Original article

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

Chaturong Putaporntip^a, Sunee Seethamchai^b, Vit Suvannadhat^c, Thongchai Hongsrimuang^a, Jetsumon Sattabongkot^d, Somchai Jongwutiwes^a

^aDepartment of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330; ^bDepartment of Biology, Faculty of Science, Naresuan University, Pitsanulok 65000; ^cSchool of Technology of Medicine, Faculty of Science and Technology, Christian University of Thailand, Nakornpathom 73000; ^dDepartment of Entomology, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand.

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

Correspondence to: Dr. Chaturong Putaporntip, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand; E-mail: fmedcpt@md2.md.chula.ac.th

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 $5' \rightarrow 3'$ 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.

FKMsp1_FK57 FKMsp1_A1_DQ220743	MKALLFLFSL	IFFVTKCQCE	TEDYNQLLVK	LDKLEGLVVD	GYELFHENKI	SIENIDAVON G.ENE	IDGNNVNALA	YKIRDIVGKY	LELQIPGHON	LIBMIRELAL
PKMsp1_PK57 PKMsp1_Al_D0220743	DANGLEYIVE	NYEEPNQLMH	VINFNYDLLR	AKINDMCARE	YCKIPEHLKI	SARELDMLER	WLGYRSPLD	NIKDDIGHME	AFINKNKETI	NN INQLITAE
FkHsp1_FK57 FkHsp1_FK57	NAGEVORPEN	GVNVTGASSD	AVANTOTPVA	NAGANINAV	PGALASPSPV	EESTPENYDQ	KKVIFQAIYN	FIFYTROLES	AQKLHQVLEK	RVKLLKEHKS
FKMsp1_FK57	IKALLEQIA	EXNNLTINNA	TTOGATTIPE	EVQKKLADLE	KQIVALAKTV	NFDHDGLFTN	VEELEYYLRE	KANNAGTLIG	FESSQSTOTP	GKAVPTLKET
PkHsp1_A1_DQ220743	VENCIPULI D	PDetvertev					A			
PKMap1_A1_DQ220743		********					······	********		******
FKHsp1_FK57 FKHsp1_A1_DQ220743	LINTLVGERF	DEFREKEAY	MREKEELERC	TYEQSINLIN	KLERGLTYLV	DITLENDVIE	DEINYFSDLE	WELENEIYEL	AKEVRENENK	LIMENKFDFS
FkMsp1_FK57 FkMsp1_A1_DQ220743	GVLELQIHKV	LMIKEIGALE	NVQNLLKNAK	LKDKLYIPSV	YRTGORPEPY	YLIVLÆKEID	KIKDFIPKIE	THIATEKAKA	PTEPVKVRAQ	SLRGASETAP
FKMsp1_FK57 FKMsp1_A1_D0220743	SEPPTATESG	STTSASTAVQ	OPTODAAQAA	QAASPVTVTQ	PTETVIQUE	PATETAGEAA	QETLPVSPTA	PAVVSEAG7E	GGEETTEVVA	QPEAAAGEAQ
FkMsp1_FK57	TPTPGAVDAS	PAAPVPAATP	OPTDAAPEAS	VPAPASSALP	ATTAFAAPAM	SKLEYLEKLL	EFLESSYACE	KHIFLTNSTM	NPELIKQYAL	TTDEEKKINE
PKMap1_RL_DQ220143	SACDELDLLF	NVONNLPSMY	SIYDTMINDL	ONTAIETAOK	EMVYNUYKNE	DIDIKIKAFL	ETLESNAA	SVTFAVVPA-	AAPVVTPAPA	EPWVTPAPAP
FKH#p1_A1_DQ220743								AP	.E	PA7
PKHap1_N1_DQ220743	E P							Н		
FkMap1_FK57 FkMap1_A1_DQ220743	17SMTEEQAN	ALGAEIEALK	REVOVALDRY	GKYKLELERF	LEKKNKISNS	KEHIKKLTSL	KNELEBELNP	LNNPTSVLKN	YIMFFNCKKE	AEACEVENTL
FkMap1_FK57 FkMap1_A1_DQ220743	SNTEILLSYY	KARAKYYIGE	PFPLATISEE	SLOKEDNYLN	LEKFRYLSEM	EGRLONNINL	EXENISYLSS	GLEHVFTELE	EIIRNKKYTG	NDRAENTTAV
PkMap1_PK57 PkMap1_A1_DQ220743	KEALQAYEEL	LPKVATQTAS	LPPVAPPAVV	PPVVPEAEAE	AFAFAFAFA .G.LPA.VSE	TSTOPATADT AVSEAVPEAE	AAPTOTPAAP .GP.S.E.	TOTPAEPATA .ADT.PI.PT	TATTGETAAA ESA.AP.TPS	PAAPAPVEVQ .TDGATA
PkMap1_PK57 PkMap1_A1_DQ220743	NAEVKAQELR - QV. AG. HYG	RGLRQSNNST EDYDRVITLP	LFGNDEDDVE	DQRENQIITG	EAENAQPENT	VPEGINEYEV	VYIKPLAGMY	NUTROCLEME	VAAFNTNITD	MLDSRLERRN
FKMap1_FK57 FKMap1_A1_DQ220743	YFLDVLDSEL	NPFRYSSOE	YIIKDPYKLL	DLEQUCKLLG	STOTICASVD	KDLITAKDOM	EYYNONGELY	KONLEAVNAQ	INEIRASVPG	EQSQLNAQKE
PkMap1_PK57 PkMap1_A1_DQ220743	ELKKYLPFLN	SIQKEYESLV	NUMBER	INCITINNEQ1E	KKETEI IVIOK	LEDYTKIDEN	LEIYKESEKE	SDVRSSGLLE	KIRNSKLINE	EESIGTVL.SQL
FKMap1_FK57 FKMap1_A1_DQ220743	LNVQTQMLNM	SSARCIDIN	VPENAACYRY	LOGTERNRCL	LOPICEVOCRC	VFASITCEEN	NGGCAPEAEC	тирризарудо	KCTKEGSEPL	PEGVPCSSSS
FRMap1_FK57 FRMap1_A1_D0220743	FLSLSFLLLI	LIFFLOMEL								

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.

