Samples from Ghana

352 samples were collected by ACD from 0–15 year old children in four villages near Winneba, a western coastal region of the country. Finger-prick blood was collected on Whatman® 31ETCHR filter paper, and DNA extraction was performed using the EZ1 BioRobot™. This study was approved by the Ministry of Health/Ghana Health Service.

Species typing PCR

DNA extracted from all samples was subjected to *Plasmodium* species typing PCR based on the nested PCR technique developed by Snounou *et al* [28] with some modifications. Oligonucleotide primers were identical to those previously described [28] but the PCR conditions were modified as follows: For the first round of PCR, 1 μl of extracted DNA was added to 14.85 μl of dH_20, 1.75 μl of each primer (rPLU5 and rPLU6 at 5 uM), 2.5 μl of AmpliTaq Gold® 10× PCR Buffer II, 2 μl of 25 mM $MgCl_2$ solution, 1 μl of dNTP mixture (2.5 mM each) and 0.15 μl of AmpliTaq Gold® in a 25 μl reaction. The following cycling conditions were applied using a GeneAmp® PCR 9700 thermocycler (Applied Biosystems, USA): 95°C for 10 min, 30 cycles of 57°C for 1 min, 72°C for 1 min, 94°C for 1 min and a final extension step of 72°C for 4 min. 1 μl of the resulting PCR product was used for the second round of PCR, with an identical reaction mix to that described for the first round (using pairs of species specific primers FAL-1 and FAL-2, VIV-1 and VIV-2, MAL-1 and MAL-2, and OVA-1 and OVA-2), and with the following cycle conditions: 95°C for 10 min, 32 cycles of 94°C for 1 min, 65°C for 1 min, and a final extension step of 65°C for 5 min. The resulting PCR products were visualized on 2% agarose gels, with the presence or absence of a band with each species primer pair indicative of the presence or absence of that species in the initial sample.

Sensitivity of species diagnosis PCR

Prior to commencement of PCR analysis of field samples, a pilot experiment was carried out to assess the sensitivity of the PCR conditions detailed above. This protocol consistently detected the presence of *P. vivax* in a dilution of genomic DNA that theoretically contained one copy of the parasite genome per μl (data not shown). Due to variation in DNA extraction technique between sample collections, a consistent volume of blood corresponding to the 1 μl of extracted genomic DNA used in the PCR cannot be given. However, it is estimated that no less than 0.5 μl of initial blood sample was used in each reaction. Therefore, the PCR detection method used in this investigation should detect *P. vivax* parasites in infections of as low as two parasites per μl of blood. Furthermore, microscopic evaluation of parasite presence was available for all samples, and these correlated well with PCR results. Although the very rare occurrence of a microscopically positive sample being found to be PCR negative did occur, the vast majority of discrepancies between microscopy and PCR diagnosis involved species misdiagnosis by microscopy, and the detection of parasite infections by PCR in microscopically negative samples, as is expected due to the greater sensitivity of the PCR technique.

Duffy status profiling

An FY* allele-specific PCR [29] was used to determine the Duffy status of the individual from Sao Tome infected with *P. vivax*. Product amplification took place in a 50 μl volume reaction containing 5 μl of 10× PCR buffer, 4 μl of 25 mM MgCl2, 0.1 mM of each dNTP, 2 μl of the two 5 μM allele specific primers, 1 μl of the 5 μM control primers and 6 units of AmpliTaq Gold® DNA polymerase (Applied Biosystems, USA). Amplification conditions were as follows; denaturation and activation of the AmpliTaqGold DNA polymerase at 96°C for 8 min, then 10 cycles of 94° for 20 s and 69°C for 1 min, leading to 25 cycles of 94°C for 20 s, 64°C for 30 s and 72°C for 1 min, followed by 5 cycles of 94°C for 20 s, 62°C for 30 s and 72°C for 1 min. Amplification of a 411 bp fragment of the ABO gene acted as the internal control for each reaction.

Results and discussion

Prevalence of P. vivax in sub-Saharan Africa

1,711 samples were positive for *P. falciparum* (1,526 single species infections, 51 with *P. ovale*, 129 with *P. malariae*, one with *P. vivax* and four with both *P. malariae* and *P. ovale*), 67 for *P. ovale* (12 single infections, 51 mixed with *P. falciparum*, and four triple infections with *P. falciparum* and *P. malariae*) 147 for *P. malariae* (14 single infections, 129 mixed with *P. falciparum*, and four triple infections with *P. ovale* and *P. falciparum*) and one for *P. vivax* (mixed infection with *P. falciparum*) (Table 2). The only *P. vivax* infected sample came from a Duffy positive individual from Sao Tome, an island off the west coast of Africa. No *P. vivax* from any other location within the continent was detected, confirming the scarcity of this parasite in Africa. When excluding samples from Rwanda, Mozambique, Angola and Sao Tome (self-selected as patients identified by microscopy with a mixed infection were excluded), *P. malariae* infections represented 8.5% of all malaria infections, and *P. ovale* 3.9%. The prevalence of both parasites varies greatly by country.

Scarcity of P. vivax

The prevalence of *P. vivax* in Africa is very low; no evidence for its presence in over 2,500 samples from nine African countries was found, with the exception of the island of Sao Tome, from which the parasite had previously been reported [10]. These results, combined with the sporadic reports of the transmission of *P. vivax* in indigenous populations [10,13,19] and the continued identification of imported cases originating in west and central Africa [15,17] indicate that a very low prevalence

Table 2: Species composition of isolates analysed by PCR (%)

Collection area	Number	*P. falciparum*	*P. ovale*	*P. malariae*	*P. vivax*
Burkina Faso[1]	108	108 (100)	6 (5.5)	8 (7.4)	0
Congo	851	341 (40.1)	11 (1.3)	8 (0.9)	0
Gabon	206	102 (49.5)	4 (1.9)	1 (0.5)	0
Ghana[1]	352	352 (100)	8 (2.3)	45 (12.8)	0
Kenya	722	459 (63.6)	35 (4.8)	84 (11.6)	0
Angola[1,2]	90	90 (100)	0	0	0
Mozambique[1,2]	90	90 (100)	0	0	0
Rwanda[1,2]	99	99 (100)	2 (2)	1 (1)	0
Sao Tome[1,2]	70	70 (100)	1 (1.4)	0	1 (1.4)
Total	*2588*	*1711*	*67*	*147*	*1*

[1] Only *P. falciparum* positive samples were available for analysis
[2] Only *P. falciparum* single species infections (diagnosed by microscopy) were analysed

of *P. vivax* is sufficient to maintain transmission. It is conceivable that in areas with very high entomological inoculation rates (EIR), such as in many areas of west and central Africa [30], even very low numbers of Duffy positive individuals may allow the continued transmission of *P. vivax*.

That low numbers of Duffy positive individuals in west and central Africa are sufficient to maintain transmission on *P. vivax*, is not surprising considering the very high basic reproduction number of malaria in this region. The basic reproductive number of a pathogen, R_0, is defined as the number of new infections arising from an infected individual introduced into a naïve population. When $R_0 > 1$, transmission is maintained in a population, but when $R_0 < 1$, transmission is interrupted and the pathogen cannot persist. A recent report showed that R_0 for *P. falciparum* malaria transmission in Africa ranges from below one to nearly 11,000 with a median value of 86, depending on geographical location [30]. Given that both *P. falciparum* and *P. vivax* are vectored by the same mosquito species, the only factor that that differentiates R_0 for both species in a given population is the human host's susceptibility to infection. In order for transmission to be blocked, the proportion of completely immune individuals (p) required in a population is given by the formula $p > 1 - 1/R_0$. Thus, in areas where Duffy negativity is present in a population at a prevalence of up to 99%, as it is in many parts of west and Central Africa [8], then *P. vivax* transmission can be expected to occur when R_0 (for *P. falciparum*) > 100, an entirely realistic value for many areas. It is entirely conceivable therefore, that *P. vivax* transmission occurs in populations in which there are a very high proportion Duffy negative individuals, given the very high *P. falciparum* R_0 values associated with west and central Africa.

