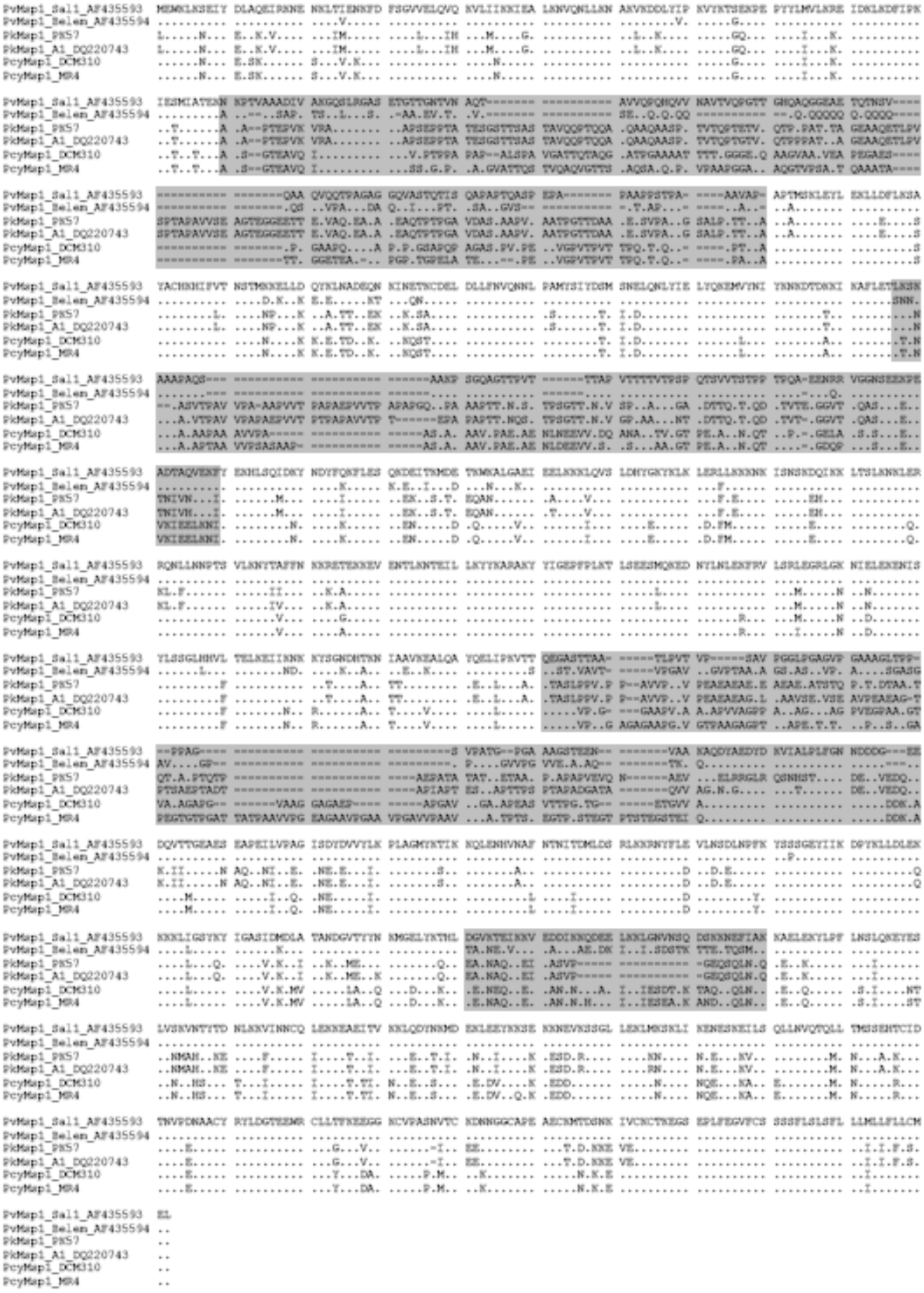

Fig. 5 Amino acid sequence comparison of the merozoite surface protein 1 of *Plasmodium vivax*, *Plasmodium cynomolgi*, and *Plasmodium knowlesi*. Boundaries of variable blocks are shaded. Dots and dashes denote identical and deleted residues, respectively (Continued).