Variable block 2

PkMspl_PK57 PkMspl_Al	TCGGTGACCCCTGCTGTAGTACCAGCAGCAGCAGCAGCAGTAGTAACACCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA
Variable block 4 PRMsp1_FX57 PRMsp1_A1	AAGCTGAAGCTGAAGCAGAACCAGCAACATCAACACAACAACCAGCAGCAGCACCAGCACCAACAACAAC
PkMspl_PK57 PkMspl_Al	GCAACAGCAACAAGCAACAAGGAGAAACAGCAGCAGCAGC
PkMspl_PK57 PkMspl_Al	CGACAAAGTAATCACTCTACC-C

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.

PcyMsp1_DCM310 PcyMsp1_MR4	MEALLFLFIF	IPPVTROQCE	TEDYRQLIVE	LDKLEALVVD	GYELFORKKL	EVECTEVENN	XMENN/NSLA VDA	YKIRDEVGKE	LELQIPGHEN	LINNIRELVM
PcyMsp1_DCM310 PcyMsp1_MR4	DNNGLICYLVE	SYEEPNQLMH	VINFHYDLLR	AKLNDMCAHE	YCKIPEHLKI	SOKELDMLKK	IVLOYPKPLD	NIKDDIGKME	AFITEMEDTI	KKINDLITAE
PcyMsp1_DCN010 PcyMsp1_MR4	NØKRMGHQIN	NVNSTOGITD	GOQIATANTE	TEGESSESTGP STQ07	GSTOTO	-PAGTGSTGT VI.AQA.A	ASPSTAGTYE V.SQ	EXAMPORTY D.A.	NIIFYTSQLE	BAQKLIEVLE
PcyMap1_DCM010 PcyMap1_MR4	KRVKVLKEHK	SIKALLDQVA	TEKGRLPSTN	PPTASLTDKQ	REAGERIADL	ERQIVAIART	VNFDMBGLFT	NABELEYYLR	EKANNAGTLI	TPESTESAIT
PcyMap1_DCM310 PcyMap1_MR4	TERTVPTMKE	TYPHGITYAL	PESTIFELIQ	KNKSEETFGD	LQNPDDGKQP	REGITISEHE	RELLORIIN	KIKLEEDKLF	DLKREYDERI	KAYEKKVEDE
PcyMap1_DCM310 PcyMap1_MR4	OPTLINIPYEG	REENTLOODK	PDQPKROREE	YMKEKKELEK	CTYEQUANLI	NELEKÇETYL	EDYNLEKDVA	NDEINYFRTM	EWKLKMEIYE	LSKEIRKNES
PcyMap1_DCM310 PcyMap1_MR4	KLTVERSFOR	BOVVELQVQK	VLINKKIEAL	RNVQNLLKNA	RVRDDLYIPK	VYRTGERPEP	VYLIVLEREI	DKLKDPIPKI	ETMITTERAK	DRIGWATOR
PcyMap1_DCMG10 PcyMap1_MR4	QSLRGASETG	VTPTFFAFAF SEGOT.MA.T	ALSPAVGA GV.TTQSTVQ	TTQTAQGAAT AQVUTTSQ	PGAAAATTTT SAQQQPVVPA	TOGGETQAAS AFA.T.Q.	VAROVERPEG TVPIAATOAA	AEBQFAGAAF .TAT7GET	QPAGAAPQPA EAG.G	GEAPQPAGAS TOPELATEPA
PcyMsp1_DCM310 PcyMsp1_MR4	PPVAPEEPVG	PVTPVTTPQP	TECRAPEAP	AAPTMSKLEY	TERTTOLTER	SYACHKHEPV	TNUTMWELL	KEYELTDOER	NKIKQSTCDE	LDLLFNVQSN
PcyMspl_0CH010 PcyMspl_NR4	LPAMYSIYDT	MINDLQNLYI	ELYQKEMLYN	IVENEDTOAR	INAPLETLTS	NAAAAAAAPA PT.	XXVVFA ,V_PE.SAAP	ABARRAVAP	AEPAEMLNEE	VVTDQANATT
PcyMsp1_DCH010 PcyMsp1_NR4	TVPOTPEIAV	TNTQTTPEAS	GELAVBOSSE .DOP.G	EEPEVKIEEL	KNIYEKHLOQ	IONINDYPER	FLEDQKENIT	RMDOTQWKAL	GVEIEELKKK	IQVELDEIVER
PcyMspl_DCH310 PcyMspl_MR4	YKLELDRPMK	RENKISMIRE	QIKELTELEN	KLORPONLLN	NPTSVLKNYT	VPPNKKREØE	REVENTLEN	TEILLEYYKA	RAKYYIGEPP	PLETLISEESM
PcyMap1_DCM310 PcyMap1_MR4	QKEDNYLNLE	RERVLER	REGNNEDLER	ENISYLSSGL	HEVPTELKEI	INNERGIOU	HARNTAAVKV	ALQAYQELLP	KVTTQEGASV	PAGGAA
FcyMap1_DCH010 FcyMap1_NR4	PVVRAPAPVV .G.VGTERAG	ASPPASSASS TP.APE.	AGROPVEOPA T. TROAPAGE	APOTVAPAGA TAPEOT.T	РО АТТЯТРАА	VAAGGAGAEP .VP.EAV	PGAAVPGAVV	PGAVVPGAGA	PERSYTTEGE .TERESTS	TOE .EOTVIITED
PcyMspl_DCM310 PcyMspl_MR4	TOWNAQOYA	EDYDEVIALP	LFGNNDDDGD	DREADQVTHG	EAESEAPEII	VPQGINEYDV	VYIKPLAGNY	KTIKKQLENH	VNALMINIID	MLDORLAXEN
PcyMsp1_DCM310 PcyMsp1_MR4	YFLDVLNSDL	NPYKYSSSGE	YIIKDPYKLL	DLEKKKKLLG	SYRYEGASVD	KOMVTANDGL	AVYORMODEY	KKHLDEVNBQ	INEVEANINK	BOREIXXIES H.B
PcyMap1_DCM310 PcyMap1_MR4	DTEXTAQUOQ	LINNOCEELQE	APALERION	EYRTLVIKVH	SYTDTLEKII	MNCQIEKKET	ETIVNKLEDY	SIMDEELDVY	KREKKELOVK	BEGLLERINN
PcyMsp1_0CM310 PcyMsp1_MR4	SKLINQEESK	KALSELLNVQ	TONLINISSEN	RCIDTWVPEN	AACYRYLDOT	BEWRCLLYPK	EDAGKCVFAP	MERCHONING	CAPEAECION	DENEIVORCH
PcyMsp1_DCH310	KEGSEPLFEG	VPCSSSSFLS	LSFLLLILLF	LLCHEL						