A number of researchers have recently suggested that *P. vivax* may be in the process of evolving mechanisms [21] that enable it to infect Duffy positive individuals. Given the extremely high EIRs and transmission dynamics of malaria parasites in sub-Saharan Africa, this scenario would appear highly unlikely given the extremely low incidences of the parasite reported here. Any *P. vivax* parasite that acquired the ability to infect Duffy negative individuals may be expected to rapidly spread throughout sub-Saharan Africa, and would be readily detectable in the population.

Discrepancy between P. vivax rates in travellers and localpopulations

Surveillance of malaria cases imported into the USA between 2001 and 2005 [31-35], reveals that 32 cases of *P. vivax* originated in four west and central African countries for which we also have species prevalence data from the local populations. In the same time period, there were 545 cases of imported *P. falciparum* from the same countries. This gives a ratio of 100:6 *P. falciparum* to *P. vivax* infections in these areas, a surprisingly high rate, especially considering that *P. malariae* and *P. ovale* are represented at ratios of 100:6 and 100:5 respectively (comparable with those of the local populations, see Table 3). How does one account, then, for the discrepancy between the imported *P. vivax* data, and the extremely low prevalence in the native population reported here?

It is possible that the geographical distribution of *P. vivax* within Africa is patchy, with sporadic areas of transmission scattered throughout the continent, possibly associated with human populations in which the Duffy negative phenotype is present at a lower frequency than elsewhere (such as on the island of Sao Tome). Travellers may preferentially visit areas of west and central Africa where there is a relatively high frequency of Duffy positive individuals

Table 3: Parasite species prevalence of traveler's malaria imported into the USA (2001-2005) from Burkina Faso, Gabon, Republic of Congo / Democratic Republic of Congo and Ghana compared to that of the local populations.

Species	Total number		Species prevalence (per 100 *P. falciparum* cases)	
	Imported to the USA[1]	Local population[2]	Imported to the USA[1]	Local population[2]
P. falciparum	545	903	-	-
P. vivax	32	0	5.9	0
P. malariae	34	62	6.2	6.9
P. ovale	26	29	4.8	3.2

[1] 2001–2005, data from [17,31-35]
[2] data from the current survey

in the local population (*e.g.* migrant workers and non-African expatriates) and where *P. vivax* is more likely to be transmitted.

Another factor that may contribute to this discrepancy is the higher transmissibility of *P. vivax* relative to other malaria parasites, and in particular relative to *P. falciparum*, under adverse conditions [9]. This has the consequence that there should be higher proportion of *P. vivax* relative to *P. falciparum* in the vector mosquitoes than there is in the corresponding human population. Consequently travellers, who are a probe of the infection rates in the local mosquitoes, can be expected to, and indeed do have (Carter and Mendis, unpublished analysis), higher proportions of *P. vivax* than are found in the endemic human populations amongst whom the travellers have briefly resided. This may explain the recent findings of Ryan et al [19], who report the presence of *P. vivax* in 0.65% of mosquitoes from an area of western Kenya with a high proportion of Duffy negativity in the local population. However, even an extremely small percentage of Duffy positive individuals in this population may be expected to support such a rate in mosquitoes.

The use of prophylactic anti-malaria drugs among travellers may also contribute to this phenomenon. Mefloquine is the recommended prophylactic drug for travellers to west and central Africa from the USA [31], and whilst effective against the blood stages of all malaria parasites, it does not affect the dormant hypnozoite stages of *P. vivax*, and will therefore not protect against relapses after cessation of drug use. This is also true of *P. ovale*, which is also capable of producing hypnozoites, and may explain the slightly higher rate of this parasite in returning travellers compared to the local populations (Table 3).

It is also probable that a small proportion of imported cases may be *P. ovale* infections rather than *P. vivax*, as is often difficult to distinguish the two species by microscopy [15]. As previously mentioned, there are an increasing number of reports detailing imported African *P. vivax* diagnosed by accurate molecular typing techniques [16,18].

In conclusion, the present study indicates that the prevalence of *P. vivax* in local populations in sub-Saharan Africa is very low, despite the frequent identification of this parasite in travellers. *P. vivax* malaria, therefore, does not constitute a health risk to the indigenous populations of west and central Africa, though Duffy positive individuals, including non-African travellers to the area, may be at risk.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
RCu, RCa and KT conceived the study and participated in its design and coordination. RCu wrote the manuscript. MN, FN, HU, RCa, UdA, AK and KT helped to draft the manuscript. RCu, HU, TM, HE and NT performed molecular parasite typing. MN coordinated and carried out sample collection in the Republic of Congo, HU and FN in Gabon, GP in Burkina Faso, HT, CK and UdA in Rwanda, PC and VdR in Sao Tome, Angola, and Mozambique, and TM, AK and TK in Ghana and Kenya. All authors read and approved the final manuscript.

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MOLECULAR & BIOCHEMICAL PARASITOLOGY

Diversity and evolution of the *rhoph1/clag* multigene family of *Plasmodium falciparum*☆

Hideyuki Iriko[a,b,1], Osamu Kaneko[a,*], Hitoshi Otsuki[a], Takafumi Tsuboi[b,c], Xin-zhuan Su[d], Kazuyuki Tanabe[e], Motomi Torii[a]

[a] *Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan*
[b] *Venture Business Laboratory, Ehime University, Matsuyama, Ehime 790-8577, Japan*
[c] *Cell-free Science and Technology Research Center, Ehime University, Matsuyama, Ehime 790-8577, Japan*
[d] *Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA*
[e] *Laboratory of Malariology, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan*

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Abstract

A complex of high-molecular-mass proteins (*Pf*RhopH) of the human malaria parasite *Plasmodium falciparum* induces host protective immunity and therefore is a candidate for vaccine development. Understanding the level of polymorphism and the evolutionary processes is important for advancements in both vaccine design and knowledge of the evolution of cell invasion in this parasite. In the present study, we sequenced the entire open reading frames of seven genes encoding the proteins of the *Pf*RhopH complex (*rhoph2*, *rhoph3*, and five *rhoph1/clag* gene paralogs). We found that four *rhoph1/clag* genes (*clag2*, *3.1*, *3.2*, and *8*) were highly polymorphic. Amino acid substitutions and indels are predominantly clustered around amino acid positions 1000–1200 of these four *rhoph1/clag* genes. An excess of nonsynonymous substitutions over synonymous substitutions was detected for *clag8* and *9*, indicating positive selection. The McDonald–Kreitman test with a *Plasmodium reichenowi* orthologous sequence also supports positive selection on *clag8*. Based on the ratio of interspecific genetic distance to intraspecific distance, the time to the most recent common ancestor of the *clag2* and *8* polymorphisms was estimated to be 1.89 and 0.87 million years ago, respectively, assuming divergence of *P. falciparum* and *P. reichenowi* 6 million years ago. In addition to a copy number polymorphism, gene conversion events were detected for the *rhoph1/clag* genes on chromosome 3, which likely play a role in increasing the diversity of each locus. Our results indicate that a high diversity of the *Pf*RhopH1/Clag multigene family is maintained by diversifying selection forces over a considerably long period.