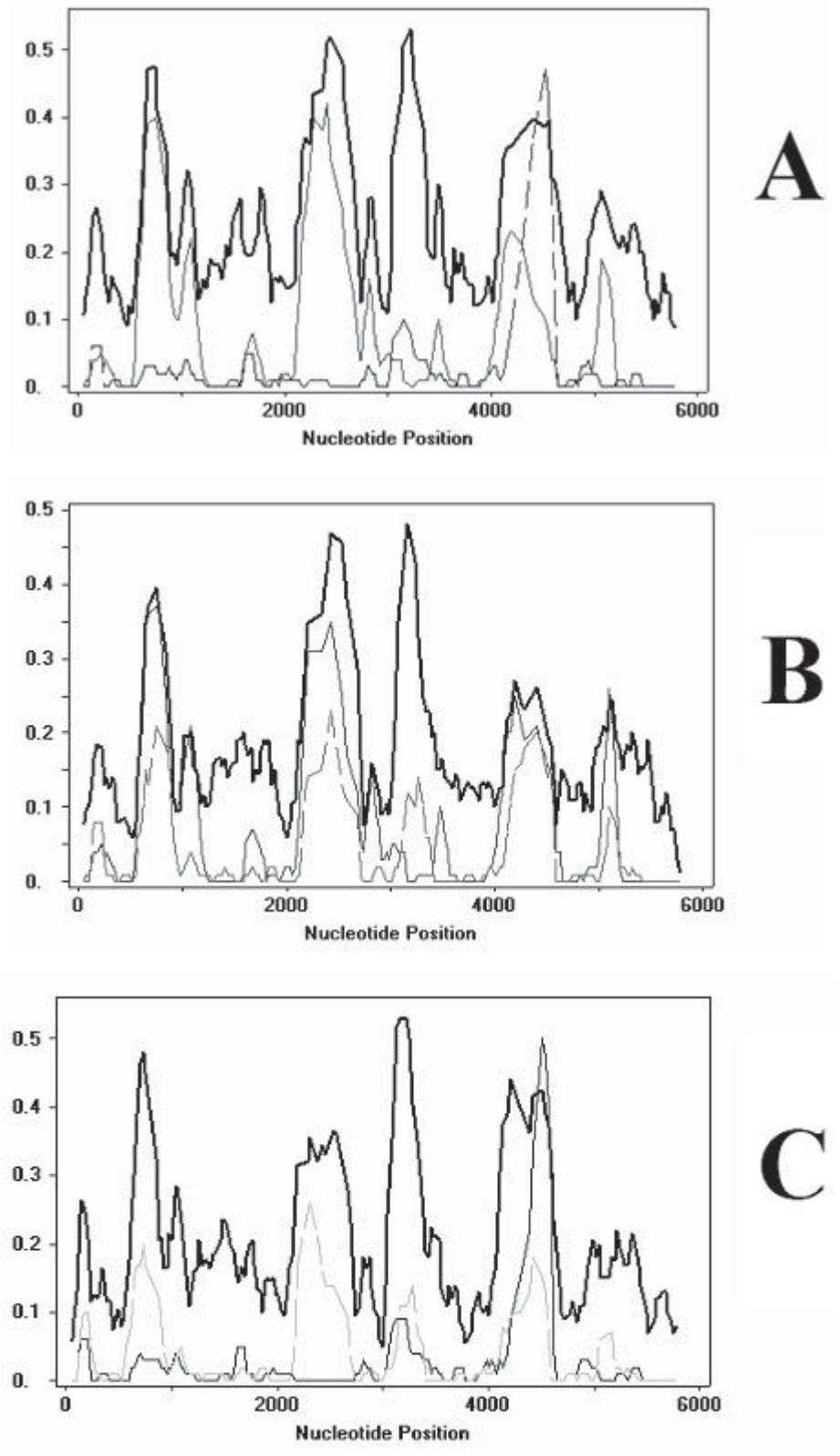

Fig. 6 Sliding window plots of nucleotide diversity (π) (thin or broken lines) and the average number of nucleotide substitutions per site between populations (Dxy) (thick lines) in pair-wise comparisons. (**A**) Pvmsp-1 (thin line) and Pkmsp-1 (broken line); (**B**) Pvmsp-1 (thin line) and Pcymsp-1 (broken line); and (**C**) Pkmsp-1 (thin line) and Pcymsp-1 (broken line).