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.

Variable bloc	k1
PoyMapl_DCM310 PoyMapl_NR4	TCTGGAAGTICTAGTTCGACTGGCCTGGTTCGACTGGCACTGGTCGACTGGCACTGGCTCGACTGGCACTGG
Variable bloc	k 2
Peutiseol DOMA10	(可知られた)れたりたりをひたりをひたつ。つつたけれてみたりをつかけれたほうたみとうかられたのうたうからなられたいたちをある。
ReyMapl MR4	TC. T. GEGETEGCA CHATS. G A. TESTETA AC. A A. T AC T. CA. G CA. GT. GG. A AC. TCT CA. T C. CA.
PeyMap1_DCM310	CONSTRACTABLE REPORTS AND A DESCRIPTION OF A DESCRIPTIONO
PcyMap1_MR4	CA. CA. C GT. GT. C G OC C AC AC T. C A. T. C C.
PeyMapl_DCM310	SECTORECONDECTOR
FeyMapl MR4	G. A.A. G. G
PeyMapi_DCH210 PeyMapi_MR4	GETISTISDISCISCISCICTISTISCISTISCISTICTISCI TCC.TAC.TG.COUTTTOSSOSGODITG
Variable bloc	k 4
Peydapi Didilo	
ACTORNOT WK4	C.GORGERGREGA
PeyMapl_DCM310	THEREBORGROUNGRACHERSCHOCKERSCHOCKER
Fryddaol MR4	С. СС С. 65, Т
PeyMapl DCM210	MC3ເພດນເຊຍລາຍການເກດແລະລາຍຄອງດາຍແລະອາດອອກການເກດແລະອາດອອກການ
PoyMap1_MR4	C. GT. CCRGRESCREAFTACCRGRESCRETA. T C
PayMapl_DCH310	GGGRARCHOGRATIKSTRAC
Peydaml MR4	MGMCMCMCMCMGMGMGMGMGMGGMGGMGGMGGMGGMGGMG

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 monkeyadapted 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 pairwise 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.