Keywords: Gene conversion; Malaria; Polymorphism; Rhoptry; Selection

1. Introduction

Malaria infects more than 300 million people and kills 1–2 million each year. Efforts have been made to develop effective malaria vaccines, but none is available so far. Malaria is caused by the obligate intracellular protozoan *Plasmodium* parasites; and entry into erythrocytes is prerequisite for the growth in the mammalian host. After contact with the erythrocyte surface, parasite discharge the content of the microorganelles called the micronemes to establish a tight junction with the erythrocyte surface molecules; parasite then invaginates into a nascent parasitophorous vacuole (PV) [1,2]. During formation of the PV, the parasite discharges the contents of another pair of microorganelles, the rhoptries [3]. The molecules located within these

Abbreviations: aa, amino acid; chr, chromosome; *clag*, cytoadherence-linked asexual gene(s); CI, confidence interval; mya, million years ago; nt, nucleotide; ORF, open reading frame(s); PCR, polymerase chain reaction; PV, parasitophorous vacuole; PVM, PV membrane; S.E., standard error; TMRCA, time to the most recent common ancestor; UTR, untranslated region(s).

☆ *Note:* Sequence data from this article have been deposited with the GenBank™/EMBL/DDBJ databases under accession numbers AB250801–AB250912.

* Corresponding author. Present address: Department of Protozoology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Tel.: +81 95 819 7838; fax: +81 95 819 7805.

[1] Current address: Division of Medical Zoology, Department of Microbiology and Immunology, Faculty of Medicine, Tottori University, Yonago, Tottori 683-8503, Japan.

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organelles play a key role in erythrocyte invasion and have been studied as vaccine targets, with the aim to induce antibodies to block invasion. One erythrocyte-binding molecule in the rhoptry is a complex of high-molecular-mass proteins called the RhopH complex [4,5]. The RhopH complex is distributed throughout the erythrocyte and PV membrane (PVM) and has been detected in ring-stage parasites [6], suggesting an important role during PV establishment. The importance of the complex has further been emphasized by the failure of attempts to disrupt the *pfrhoph3* gene locus, suggesting its necessity for parasite survival [7].

The RhopH complex comprises three distinct components: RhopH1, RhopH2, and RhopH3 [8–12]. The genes encoding RhopH1 are members of the *rhoph1/clag* gene family, which was originally defined by the cytoadherence-linked asexual gene (*clag*) on chromosome 9 in *Plasmodium falciparum* (*clag9*) and consists of at least three members; *clag2*, *3.1*, and *9* [13–15]. Although not yet determined experimentally, molecules encoded by *clag3.2* and *8* are likely parts of the RhopH complex as judged by their similarity in amino acid sequence and transcription pattern with other members [15]. Because only one RhopH1/Clag participates to form a single RhopH complex [15,16], five types of *Pf*RhopH complex are expected to exist, each of which contains one *rhoph1/clag* gene product. In this report we employ 'RhopH1/Clag' (protein) and '*rhoph1/clag*' (gene) as the family name, and 'Clag' (protein) and '*clag*' (gene) for each member.

Erythrocyte-binding proteins discharged from *P. falciparum* merozoites are considered to be targets of host immune responses. Strong diversifying selections on microneme proteins have been detected (e.g., AMA-1 and EBA-175), suggesting that polymorphism of these proteins has been maintained to evade host immunity in parasite populations [17,18]. Antibodies against the *Pf*RhopH complex partially inhibit the growth of *P. falciparum* in vitro and in vivo, consistent with its potential as a vaccine target [19–21]. Although the RhopH complex has been shown to induce host protective immunity and is likely to be under host immune pressure, the genetic diversity and immunologic characteristics of this complex are not fully understood. Here, we analyzed sequence polymorphism in five *rhoph1/clag* members, *rhoph2*, and *rhoph3* and show that some of the *rhoph* genes are under positive/diversifying selection. In addition, we assessed a population genetic mechanism that might drive the evolution of the *rhoph1/clag* multigene family.

2. Materials and methods

2.1. Malaria parasites

All cloned lines of *P. falciparum* were maintained in vitro, essentially as described previously [22]. The parasite lines examined originated from Southeast Asia (Dd2, FVO, Camp, T9/96, T9/102, K1, and Thai838), Papua New Guinea (MAD20), Central and South America (HB3, 7G8, DIV17, DIV29, DIV30, PC49, PC54, Santa Lucia, and Haiti), and Africa (RO33, 123/5, 128/4, SL/D6, LF4/1, 102/1, M2, M5, Fab9, 713, P13, and KMWII) and have been previously described [23–25]. Their geographic origins have also been previously described [26].

2.2. DNA and RNA isolation

Genomic DNA was obtained as described previously [24]. Total RNA was isolated from schizont stage-enriched HB3 and Dd2 parasite lines using the RNeasy mini kit (Qiagen, Valencia, CA). Complementary DNA was synthesized using random hexamers and an Omniscript reverse transcription kit (Qiagen) after DNase treatment.

2.3. Polymerase chain reaction (PCR) amplification and sequencing

Nucleotide sequences corresponding to open reading frame (ORF) were determined for five *pfrhoph1/clag* genes, *rhoph2*, and *rhoph3* in four parasite lines: Dd2, HB3, 7G8, and FVO. DNA fragments were PCR amplified with KOD-Plus DNA polymerase (Toyobo, Japan) using a panel of oligonucleotides specific for the genes (Supplemental Table 1) and sequenced directly using an ABI PRISM® 310 genetic analyzer (Applied Biosystems, Foster City, CA) or sequenced after cloning into pGEM-T Easy® plasmid (multiple plasmid clones sequenced for each DNA fragment; Promega, Madison, WI). To PCR amplify DNA fragments including the entire ORF of *clag3.1* or *3.2*, LA *Taq* DNA polymerase (TaKaRa, Japan) was used with oligonucleotide primers 3.1F (5′-TGTGCAATATATCAAAGTGTACATGC-3′) and 3.1R (5′-TAGAAAATATTAGAATTGCTATTATGTAC-3′) or 3.2F (5′-AATAGTTGAGTACGCACTAATATGTC-3′) and 3.2R (5′-ACACAAATTCTTAATAATTATATAAAACC-3′), respectively. A highly polymorphic region identified in *clag2*, *3.1*, *3.2*, and *8* in this study was further analyzed by increasing the number of parasite lines ($n = 25$) from different geographic areas.

2.4. Plasmodium reichenowi sequences

A TBLASTN search was performed against the *P. reichenowi* preliminary genome shotgun database (Dennis strain; Sanger Centre, UK) using Clag2, 3.1, 3.2, and 8 amino acid sequences as queries. For *prclag2* and *prclag8*, sequences were assembled using SeqMan II accompanied with Lasergene software (DNASTAR Inc., Madison, WI) with manual corrections. Regions covered by at least two independent reads and showing identical sequences were selected and used for analysis (Supplemental Figs. S1 and S2). The generated sequences were 3273 bp long for *prclag2*, corresponding to nucleotide (nt) positions 193–870, 1021–1902, 2458–3432, and 3448–4185 of *pfclag2* (3D7), and 2175 bp long for *prclag8*, corresponding to nt positions 1459–4173 of *pfclag8* (3D7). For Clag3 orthologs in the *P. reichenowi* genome, only sequences possessing homology with the 5′ untranslated region (UTR) (reich908g11.plk) or 3′ UTR (reich1194c08.plk and reich289f06.qlk) were used.