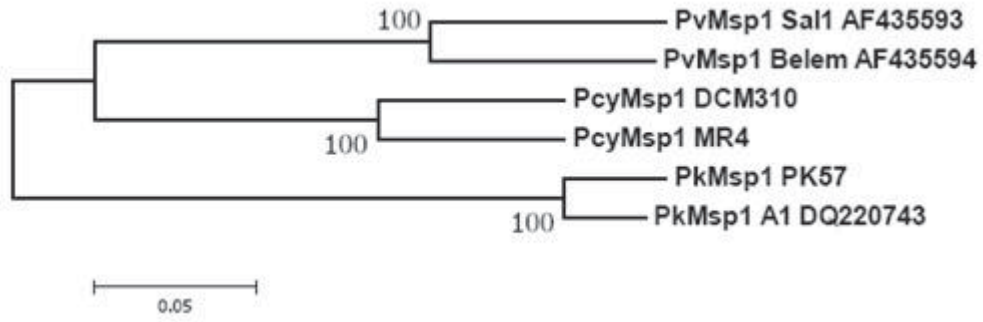

Fig. 7 Neighbor-joining tree based on the six taxa of the merozoite surface protein 1 genes. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Selection on the msp-1 locus

Estimation of the proportion of substitutions that are due to adaptive evolution using the number of synonymous and nonsynonymous polymorphisms and substitutions in a McDonald and Kreitman test by pair-wise comparison of the msp-1 genes of each species is shown in **Table 1.** Regions equivalent to variable blocks of Pvmsp-1 were excluded from analysis because of ambiguity in sequence alignment due to variation in the number of repeat units in these regions. The neutrality indices of Pvmsp-1 when using Pkmsp-1 as an out-group gave significant values for blocks 1 and 9. Block 9 also showed evidence of departure from neutrality when comparison was made between Pvmsp-1 and Pcymsp-1. Likewise, block 1 seems not to be evolved neutrally when Pkmsp-1 was compared with Pcymsp-1. Therefore, evidence of selection occurred in the evolutionary history of the msp-1 locus of *P. vivax, P. knowlesi* and *P. cynomolgi*.

Table 1. The McDonald and Kreitman test for *P.vivax, P.knowlesi* and *P.cynomolgi.*

	Block	Fixed difference between species		Polymorphic within species		Neutrality Index	p
		Synonymous	Nonsynonymous	Synonymous	Nonsynonymous		
P.vivax vs	1	38	40	3	12	3.800	0.0493*
P.knowlesi	3	8	4	4	3	1.500	1.0000
	5	84	92	14	13	0.848	0.8365
	7	28	19	12	12	1.474	0.4606
	9	72	37	15	19	2.465	0.0274*
	11	41	33	5	4	0.994	1.0000
	13	56	55	2	1	0.509	1.0000
	all	271	225	53	63	1.432	0.0981
P.vivax vs	1	19	30	7	11	0.995	1.0000
P.cynomolgi	3	2	2	5	3	0.600	1.0000
	5	58	72	16	8	0.403	0.0737
	7	13	13	11	12	1.091	1.0000
	9	58	22	12	19	4.174	0.0018*
	11	32	22	2	3	2.182	0.6414
	13	29	51	2	2	0.56	0.6236
	all	182	161	53	56	1.194	0.4424
P.knowlesi vs	1	35	29	5	14	3.379	0.0378*
P.cynomolgi	3	10	4	1	0	0.000	1.0000
	5	68	84	9	9	0.810	0.8033
	7	20	16	5	0	0.000	1.0000
	9	48	39	9	4	0.547	0.3852
	11	24	30	7	1	0.114	0.0529
	13	42	46	4	3	0.685	0.7089
	all	205	202	36	28	0.789	0.4209

* Significant departure from neutral expectation.

Discussion

The process of merozoite invasion of erythrocytes is crucial for establishment of intraerythrocytic development of malarial parasites. The malarial merozoite possesses specific structures such as the filamentous surface coat that differs in thickness depending on the malarial species and the secretory organelles of the rhoptries, micronemes and dense granules participating in this process. The repertoire of proteins in these locations has been identified: one of these is a high molecular weight surface glycoprotein, designated msp-1. Apparently, msp-1 is expressed on the surface of merozoites in all species of *Plasmodium*. Attempts to disrupt the msp1 coding sequence by gene targeting did not result in recovery of viable parasites, suggesting that this protein is essential in parasite survival [30]. Despite the immunological evidence of msp-1 as a prime target for malaria vaccine development, its function in merozoite invasion is not fully elucidated [31].