PvMap1_Sal1_AF435593 PvMap1_Belen_AF435594 PkMap1_PK57 PkMap1_AL_D0220743 PcyMap1_DCM310 PcyMap1_MM4	MEALLFLFSF	IFFVTNCQCE	TESYNQLVAK DLV. DLV. DLV. DLV.	LDELEALVVD	GYELFHNNNL N.I Q.	GENDINVETN DA. SLDN.DAVQ. .LEN.NEVQ. EVK.P.DN. EVK.TE.NN.	ASANNNNNNQ IDG.N IDG.N A.E.N VDA.N	VSVLTSNIRN .NA.AYD .NA.AYD .NS.AYD .NS.AYD	FLSNFLELQI .VG IVG.Y IVG.Y .VG	PGHTDLLHLI GNM. NM. SNM.
PvHap1_Sal1_AF435593 PvHap1_Belem_AF435594 PkHap1_PX57 PkHap1_AL_D0220743 PcyHap1_DCH310 PcyHap1_DCH310 PcyHap1_M84	PELAVEPNGI F LDA.L LDA.L VMDN.L VMDN.L	KYLVESYEEF	NQLMHVINEH	YDLLPANLHD	MCANDYCKIP	BHLNISDNEL	DMLAXVVLGY	REPLONIED	IGRLETFITK M.AN. M.AN. M.A	NNITINNISD .E.S.NN .E.N.NQ .E.N.NQ .DK.N. .E.K.NE
PvHsp1_Sal1_AF435593 PvHsp1_Belen_AF435594 PkHsp1_PK57 PkHsp1_AL_D0220743 PcyHsp1_DCH310 PcyHsp1_DCH310 PcyHsp1_M84	LIIAENKKRS SDAG TA.IV TA.IV TVN VTGN	GHPTTTTNGA .QS.NT INGV.VT INGV.VT QINNV.ST QINNV.ST	GTQPANGSIA .A.NN .ASSD .ASSD .GSTD .ASVD	AASSETTQIS	GSSNSGSSST AQGST.NTE. AVA.T.TFVA AVA.T.TFVA SQIATANTE. TQTTRTES	GSSNSGSSST .TRS.AN. AAAGAAAAAV AAAGAAGAAV GS.S.TGP -TQSTG.	G33GTG3T L.G.D.T P P TDGA	GTGQSPPAAA VV.T.SP. AIASPSP AIASPSP -PAGTGSTGT VSAATGQ.G.	DASSTNANYE APED.D VEPE.D VEP.PE.D ASP.AGT. VSAGT.Q	ABBIIYQAVY E.E.M.Q.V.F.I. Q.V.F.I. E.N.F.I. D.A.F.I.
PvHsp1_Sal1_AF435593 PvHsp1_Belem_AF435594 PkHsp1_PNS7 PkHsp1_Al_DO220743 PcyHsp1_DCH310 PcyHsp1_MM4	NTIFTTNQLQ .GS.E .FD.E .FS.E .IS.E .IS.E	EAQNLIAVLE MQ 	SRVEVLEEHE	DINVLISOVA G. A E S A I S A D S A D	NENENLPSDY A. K. K.N T. NN. TTNN V. NN. TTNN T. GN TN T. G TN	PHTTNLTN-V TTN.PDSQ AT.GGA.T-I AT.GGA.T .P.ASD-A .P.ASD	HNEAESNIAE Q.A.QKD PE.VQKD QE.VQKD QQKD QQKD	LENNIEATAX 	MM	TDAEELEYYL .NV .N
PvHsp1_Sal1_AF435593 PvHsp1_Belem_AF435594 PkHsp1_PK57 PkHsp1_AL_D0220743 PcyHsp1_DCH310 PcyHsp1_ME4	RENARMAGTI	IIPESTNSAG .GSQ.T. .GSQ.T. .TI .TI	TPGRTVPTLK A. A. .TEM. .TEM.	ETTPHGISTA 	LAENSIYELI .P.RT .P.RT .P.ST.F .P.ST.F	ENIGSDETTG F.E.S. Q.NA.E Q.NS.E	DLQNPDDGNQ	PREGILINET .NI .NI.S.H I.S.H	NRSELLENIM	NNINIEEDNL SL.E. SL.F.
PvMap1_Bal1_AF435593 PvMap1_Balan_AF435594 PkMap1_PR57 PkMap1_A1_DQ220743 PcyMap1_DCM310	PNLSNEYEEK	YEVYEASVNE MEQ.NQQD MEQ.NQQD I.AK.ED	FEPAFNHFYE .L.TLEY .L.TLEY .Q.TLH	ARLDNTLVEN GKG3 GKGT GEDGD	NFDDFNNKRE ET EN ESA QA	ATMEENSKLE KEE EKEE EKEE.	SCSTEONSNL K.TSI K.TSI K.TSI K.TA	INNLANQLTY	LEDYVLRSDI .VT.KV .VT.KV .VT.KV	ADDEINHFSF TENYD TENYD TENYD

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.