2.5. Sequence alignment and analysis

The entire ORFs for the 7 *Pf*RhopH complex-related genes (5 *rhoph1/clag* genes, *rhoph2*, and *rhoph3*) in four culture-adapted

P. falciparum lines—Dd2 (Southeast Asia), 7G8 (Brazil), HB3 (Honduras), and FVO (Vietnam)—were aligned with those retrieved from a genome database (3D7 line, presumably African in origin) using a CLUSTAL W program [27] with manual corrections; nucleotide diversity (π) and its standard error (S.E.) were computed with the Jukes and Cantor method using MEGA 3.1 software [28] after excluding insertions/deletions (indels) and highly polymorphic regions. The mean numbers of synonymous substitutions per synonymous site (d_S) and nonsynonymous substitutions per nonsynonymous site (d_N) and their standard errors were computed using the Nei and Gojobori method [29] with the Jukes and Cantor correction, implemented in MEGA 3.1. The statistical difference between d_S and d_N was tested using a one-tailed Z-test with 500 bootstrap pseudosamples using MEGA 3.1. A value of d_N significantly higher or lower than d_S at the 95% confidence level was taken as evidence for positive or purifying selection, respectively. The d_N:d_S ratio was evaluated using a sliding window method (50 bases with a step size of 10 bases) in DnaSP 4.0 [30]. Positive selection was also evaluated using the McDonald–Kreitman test [31]. Before estimating the time to the most recent common ancestor (TMRCA) for *P. falciparum clag2* and *8* polymorphism, the evolutionary rate constancy of *clag2* and *8* between *P. falciparum* and *P. reichenowi* was validated using a *Plasmodium yoelii* ortholog *Py*RhopH1A (accession number AB060734) as an outgroup using Tajima's relative rate test [32] implemented in MEGA 3.1. Mean and 95% confidence intervals (CI) for estimated TMRCA were computed based on the model assuming the distribution of the distance and the substitution rate were Gamma-distributed [33]. Gene conversion was evaluated for each exon using an algorithm by Betrán et al. [34] implemented in DnaSP 4.0.

Unrooted dendrograms of the *pfrhoph1/clag* members were constructed using the neighbor-joining and maximum parsimony methods in MEGA 3.1, and Tajima's relative rate test was used to evaluate the evolutionary rate among members. Indels and highly polymorphic regions could not be satisfactorily aligned and were therefore excluded from the analysis. The sequences (3D7 parasite line) used to construct trees and the evolutionary rate were as follows: nt positions 154–312, 331–573, 727–1122, 1207–1266, 1324–1560, 1609–2988, 3004–3288, and 3382–3924 for *clag3.2*; nt positions 160–318, 337–579, 733–1128, 1213–1272, 1330–1566, 1615–2994, 3010–3294, and 3388–3930 for *clag3.1*; nt positions 223–381, 400–642, 799–1194, 1279–1338, 1390–1626, 1696–3075, 3091–3375, and 3553–4095 for *clag2*; nt positions 130–288, 307–549, 706–1101, 1186–1245, 1300–1536, 1606–2985, 3001–3285, and 3415–3957 for *clag8*; and nt positions 82–240, 265–507, 652–1047, 1132–1191, 1276–1512, 1582–2961, 2977–3261, and 3394–3936 for *clag9*.

3. Results

3.1. Polymorphism of the PfRhopH complex-related genes

All seven *Pf*RhopH complex-related genes showed greater nucleotide diversity levels than the average (+2 S.E.) of 204 ORFs on *P. falciparum* chromosome (chr) 3 [35] (Table 1). Among the seven genes, *clag2*, *3.1*, *3.2*, and *8* are highly polymorphic with nucleotide diversity ($\pi = 0.0053$–0.0164) comparable to malaria vaccine candidate antigen protein genes such as *eba-175* ($\pi = 0.0030$) and *ama-1* ($\pi = 0.0166$) [17,18]. The observed nucleotide diversity levels of *clag2*, *3.1*, and *3.2* should be taken as minimum estimates, because indels and highly polymorphic regions were excluded from this analysis to obtain reliable alignments. The highly polymorphic nature of four *rhoph1/clag* genes at the nucleotide level extends to the amino acid level, which is represented by high d_N values (Table 1). Thus, the genes encoding RhopH1/Clag are more polymorphic than RhopH2 and RhopH3.

Among the four RhopH1/Clag showing high polymorphism (Clag2, 3.1, 3.2, and 8), the majority of polymorphic sites are clustered in a region at amino acid (aa) positions 1000–1200

Table 1
Nucleotide diversity of the *Pf*RhopH complex genes[a]

Gene	*n*	Indel	Sites	π	π (S.E.)	d_N	d_N (S.E.)	d_S	d_S (S.E.)	d_N/d_S	*P*[b]
clag2[c]	5	(+)	4,317	0.0053	(0.0008)	0.0032	(0.0007)	0.0133	(0.0028)	0.24	(0.0003)
clag3.1[c]	5	(+)	4,140	0.0164	(0.0015)	0.0062	(0.0011)	0.0582	(0.0058)	0.11	($<10^{-10}$)
clag3.2[c]	5	(+)	4,134	0.0138	(0.0011)	0.0063	(0.0011)	0.0445	(0.0050)	0.14	($<10^{-10}$)
clag8	5	(−)	4,182	0.0066	(0.0007)	0.0065	(0.0011)	0.0069	(0.0020)	0.94	ns
clag9	5	(−)	4,020	0.0009	(0.0003)	0.0011)	(0.0004)	0.0000	(0.0000)	∞	0.002
rhoph2	5	(−)	4,134	0.0009	(0.0003)	0.0010	(0.0004)	0.0005	(0.0005)	2.00	ns
rhoph3	5	(−)	2,691	0.0013	(0.0004)	0.0012	(0.0005)	0.0015	(0.0010)	0.80	ns
clag2[d]	24	(+)	522	0.0131	(0.0032)	0.0114	(0.0042)	0.0192	(0.0074)	0.60	ns
clag8[d]	26	(−)	585	0.0267	(0.0042)	0.0305	(0.0060)	0.0132	(0.0061)	2.31	0.020
Chr 3[e]	5		202,069	0.00044	(0.00006)	0.00039	(0.0060)	0.00068	(0.00010)	0.57	

[a] *n*, Number of sequences sampled; sites, sites analyzed excluding noncoding sequences and alignment gaps; π, pairwise nucleotide diversity; d_N, number of nonsynonymous substitutions over numbers of nonsynonymous sites; d_S, number of synonymous substitutions over numbers of synonymous sites; S.E., standard error computed using the Nei-Gojobori method with Jukes-Cantor correction. S.E. was estimated using the bootstrap method with 500 replication.

[b] *P*-value indicates that d_N is significantly greater than d_S. Those shown in parenthesis indicate that d_S are significantly greater than d_N. The statistical difference between d_S and d_N was tested using an one-tail Z-test with 500 bootstrap pseudosamples implemented in MEGA 3.1. ns indicate not significant ($P > 0.05$).

[c] For optimal sequence alignment, nt 3433–3435 was excluded from *clag2*, nt 3337–3447 from *clag3.1*, and nt 88–99 and 3343–3444 from *clag3.2* for the analysis. Nucleotide numbering are after the 3D7 line sequences.

[d] nt 3022–3606 of *clag8* and nt 3106–3420 and 3436–3642 of *clag2* were used.

[e] Data from 204 ORF on *P. falciparum* chr 3 using five parasite lines [35].

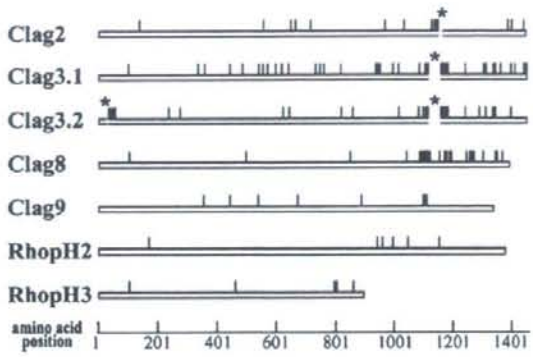

Fig. 1. Locations of amino acid polymorphism of seven components of the *Pf*RhopH complex among five *P. falciparum* parasite lines (3D7, HB3, Dd2, FVO, and 7G8). Indels are shown as gaps with asterisks (aa position 1145 for Clag2, aa positions 1113–1149 for Clag3.1, and aa positions 30–33 and 1115–1148 for Clag3.2). Numbers are those of 3D7 line sequences.

(Fig. 1). In addition, numerous polymorphic sites in this region have more than one amino acid substitution, whereas most polymorphisms in the other regions are dimorphic (at both nucleotide and amino acid levels). Most indels are also located in this region (Fig. 1, asterisks). Thus, the region at aa positions 1000–1200 of RhopH1/Clag is the most highly polymorphic region of the *Pf*RhopH complex.