The occurrence of cross species transmission of *P. knowlesi* from macaque monkeys to humans has raised public concern over aspects of malaria control and the health hazard for travelers in endemic areas [3, 4]. Furthermore, *P. knowlesi* has been reportedly implicated in the mortality of infected individuals in Sarawak Island [32]. Although *P. knowlesi* differs biologically from *P. vivax*, the merzoites of both species require Duffy receptor on the erythrocyte membrane for invasion [33]. Moreover, our analysis has shown that the msp-1 of both species shared a relatively high sequence similarity, suggesting that structure-related functions of both proteins could be conserved after the divergence from a common ancestor.

Our previous analysis has shown that Pvmsp1 can be partitioned into 13 blocks, consisting of seven conserved and six variable blocks, as inferred from homology of the deduced amino acid sequences and nucleotide diversity among haplotypes. Like msp1 of *P. falciparum*, nucleotide substitutions in conserved blocks of Pvmsp1 are basically dimorphic, i.e. one of the two nucleotides at a position wherever substitutions occur, and that various combinations of these substitutions have created microheterogeneity in the regions [23]. Four of the six variable blocks are characterized by repeat motifs containing two or more basic sequence types and several novel types, resulting in size and sequence polymorphism among haplotypes. The extensive sequence variation in Pvmsp1 is partly attributable to frequent meiotic intragenic recombination as evidenced by a rapid decline in significant linkage disequilibrium tests between pairs of loci with increasing molecular distance. In addition, several novel types were apparently generated by recombination between the basic sequence types, spanning from variable blocks at the 5' to the 3' portions of the gene [21-23]. On the other hand, the rate of nonsynonymous substitutions per nonsynonymous sites significantly outnumbers that of synonymous substitutions per synonymous sites in six segments within both conserved and variable blocks of Pvmsp1. This implies that positive selection, such as host immune pressure, has enhanced sequence diversification in Pvmsp1 at the amino acid level [23]. Although the extent of sequence variation in msp-1 of *P. knowlesi* and *P. cynomolgi* seems to be underestimated in our analysis because of the limited number of isolates used, two variable regions in Pkmsp-1 and four variable regions in Pcymsp-1 are located at equivalent regions of variable blocks of Pvmsp-1, suggesting that structural similarity occurred in this gene and functional conservation of msp-1 in erythrocyte invasion across plasmodial species [34].

Analysis of the evolutionary history of Pvmsp-1 has revealed that polymorphism in this gene exists much longer than expected under selective neutrality and is consistent with balancing selection acting to maintain polymorphism at this locus [35]. Based on the estimation of mutation rate for Pvmsp-1 and the assumption of host-parasite co-speciation, Pvmsp-1 and Pkmsp-1 seem to have diverged along with their respective hosts around 4.5 to 5.6 million years ago, making the divergence contemporary with the period of macaque radiation in Asia [35]. Our present analysis has shown that the pattern of the average number of nucleotide substitutions per site between populations in pair-wise comparisons along the entire coding regions of Pvmsp-1, Pkmsp-1 and Pcymsp-1 is similar in profile for each pair of comparisons, implying that these genes have evolved from a common ancestor. This supports our previous study [35]. However, after speciation, the msp-1 locus of each species could have evolved at a different rate as evidenced by the Tajima's relative rate test. Meanwhile, the McDonald and Kreitman test, in which the ratio of nonsynonymous to synonymous polymorphisms within species is compared to the ratio of the number of nonsynonymous and synonymous fixed differences between species, has revealed a significant excess of

nonsynonymous polymorphisms in blocks 1 and 9 of Pvmsp-1 and equivalent regions over either of these blocks in Pkmsp-1 and Pcymsp-1, suggestive of positive selection on these msp-1 loci. It seems plausible that host immune pressure could have shaped msp-1 polymorphism and that the intensity of selective pressure could differ among different host species.

Interestingly, Pvmsp-1 seems to be genetically more related to Pcymsp-1 than to Pkmsp-1 although naturally acquired human infection with *P. cynomolgi* has not been reported. Nevertheless, experimental and accidental infections of *P. cynomolgi* in humans have resulted in symptomatic malaria [7]. Therefore, it seems likely that in certain areas, where macaques harbor *P. cynomolgi* in their blood stream, human cases could be infected but they might not yet have been discovered. It is noteworthy that intraerythrocytic stages of *P. cynomolgi* are highly reminiscent of those of *P. vivax*, an issue that hinders definite microscopy-based diagnosis [36]. Further surveillance using appropriate diagnostic tools is undoubtedly required to address this issue.

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