PvMap1_Sal1_AF435593 PvMap1_Belen_AF435594 PkMap1_PX57 PkMap1_PX57 PcyMap1_DCM310 PcyMap1_DCM310 PcyMap1_MR4	MEMNLASELY LN. LN. N.	DLAQEIPENE EK.V E.SK. E.SK.	NELTIENETD V IM SV.E SV.E	FIGWVELQVQ	NULIINNIEA	LENVONLLEN	ARVEDDLYIP	KVINTSENPE GQ GQ GQ G.	PTYLMVLSRE IK. IK. IK. IK.	IDELEDETEE
PvMap1_Sal1_AF435593 PvMap1_Belem_AF435594 PkMap1_PK57 PkMap1_At_D0220743 PcyMap1_DCK310 PcyMap1_MR4	IESMIATESN 	NPTVAAADIV SAP. .APTEPVN .APTEPVN .SGTEAVQ .SGTEAVQ	ANGQSLRGAS TS.L.S. VRA I I.	ETGTTCHTVN AA.EV.T. APSEPPTA V.PTPPA SS.G.P.	AQT	TAVQOPTOOA TAVQOPTOOA VGATTQTAQG TVQAQVGTTS	AVVQPOHQVV SEQ.Q.QQ .QAAQAASP. .QAAQAASP. .ATPGAAAAT .AQSA.Q.P.	NAVIVQSGIT TVTQSTETV. TVTQSTGTV. TTT.GGGE.Q VPAAPGGA	GHQAQGGEAE Q.QQQQQ QTP.PAT.TA QTPPPAT.A AAGVAA.VEA AQGTVPSA.T	TQTNSV Q-QQQQ GEAAQETLPV GEAAQETLPV PEGAES
PvMap1_Sal1_AF435593 PvMap1_Belen_AF435594 PkMap1_PK57 PkMap1_AL_D0220743 PeyMap1_DCH310 PeyMap1_MR4	SPTAFAVVSE SPTAFAVVSE	QAA .QS AGTEGGEETT AGTEGGEETT .P. .TT.	QVQQTFAGAG VFADA E.VAQ.EA.A E.VAQ.EA.A GAAFQA GGETEA	GQVASTQTIS Q.IPT. .EAQTFTFGA .EAQTFTFGA P.P.GSAPOF PGP.TGPELA	QAPAPTOASP .SAGVS VDAS.AAPV. VDAS.AAPV. AGAS.PV.PE TEPE	EPA AATPGTTDAA AATPGTTDAA VGPVTPVT VGPVTPVT	PAAPPSTPA- T.AP .E.SVPAG .E.SVPAG TPQ.T.Q TPQ.T.Q	AAVAP- SALP.TT.A SALP.TT.A SALP.TT.A PT.A	APTMSKLEYL A A	ENLLDF183A
PvMap1_Sal1_AF435593 PvMap1_Belam_AF435594 PkMap1_PK57 PkMap1_A1_D0220743 PeyMap1_DCM310 PeyMap1_DCM310 PeyMap1_MM4	YACHENIIFVT	NSTMENELLD D.KK NPK NPK NK	QYNLNADEON E.ENT A.TTEX A.TTEX K.E.TDX. K.E.TDX.	NINETNODEL QN .K.SA .K.SA .NQST	DLLFNVONNL	PANYSIYDSM .ST. .ST. .T.	SNELQNLYTE I.D I.D I.D I.D	LYQKEMVYNI	TENEDTDENI 	NAFLETINGN SNN. N N N
PvMap1_Sal1_AF435593 PvMap1_Belem_AF435594 PkMap1_PS57 PkMap1_PS57 PvMap1_DCM310 PvyMap1_DCM310 PvyMap1_M64	AAAPAQS ASVTPAV ASVTPAV 	VPA-AAPVVT VPAPAEPVVT AVVPA	PAPAEPVVTP PTPAPAVVTP	AASP APAPGQPA TEPA AS.A. AS.A.	SGQAGTTPVT AAPTT.N.S. PAPTT.NQS. AAV.PAE.AE AAV.PAE.AE	TPSGTT.N.V TPSGTT.N.V NLNEEVV.DQ NLDEEVV.S.	VTTTTVTPSP SPAGA GP.AANT ANATV.GT .SAA.GT	QTSVVTSTPP .DTTQ.T.QD .DTTQ.T.QD PE.A.N.QT PE.A.N.QT	TPQA-EENDR Q. .TVTE.GGVT .TVTGGVT PGELA GDQP	VGGNSEEXPE .QASE. .QASE. .S.SE. SE.
PvMap1_Sal1_AF435593 PvMap1_Belem_AF435594 PkMap1_Px57 PkMap1_Px57 PvMap1_DCR510 PvyMap1_DCR510 PvyMap1_M84	ADTAQVENFY TNIVNI. TNIVNI. VNIEELSNI. VNIEELSNI.	EXHLSQIDKY	NDYFONFLES	QNDEITNMDE N.E.ID EXS.T. EXS.T. END END	TEMEALGAEI NE. EQAN QV. .QV.	EELNNKLQV3 .AV .TV .TT.	LDHYGNYNLN	LERLLNXNNK F.E F.E .D.FM .D.FM	ISNSNDQINK EH EH E	LT3LNNNLER
PvMap1_Bal1_AF435593 PvMap1_Belen_AF435594 PkMap1_PK57 PkMap1_AL_DQ220743 PeyMap1_DCH310 PeyMap1_MR4	RQNLLNNPTS NL.F	VLXNYTAFFN IV .VV	NNRETENNEV K.A K.A 	ENTLENTEIL	LETTEARAET	YIGEPUPLAT	LSEESMONED	NYLNLENFRV	LSRLEGRLGK MN MN MN IN	NIELEMENIS
PvMap1_Sal1_AF435593 PvMap1_Belem_AF435594 PkMap1_PK57 PkMap1_AL_D0220743 PeyMap1_DCK310 PeyMap1_MR4	YLSSGLHHVL F F F	VELNEIINNK ND. N.	KYSGNDHTNN 	IAAVKEALQA EK TT TT TV TV	YQELIPHVTT S .E.LA. .E.LA. L	CEGASTTAA- ST.VAVT- .TASLP9V.P .TASLP9V.P VP.G-	TLEVT VPGAV PAVVPV PAVVPV GAAPV.A AGAGAAPG.V	VPSAV GVFTAA.A PEAEAEAE.E PEAEAEAG.L A.APVVAGPP GTPAAGAGPT	PGGLPGAGVP GS.ASVP. AEAE.ATSTQ .AAVSE.VSE AAGAG AFE.T.T.	GAAAGLTPP- ASGASG P.T.DTAA.T AVPEAEAG-T PVEGDAA.GT PSGA
PvMap1_Sal1_AF435593 PvMap1_Belam_AF435594 PkMap1_P857 PkMap1_DCH310 PcyMap1_DCH310 PcyMap1_Sal1_AF435593 PvMap1_Belam_AF435594 PkMap1_P857 PkMap1_P857 PkMap1_P857 PkMap1_P857 PkMap1_P857 PkMap1_P857 PkMap1_DCH310 PcyMap1_DCH310	YLSSGLHHVL 	VAAG TATPAAVVPG	KY2GHDHTNN 	IAVXIALQA .E. K. TT. T. V. T. V. T. V. T	YQELIPKVTT S.E.L.A. E.L.A. E.L.A. P.GVTG-PGA P.GVTG TAT.ETAA. ES.APTTS GAAPEAS A.TFTS.	QEGASTTAA- .ST.VAVT- .TASLPPV.P VP.G- VP.G- VE.A.AQ- P.AFASPEEV PTAFADGATA VTTPG.TG- EGTP.STEGT		VPSAV GVPTAA.A PEAEAEAE.E PEAEAEAE.L A.APVVAGPP GTPAAGAGPT NAQDYAEDVD QELRRGLR AG.N.G Q	PGGLBGACVP GR.ASVP. AEAE.ATSTQ .AAVSE.VSE AACAG .APE.T.T. NVIALPLFGN QGNHST	GAAALTPP- ASGASG P.T.DTAA.T AVPEAEAG-T PVEGPAA.GT PS.GA NDDDGEE DEVEDQ DEVEDQ DEVEDQ DEVEDQ DEVEDQ