3.2. Gene conversion between clag3.1 and 3.2

Of interest, *clag3.1* and *3.2* share some polymorphic sites. Because *clag3.1* and *3.2* have 96.7% nucleotide identity (3D7 parasite line) and are located on chr 3 and separated by only 10 kb harboring one putative ORF (PFC0115c) (Fig. 2A), we assessed gene conversion between these two loci. Using an algorithm by Betrán et al. [34], we identified multiple gene conversion tracts located at nt positions 1314–1353, 1447–1452, 1612–1659, 1702–1785, 1852–1983, and 2148–2208 in 3D7 *clag3.1*; nt positions 3824–4240 in HB3 *clag3.1*; nt positions 189–247 in 7G8 *clag3.1*; nt positions 813–817 and 3821–4182 in 3D7 *clag3.2*; nt positions 88–151 in HB3 *clag3.2*; and nt positions 3320–3755 in 7G8 *clag3.2* (Fig. 3). The detected conversion tracts had less than 5% informative nucleotides showing a mosaic origin, indicating that the probability of these tracts being involved in a recombination event more than once is negligible [34]. No gene conversion was detected between the other *rhoph1/clag* genes.

Because gene conversion potentially accelerates nucleotide diversity, we evaluated the evolutionary rates of *clag3.1* and *3.2*. Results showed that *clag2*, *3.1*, and *3.2* form a single clade and *clag8* another (Fig. 4); thus we performed Tajima's relative rate test using *clag8* as an outgroup and found that the evolutionary rates between *clag3.1* and *2* and between *clag3.2* and *2* were significantly different for all combinations of the sequences from five parasite lines. Because *clag3.1* and *3.2* were more diverse than *clag2*, *clag3.1* and *3.2* appear to have evolved more rapidly than *clag2*.

3.3. Amino acid polymorphism of the region around aa positions 1000–1200 of Clag2, 3.1, 3.2, and 8

Because extensive polymorphisms were observed around aa positions 1000–1200 in Clag2, 3.1, 3.2, and 8, we further analyzed polymorphism in this region with additional sequences from parasite lines originating worldwide. Alignment of Clag2 sequences showed multiple amino acid substitutions per site at multiple sites, e.g., five amino acids at aa position 1139 (K, R, S, G, and I). Indels were also observed (Supplemental Fig. S3). Clag8 has even higher levels of amino acid substitutions at between 1077 and 1136; five different amino acids (I, S, R, G, and N) at 1100, seven at 1101 (D, S, T, E, N, I, and K), six at 1104 (S, N, I, K, R, and T), and five at 1105 (G, D, T, S, and N) (Supplemental Fig. S4). Clag3.1 and 3.2 are also highly polymorphic (Fig. 5), which will be discussed later.

3.4. Copy number polymorphism of rhoph1/clag genes on chr 3

Notably, when PCR amplification was performed to obtain DNA fragments of the entire ORFs of *clag3.1* or *3.2*, 17 parasite

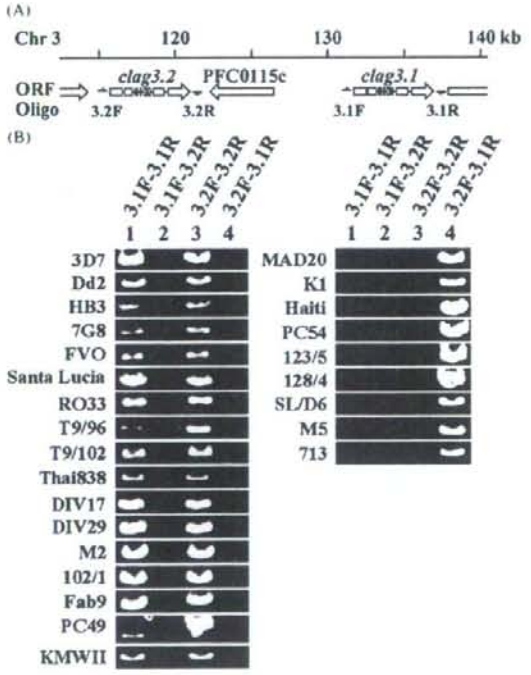

Fig. 2. Copy number polymorphism of *rhoph1/clag* genes on chr 3. (A) Genome organization around *clag3.2* and *3.1* gene loci on chr 3. The locations of the oligonucleotide primers are indicated. Oligonucleotide 3.2F and 3.1F were designed on the 5′ UTR of *clag3.2* and *3.1*, respectively. Oligonucleotide 3.2R and 3.1R were designed on 3′ UTR of *clag3.2* and *3.1*, respectively. (B) PCR-amplified DNA fragments of 26 *P. falciparum* lines with different combinations of oligonucleotides.

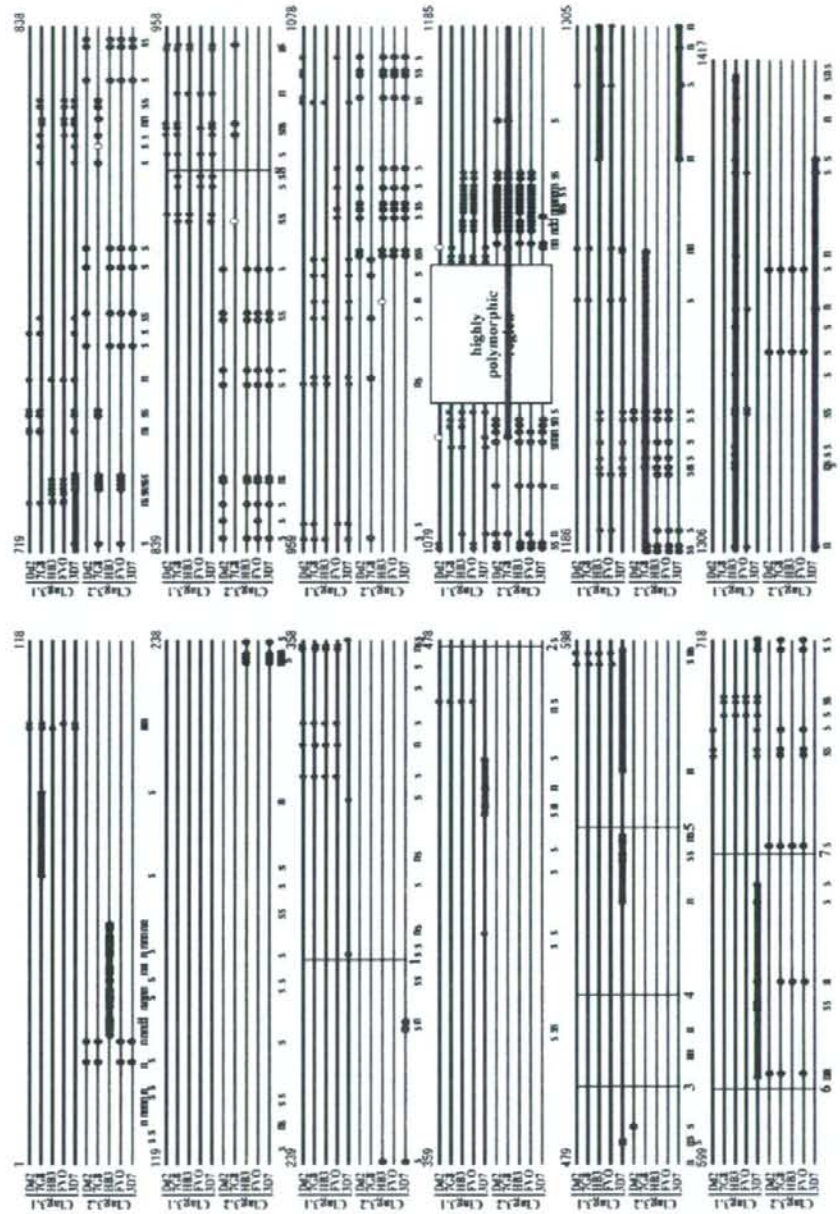

Fig. 3. Gene conversion tracts in *clag3.1* and *clag3.2*. Polymorphic codons (circles) in the coding sequences of Clag3.1 and 3.2 were compared in five *P. falciparum* lines. Clag3.1, black bar; Clag3.2, gray bar. Polymorphisms matching the paralogous sequence are shown in gray or black circles, respectively, and rare polymorphisms by an open circle. Exons are separated by vertical bar with the intron number at the bottom. Polymorphic sites that differ between consensus sequences are shown below the line classified as nonsynonymous (n), synonymous (s), and deletion (d). Gene conversion tracts identified using algorithm by Betrán et al. [34], wide gray bars.

lines showed the 2 expected positive bands with the primer sets 3.1F–3.1R and 3.2F–3.2R, whereas 9 parasite lines showed a positive band only with the primer set 3.2F–3.1R, which suggests that these 9 parasite lines possessed a hybrid gene with *clag3.2* sequence at the 5′ UTR and *clag3.1* sequence at the 3′ UTR (Fig. 2B). DNA fragments were not amplified with other primer combinations, indicating that artificial amplification due to primer mispairing was negligible. This is consistent with a

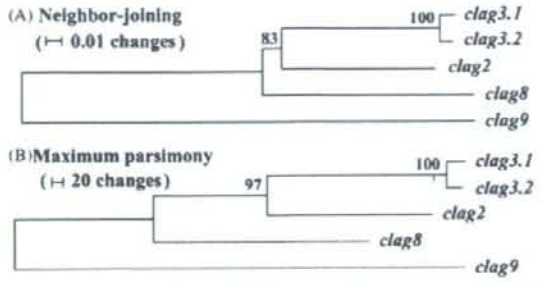

Fig. 4. Unrooted dendrograms of *pfrhoph1/clag* genes using nucleotide sequences from the 3D7 parasite line. The trees were constructed by the neighbor-joining and maximum parsimony methods using MEGA 3.1. Numbers on branches indicate bootstrap values (500 pseudoreplicates).

recent report by Chung et al. [36], who found that some parasite lines possess only a single *rhoph1/clag* on chr 3 by Southern blot hybridization. We here designate this *clag3* gene as *clag3h* (*clag3 hybrid*; Clag3H for protein). In addition, we obtained two distinct sequences for *clag3.1* from the KMWII parasite line (Fig. 5), using several cloned plasmids after experiencing difficulty in direct sequencing of PCR products. Sequences for *clag2*, *3.2*, and *8* were easily obtained from the KMWII line by direct sequencing of the PCR products, supporting the assumption that this line was a clone. Thus, the KMWII line appears to possess at least three *clag3*-related sequences in the genome. This data suggests that the number of *clag3*-related sequences in *P. falciparum* varies from one to at least three.

To deduce the direction of the one-gene to two-gene (or vice versa) change, we searched *P. reichenowi* orthologs in the genome database and found one sequence read (reich908g11.plk) showing high similarity with the sequence around the start codon of *clag3.1* and *3.2*. We also found two reads (reich289f06.qlk and reich1194c08.plk) showing strong homology with the sequence around the stop codons of *clag3.1* and *3.2*. Comparison of the nucleotide sequences at the UTR revealed that reich908g11.plk and reich289f06.qlk were similar to the *pfclag3.2* sequence and that reich1194c08.plk was similar to the *pfclag3.1* sequence (Fig. 6). Thus, duplication of *clag3.1* and *3.2* gene loci appears to predate the divergence of *P. falciparum* and *P. reichenowi*, suggesting that a single *rhoph1/clag* (*clag3h*) found in some *P. falciparum* lines is likely a result of an unequal crossover between two closely related genes. Notably, Clag3H had characteristic amino acids that were not observed in Clag3.1 and 3.2. For example, Ala at 1116 was found in three of nine Clag3H (30%). If Clag3H originated recently, for example during culture, the amino acid allele observed in Clag3H would also exist in Clag3.1 or 3.2; however, Ala at 1116 was not found in a total of 36 sequences of non-Clag3H protein sequences. Three in nine Clag3Hs is a significant excess compared to zero Ala at 1116 in 36 non-Clag3H sequences by Fisher's exact test ($P=0.013$). This suggests that at least some Clag3H have accumulated some unique amino acid substitutions since their creation.

3.5. Selection on the PfRhopH complex

Positive selection was evaluated by comparing synonymous and nonsynonymous substitutions (Table 1). A significant excess of d_N over d_S was observed for *clag9* (entire ORF of five parasite lines) and for *clag8* (highly polymorphic region at nt positions 3022–3606 of 26 parasite lines), suggesting positive selection acting on these genes. A sliding window plot of d_N:d_S ratios revealed that *clag2* and *8* had the highest peaks, around nt positions 3000–3600 (Fig. 7). It should be noted that the corresponding regions of *clag3.1* and *3.2* are the regions showing highly extensive polymorphism with indels (asterisks in Fig. 7), thereby preventing evaluation of d_N:d_S ratios in this region. The peak at the N-terminus of *clag3.2* is due to introduction of part of the *clag3.1* sequence into the HB3 line *clag3.2* by gene conversion (see Fig. 3).

Positive selection was further evaluated by the McDonald–Kreitman test using *P. reichenowi* orthologs for *clag2* and *8*. Significant excess of intraspecific nonsynonymous substitutions over synonymous substitutions was observed in *clag8* as compared with interspecies fixed differences of nonsynonymous and synonymous changes, suggesting positive selection (Table 2).

3.6. Early origin of the clag2 and 8 polymorphism

We estimated the TMRCA for *clag2* and *8* polymorphism using aligned regions. Distances of synonymous single-nucleotide polymorphisms are 0.0139 ± 0.0031 for *clag2* and 0.0106 ± 0.0030 for *clag8*. Distances between *P. falciparum* and *P. reichenowi* are 0.0455 ± 0.0082 and 0.0748 ± 0.0120 for *clag2* and *8*, respectively. Assuming that the divergence time of *P. falciparum* and *P. reichenowi* was 6 million years ago (mya) [37,38], the estimated TMRCA of the polymorphism of *clag2*

Table 2
The McDonald–Kreitman test of selection for *Plasmodium falciparum clag2* and *8*

Locus	n[a]	No. of sites	Fixed differences between species		Polymorphic sites within *P. falciparum*		
			Syn	Nsyn[b]	Syn	Nsyn	P[c]
clag2	5	3273	23	47	18	12	(0.011)
clag8	5	2715	36	55	11	38	0.030

[a] *n*, Number of *P. falciparum* lines used.
[b] Syn, synonymous; Nsyn, nonsynonymous substitutions.
[c] Fisher's exact test (one-tailed) was used. *P*-value indicates that Nsyn are significantly greater than Syn. Value in parenthesis indicates that Syn are significantly greater than Nsyn.