PvMap1_Sal1_AF435593 PvMap1_Belam_AF435594 PvMap1_ReS7 PkMap1_RS7 PvMap1_DCM310 PvMap1_MR4 PvMap1_Sal1_AF435593 PvMap1_Sal1_AF435594 PkMap1_Sal1_AF435594 PkMap1_DCM310 PvMap1_CM310 PvMap1_Sal1_AF435593 PvMap1_Belam_AF435594 PkMap1_PS7 PkMap1_PS7 PkMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310	YLSSGLHIVL F F F F F F F F F F F F F F F F F F F	TELAEIINNK 	NTSCHENTYN 	IAAVYEALQA .E. X. TT TT T. V. T. V. T. V. T. V. T. V. T. AEPATA APIAPT APIAV PLACHINTIK 	YQELIPAVTY 	CELSATTAA .ST. VANT- .ST. VANT- .TASLPPV.P VP.G AAGSTEEN VVE.A.AQ- P.APASVENO P.APASVENO PTRSADGATA VTTRG.TC- ELTP.STELT NTNITCHLDS 		VPBAV .GVPTAA.A PEARARE.E PEARARE.E PEARARE.L A.APVVARDYD QELRRGLR AG.N.G A.Q. VLNSDLNPFK .D.E. .D.E. .V.Y	PGGLPGACVP GSLAS.VP. AEAE.ATSTQ .AAVIE.VIE A.NGAG .APE.T.T. NVIALPIFGN QSNHET T YSSSGEVIIK .P	GAAALITPP- ASGARG P.T.DTAA.T AVPERAEAC- P.VERPAEAC- P.VERPAEAC- DE.VEDQ. DE.VEDQ. DE.VEDQ. DDN.A DPYNLLDLEN QQ
PvMap1_Sal1_AF435503 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PcyMap1_DCM310 PcyMap1_DCM310 PcyMap1_MEM PvMap1_Sal1_AF435593 PvMap1_Sal1_AF435593 PvMap1_Sal1_AF435593 PcMap1_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_DCM310 PcyMap1_MEM PvMap1_MEM PvMap1_MEM PvMap1_Sal1_AF435593 PvMap1_MEM PvMap1_MEM PvMap1_MEM PvMap1_MEM	YLSSGLHHVL F F F F F F F F F F F F F	TELAEIINNE 	NTSCHONTON 	IAAVNEALQA .E. K. TT. TT. V. T. V. T. V. T. V. T. V. T. V. T. V. T. V. T. APGAVADAAV PLAGMINTIK S. S. S. S. S. S. S. S. S. S. S. S.	YQLLPAVTF 	QEGARTTAA .ST.VAVT- .ST.VAVT- .TASLPPV.P VP.G AAGSTEEN- VVE.A.AC P.APAPUNO PTAPADGATA VTTG.TC- EGTP.STEDT NTNITCMLDG I 	PAVVP.VP.VP PAVVP.VP.VP.VP.VP.VP.VP.VP.VP.VP.VP.VP.VP.	VP	PGGLPGACVP GSLNS.VP. AEAE.ATSTQ .AAVIE.VIE APE.T.T. NVIALPLIGN QSNHET T.T. YISISGEVIIK .P. NAELENVLPF E.K. E.K. E.Q. E.Q.	GAAALITPP ASGAAG P.T.DTAA.T AVPENIAG-T PVEDPAA.GT .P.S.GA NDDDGEE DE.VEDQ. DE.
PvMap1_Sal1_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435594 PvMap1_DCK310 PvMap1_DCK310 PvMap1_DCK310 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_DCK310 PvMap1_MEM4 PvMap1_Sal1_AF435593 PvMap1_Sal1_AF435593 PvMap1_Belam_AF435594 PvMap1_Belam_AF435594 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435594 PvMap1_BS7 PvMap1_BS7 PvMap1_BS7 PvMap1_BS7 PvMap1_DCK310 PvMap1_DCK3	YLSSGLHHVL 	TELAEIINNE 	NISCHENTEN K.A. .TA. .TA. .TA. RA. RA. RA. GAGAES- EAGAAVPGAA ISDIDAVTLE .NE.E.I. .NE.E.I	IAAVNEALQA .E.N. TT. TT. V. T. V. T. V. V. T. V. T. V.	YQELIPAVTY 	CELGATTAA- .ST.VANT- .ST.VANT- .TASLPPV.P .TASLPPV.P VP.G AAGSTEEN- VVE.A.AC- P.AFAPUEND PTAFADGATA VTTPG.TE- EGTP.STEGT NTNITCHLDS I I I I I I 	P-AVVP.VP.AVVP.VP.AVVP.VP.AVVP.VP.AVVP.VP.AVVP.VP.AVVP.VP.AVAAAAPG.V P-AVVP.AAAAAPG.V 	VP	PGGLPGAVVP AEAE.ATSTQ .AAVJE.VSE A.AGAG A.PE.T.T. NVIALPLFGN GENNET T.T. YSBSGEVIIK P NAELEXYLPF E.K. E.Q. QLLNVQTQLL M. E.M. M. E.M. M.	GAARLTPP ASGARG P.T.DTAA.T AVPERATAT VIERPAA.GT .P.S.GA NDCDGEE DE.VEDQ. DE.VE.
PvMap1_Sal1_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_DCM310 PvyMap1_DCM310 PvyMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_DCM310 PvyMap1_DCM310 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_Belam_AF435593 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310 PvMap1_DCM310	YLSSGLHHVL 	TELAEIINNE 	NYSCHONYNN K.A. .T.A. .T.A. .R.A. R.A. .A. R.A. .A. .A.	IAAVNEALQA .E. K. TT. TT. V. T. V. T. V. T. V. T. V. T. V. T. V. T. V. T. APLAPI APLAPI APLAPI PLAGMINTIN PLAGMINTIN S. S. S. S. S. S. S. S. S. S. S. S. S.	YQELIPRVTF 	QEGANTTA- ST.VAVT- .TASLPPV.P .TASLPPV.P .VP.G AAGSTEEN- VVE.A.AQUP P.APAPUP P.APAPUP P.APAPUP TATEG.TG- EDDINGOEE 		VPBAV -GUPTAA.A PEARAEAE.E PEARAEAE.E PEARAEAE.E PEARAEAE.E PEARAEAE.E PEARAEAE.E PEARAEAE.E PEARAEAE.E PEARAEAE.E PEARAEAE.E N.E.LBRGLR AG.N.G. -GEDGIOLN.Q -GEDGIOLN	PGGLPGACVP AEAE.ATSTQ .AAVJE.VSE AEAE.ATSTQ .AAVJE.VSE .APE.T.T. NVIALPLFGN QSNHET .T. YSSSGEVIIK .P. NAELENYLPF .E. K. .E. Q. QLINVQTQLL M. E M. SSSFLSLSTL	GAAALITPP ASGARG P.T.DTAA.T AVPERALAG-T PVERDAA.GT .P.S.GA NEDDGGEE DE.VEDQ. DE