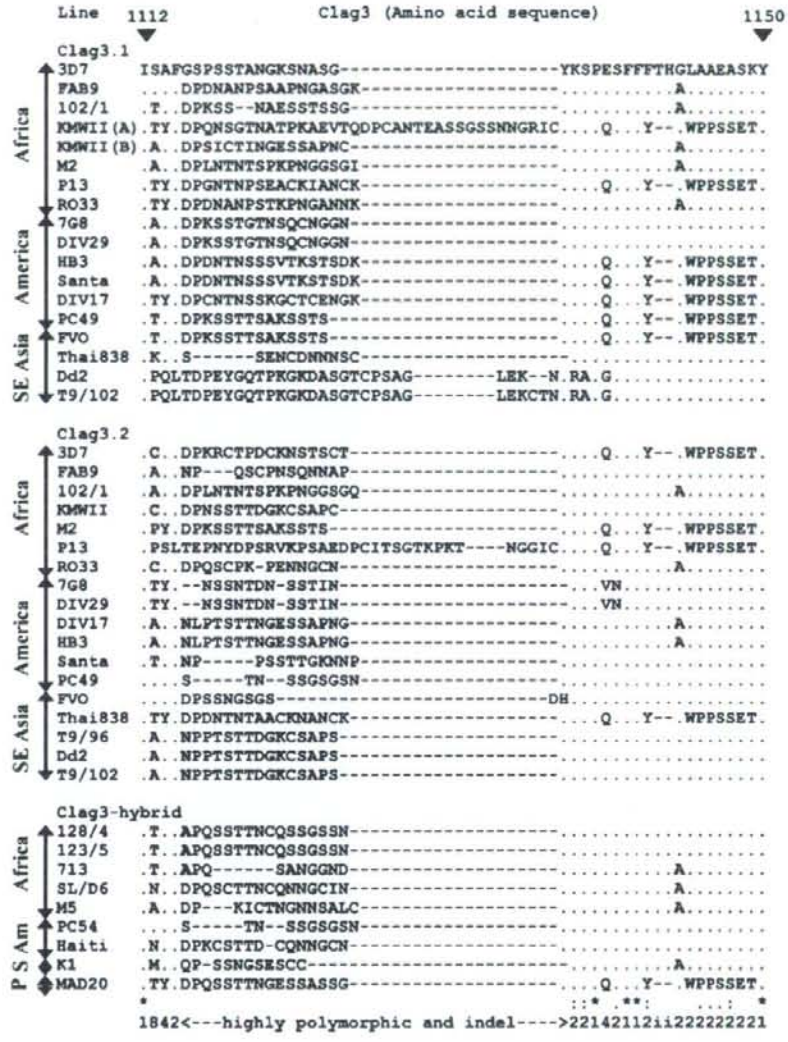

Fig. 5. Polymorphism of Clag3. An amino acid region 1112–1150 (after 3D7 line Clag3.1 sequence) of *P. falciparum* (27 lines) was aligned. Geographic origins are shown at left: SE Asia or S, Southeast Asia; Am, America; P, Papua New Guinea; Santa, Santa Lucia cloned line. Identical, conserved, or semiconserved residues in the alignment are indicated with asterisk, colon, or period, respectively. The number of amino acid replacements at each position and the region with indels are shown at the bottom. Cys residue at aa position 1113 and Asn and Ala residues at aa position 1116 are masked.

and *8* are 1.89 (95% CI, 1.02–3.18) and 0.87 (95% CI, 0.42–1.54) mya, respectively.

4. Discussion

4.1. Diversifying selection on the rhoph1/clag gene loci

The present study revealed that the RhopH1/Clag-encoding genes *clag2*, *3.1*, *3.2*, and *8* contain a highly polymorphic region, particularly at nt positions 3000–3600. Diversifying selection increases nucleotide diversity (π), and an excess of d_N to d_S is indicative of positive selection favoring amino acid replacement [39]. Thus, the observed excess of d_N to d_S at nt positions 3000–3600 of *clag8* suggests that the polymorphism in *clag8* is positively maintained. An excess of d_N to d_S was also observed for *clag9*, indicating that this gene is also under positive selection. The most polymorphic region, in which positive selection was detected for *clag8*, was excluded from *clag2*, *3.1*, and *3.2* due to extensive sequence variation that made sequence alignment unreliable. Further analysis is required to evaluate positive selection on these three *rhoph1/clag* genes.

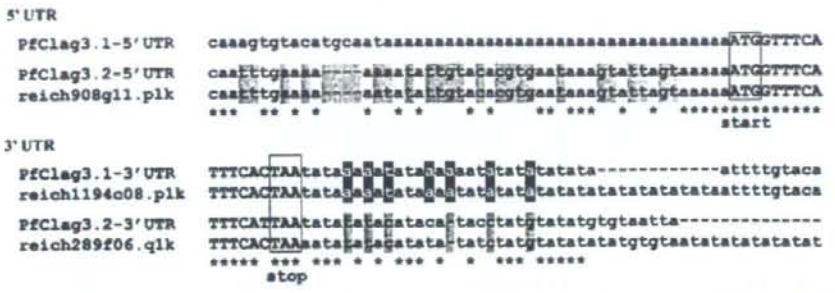

Fig. 6. Nucleotide sequence alignment of the 5′ and 3′ UTRs for *P. falciparum clag3.1* and *3.2* genes and *P. reichenowi* orthologous sequences. A *P. reichenowi* sequence possessing homology with *pfclag3.1* was not found in the current database. Nucleotide sequences corresponding to the ORF and the UTR are shown with upper case and lower case letters, respectively. Putative start and stop codons are boxed. Characteristic nucleotides are displayed in reverse (*clag3.1*) or masked with gray (*clag3.2*).

To date, observation of such high levels of polymorphism for *clag2*, *3.1*, *3.2*, and *8* ($\pi = 0.0053$–0.0164; $d_N = 0.0032$–0.0065) has not been reported for other known malaria rhoptry protein genes. The high polymorphism observed in *clag2*, *3.1*, and *3.2* is consistent with the observation by Kidgell et al. [40] based on the hybridization of genomic DNA from a panel of parasite lines to an oligonucleotide array for the *P. falciparum* genome. In addition, the polymorphism levels are comparable to those of the microneme proteins such as *eba-175* ($\pi = 0.0030$; $d_N = 0.0037$) and *ama-1* ($\pi = 0.0166$; $d_N = 0.0207$), which are exposed to host immune responses [17,18]. Rhoptry proteins are released into the PV and are considered to be minimally exposed to host immunity. If RhopH1/Clag polymorphism is generated by host immune pressure, the questions arises as to how RhopH1/Clag is exposed to host immunity. There are a few possible explanations. First, RhopH1/Clag may be released from the merozoites before attachment to the erythrocyte surface, thereby becoming a target of host immunity. Second, the RhopH complex, which is released into PVs, may be leaked to the surface of infected erythrocytes through the junction between invading parasite and the erythrocyte membrane. Leaked RhopH complex, and therefore parasite-infected erythrocytes, are then potential targets of host immunity. Indeed, the *Pf*RhopH complex and rhoptry-associated protein 2 (RAP-2, RSP-2), another malaria rhoptry protein, have been detected on the erythrocyte surface upon parasite attachment to erythrocytes [41,42]. The last possibility is that RhopH1/Clag, after release into the PV, may be distributed to the parasite-derived membranous network (i.e., Maurer's clefts) in the erythrocyte cytosol, where it is exposed to host immunity. RhopH2 and RhopH3 have recently been observed in materials deriving from Maurer's cleft by proteome analyses, consistent with this possibility [43,44].

There are no obvious associations between particular haplotypes and their geographic origins, and most haplotypes co-exist in different geographic areas, similar to other known polymorphic antigens such as MSP-1 [45,46]. RhopH1/Clag polymorphism might be maintained in natural parasite populations to evade host immunity. Using a T-cell epitope prediction algorithm (SYFPEITHI software) [47], we found that binding of a predicted T-cell epitope peptide to a particular HLA allotype was dramatically affected by the RhopH1/Clag polymorphism in silica. For example, aa positions 1094–1108 of 3D7 line Clag8 (KRISTSIDHISGGKW) was predicted as a T-cell epitope of HLA-DRB1*1101 with a score of 22, but the score for the corresponding region of Camp line Clag8 (MRISSTSTYISNNEW) was 0, emphasizing a potential involvement in immune evasion of RhopH1/Clag polymorphism. We consider the algorithm useful because the score of HLA-DRB1*0701 for the *Pf*CSP Th2R domain, a well characterized malaria polymorphic T-cell epitope peptide, is 22 for the K1 parasite line (KIQYSLSTEWSPCSV) but only 12 for that of the 3D7 line (KIQNSLSTEWSPCSV).