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*.

	Block	Fixed different Synonymous	ce between species Nonsynonymous	Polymorphic Synonymous	within species Nonsynonymous	Neutrality Index	р
P vivax vs	1	38	40	3	12	3 800	0.0493*
P.knowlesi	3	8	4	4	3	1.500	1.0000
1 11110 11 1001	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
<i>P.vivax</i> vs	1	19	30	7	11	0.995	1.0000
P.cynomolgi	3	2	2	5	3	0.600	1.0000
2 0	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

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

* 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.

Acknowledgements

This work received financial support from The Thailand Research Fund (No. RMU5080002) and Molecular Biology Research Fund (No. MB007/48) from the Faculty of Medicine, Chulalongkorn University to C. Putaporntip. The authors have no conflict of interest to declare.

References

- 1. Breman JG, Egan A, Keusch GT. The intolerable burden of malaria: a new look at the numbers. Am J Trop Med Hyg. 2001;64 (1-2 Suppl):4-7.
- 2. Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of Plasmodium vivax malaria. Am J Trop Med Hyg. 2001;64 (1-2 Suppl):97-106.
- 3. Jongwutiwes S, Putaporntip C, Iwasaki T, Sata T, Kanbara H. Naturally acquired Plasmodium knowlesi malaria in human, Thailand. Emerg Infect Dis. 2004; 10:2211-3.
- Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired Plasmodium knowlesi infections in human beings. Lancet. 2004;363:1017–24.
- 5. Fandeur T, Volney B, Peneau C, De Thoisy B. Monkeys of the rainforest in French Guiana are natural reservoirs for P. brasilainum/P. malariae malaria. Parasitology. 2000;120:11-21.

 Deane LM, Deane MP, Ferreira Neto J. Studies on transmission of simian malaria and on a natural infection of man with Plasmodium simium in Brazil. Bull World Health Organ. 1966;35:805-8.

Selective pressure on malarial merozoite surface protein-1

- 7. Coatney GR, Elder HA, Contacos PG, Getz ME, Greenland R, Rossan RN, et al. Transmission of the M strain of Plasmodium cynomolgi to man. Am J Trop Med Hyg. 1961;10:673-8.
- Coatney GR, Chin W, Contacos PG, King HK. Plasmodium inui, a quartan-type malaria parasite of Old World monkeys transmissible to man. J Parasitol. 1966;52:660-3.
- 9. Kissinger JC, Collins WE, Li J, McCutchan TF. Plasmodium inui is not closely related to other quartan Plasmodium species. J Parasitol. 1998;84:278-82.
- Escalante AA, Freeland DE, Collins WE, Lal AA. The evolution of primate malaria parasites based on the gene encoding cytochrome b from the linear mitochondrial genome. Proc Natl Acad Sci USA. 1998; 95:8124-9.
- Escalante AA, Cornejo OE, Freeland DE, Poe AC, Durrego E, Collins WE, Lal AA. A monkey's tale: the origin of Plasmodium vivax as a human malaria parasite. Proc Natl Acad Sci USA. 2005;102:1980-5.
- Holder AA. Proteins on the surface of the malaria parasite and cell invasion. Parasitology. 1994;108 Suppl: S5-18.
- Blackman MJ, Heidrich HG, Donachie S, McBride JS, Holder AA. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J Exp Med. 1990;172:379-82.
- Siddiqui WA, Tam LQ, Kramer KJ, Hui GS, Case SE, Yamaga KM, et al. Merozoite surface coat precursor protein completely protects Aotus monkeys against Plasmodium falciparum malaria. Proc Natl Acad Sci USA. 1987;84:3014-8.
- Egan AF, Morris J, Barnish G, Allen S, Greenwood BM, Kaslow DC, et al. Clinical immunity to Plasmodium falciparum malaria is associated with serum antibodies to the 19-kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1. J Infect Dis. 1996;173: 765-9.
- McBride JS, Newbold CI, Anand R. Polymorphism of a high molecular weight schizont antigen of the human malaria parasite Plasmodium falciparum. J Exp Med. 1985;161:160-80.
- Tanabe K, Mackay M, Goman M, Scaife JG. Allelic dimorphism in a surface antigen gene of the malaria parasite Plasmodium falciparum. J Mol Biol. 1987;

195:273-87.

- Jongwutiwes S, Tanabe K, Kanbara H. Sequence conservation in the C-terminal part of the precursor to the major merozoite surface proteins (MSP1) of Plasmodium falciparum from field isolates. Mol Biochem Parasitol. 1993;59:95-100.
- Jongwutiwes S, Tanabe K, Nakazawa S, Yanagi T, Kanbara H. Sequence variation in the tripeptide repeats and T cell epitopes in P190 (MSA-1) of Plasmodium falciparum from field isolates. Mol Biochem Parasitol. 1992;51:81-9.
- Hughes AL. The evolution of amino acid repeat arrays in Plasmodium and other organisms. J Mol Evol. 2004; 59:528-35.
- Putaporntip C, Jongwutiwes S, Tanabe K, Thaithong S. Interallelic recombination in the merozoite surface protein 1 (MSP-1) gene of Plasmodium vivax from Thai isolates. Mol Biochem Parasitol. 1997;84:49-56.
- 22. Putaporntip C, Jongwutiwes S, Seethamchai S, Kanbara H, Tanabe K. Intragenic recombination in the 3' portion of the merozoite surface protein 1 gene of Plasmodium vivax. Mol Biochem Parasitol. 2000;109: 111-9.
- Putaporntip C, Jongwutiwes S, Sakihama N, Ferreira MU, Kho WG, Kaneko A, et al. Mosaic organization and heterogeneity in frequency of allelic recombination of the Plasmodium vivax merozoite surface protein-1 locus. Proc Natl Acad Sci USA. 2002;99:16348-53.
- Sakihama N, Mitamura T, Kaneko A, Horii T, Tanabe K. Long PCR amplification of Plasmodium falciparum DNA extracted from filter paper blots. Exp Parasitol. 2001;97:50-4.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997;25: 4876-82.
- Nei M, Miller JC. A simple method for estimating average number of nucleotide substitutions within and between populations from restriction data. Genetics. 1990;125:873-9.

- 27. Tajima F. Evolutionary relationship of DNA sequences in finite populations. Genetics. 1983;105:437-60.
- McDonald JH, Kreitman M. Adaptive protein evolution at the Adh locus in Drosophila. Nature. 199;351: 652-4.
- 29. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA. 2004;101:11030-5.
- 30. O'Donnell RA, de Koning-Ward TF, Burt RA, Bockarie M, Reeder JC, Cowman AF, et al. Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. J Exp Med. 2001;193: 1403-12.
- Cowman AF, Baldi DL, Duraisingh M, Healer J, Mills KE, O'Donnell RA, et al. Functional analysis of Plasmodium falciparum merozoite antigens: implications for erythrocyte invasion and vaccine development. Philos Trans R Soc Lond B Biol Sci. 2002;357:25-33.
- 32. Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, et al. Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. Clin Infect Dis. 2008;46:165-71.
- Chitnis CE, Miller LH. Identification of the erythrocyte binding domains of Plasmodium vivax and Plasmodium knowlesi proteins involved in erythrocyte invasion. J Exp Med. 1994;180:497-506.
- O'Donnell RA, Saul A, Cowman AF, Crabb BS. Functional conservation of the malaria vaccine antigen MSP-119 across distantly related Plasmodium species. Nat Med. 2000;6:91-5.
- 35. Putaporntip C, Jongwutiwes S, Iwasaki T, Kanbara H, Hughes AL. Ancient common ancestry of the merozoite surface protein 1 of Plasmodium vivax as inferred from its homologue in Plasmodium knowlesi. Mol Biochem Parasitol. 2006;146:105-8.
- Coatney GR, Collins WE, Warren M, Contacos PG The primate malarias [original book published 1971] [CD-ROM]. Version 1.0. Atlanta: Centers for Disease Control and Prevention. 2003.