4.2. Evolution of PfRhopH1/Clag polymorphism of the extant P. falciparum population

Gene conversion has been reported for other *P. falciparum* loci, such as *falcipain* 2 [48] and *var* [49], as a source of genetic diversity. In this study, we show that *clag3.1/3.2*, which interchange their sequences by gene conversion, evolved more rapidly than *clag2*. The precise function of the RhopH complex remains unknown, and thus whether the gene conversion observed was functionally advantageous or neutral is also unknown; however, gene conversion can be a mechanism for antigenic variation to evade host immunity. Some examples include the *vsg* gene *of Trypanosoma brucei*, causative agent of African sleeping sickness, the *ves* gene of a cattle parasite *Babesia bovis* [50], and *var* genes in *P. falciparum* [51].

Based on the shared hybridization pattern between *clag3.1* and *3.2*, Chung et al. [36] proposed that these genes are alleles of the same locus; however, because the origin of two *rhoph1/clag* loci on chr 3 appear to predate the *P. falciparum–P. reichenowi* divergence, these should be categorized as paralogous genes but not the same gene. Shared features between these loci detected by Southern blot hybridization by Chung et al. can be simply explained by the gene conversion identified in this study. *clag3h* could be generated by an unequal crossover between *clag3.1* and *3.2* of a set of chromosomes during meiosis. If such crossover had occurred, parasite lines possessing three *rhoph1/clag* on chr 3 would be expected, with a molecule having its 5′ end derived from *clag3.1* and its 3′ end from *clag3.2* (Supplemental Fig. S5, model 1); however, this type of *rhoph1/clag* was not detected in this study. KMWII line appears to possess 3 *rhoph1/clag*

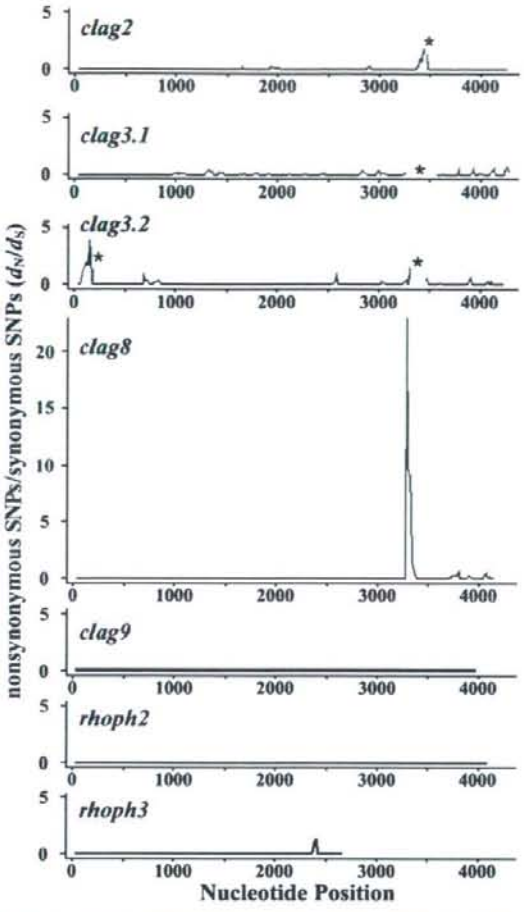

Fig. 7. Sliding window plot of d_N/d_S ratio for seven genes of the *Pf*RhopH complex. For optimum sequence alignment, nt positions 3433–3435 was excluded from *clag2*, nt positions 3337–3447 from *clag3.1*, and nt positions 88–99 and 3343–3444 from *clag3.2* for the analysis (asterisks), because these regions were highly polymorphic with indels. Nucleotide numbers are those of 3D7 line sequences. Window length is 50 bp, and step size is 10 bp.

genes on chr 3, but the third *rhoph1/clag* on chr 3 appears not to be generated by the mechanism described above, because this *rhoph1/clag* was obviously a duplicated *clag3.1* gene amplified with the *clag3.1*-specific primer set. Thus, *clag3h* is more likely a product of a recombination event between *clag3.1* and *3.2* on the same chromosome (Supplemental Fig. S5, model 2). Because a unique amino acid of Clag3H (e.g., Ala at aa position 1116) suggests a relatively old origin of *clag3h*, recombination events between *clag3.1* and *3.2* might be rare in the natural population.

Four highly polymorphic *rhoph1/clag* genes contained unexpectedly large numbers of synonymous substitutions. Based on the ratio of interspecific distance to intraspecific distance, the TMRCA of the polymorphism of *P. falciparum clag2* and *8* were estimated to be 1.89 (95% CI, 1.02–3.18) and 0.87 (95% CI, 0.42–1.54) mya, respectively. Although there is still controversy surrounding its accuracy, TMRCA of the extant *P. falciparum* population was estimated to be approximately 0.1–0.2 mya based on the genetic distance in nuclear genome housekeeping genes between *P. falciparum* and *P. reichenowi* ([52], Tanabe, unpublished data). Thus, polymorphism of *clag2* and *8* appears to be generated between the divergence of *P. falciparum* and *P. reichenowi* and TMRCA of the extant *P. falciparum* populations. Early origins of the polymorphism have been suggested for merozoite surface proteins *Pf*MSP-1 and *Pf*MSP-2, for which the origin of the polymorphism was proposed to predate the *P. falciparum–P. reichenowi* divergence (thus termed 'ancient origin'), or TMRCA of the extant *P. falciparum* population, respectively [53,54]. Early origins of the polymorphism older than TMRCA of extant *P. falciparum* populations would suggest that *rhoph1/clag* polymorphisms confer an advantage to the parasite and were positively selected for during the recent evolution of *P. falciparum*.

In summary, four factors appear to affect current *rhoph1/clag* polymorphism; (i) older origin than TMRCA of the extant *P. falciparum* population; (ii) gene conversion and (iii) copy number polymorphism for *rhoph1/clag* on chr 3; and (iv) positive diversifying selection. Multigene families play important roles in many aspects of malaria biology, e.g., responsibility for redundancy of erythrocyte invasion or antigenic variation of parasite-infected erythrocytes. Given the abundance of multigene families in the *P. falciparum* genome [55], combination of the mechanisms described in this study can be a powerful driving force to generate high biologic redundancy for parasite survival.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2007.11.004.

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Short communication

Disruption of the *Plasmodium berghei* 2-Cys peroxiredoxin TPx-1 gene hinders the sporozoite development in the vector mosquito

Kazuhiko Yano[a], Hitoshi Otsuki[b], Meiji Arai[c,1], Kanako Komaki-Yasuda[a,d], Takafumi Tsuboi[e], Motomi Torii[b], Shigeyuki Kano[a], Shin-Ichiro Kawazu[a,d,f,*]

[a] Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan
[b] Department of Molecular Parasitology, Ehime University School of Medicine, Toon, Ehime 791-0295, Japan
[c] Division of Medical Zoology, Department of Infection and Immunity, Jichi Medical University School of Medicine, Shimotsuke, Tochigi 329-0498, Japan
[d] Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, 5, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan
[e] Cell-Free Science and Technology Research Center, Ehime University, Matsuyama, Ehime 790-8577, Japan
[f] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, 2-13 Inada-cho, Obihiro, Hokkaido 080-8555, Japan

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ABSTRACT

To investigate the physiologic role of cytosolic 2-Cys peroxiredoxin of *Plasmodium berghei* (PbTPx-1), we infected the vector mosquito *Anopheles stephensi* with a parasite carrying a targeted knockout of *pbtpx-1* (Prx-KO). The number of Prx-KO midgut oocysts at 14–15 days post-feeding (pf) was comparable to that of the parent strain (WT); however, the numbers of sporozoites that formed in midgut oocysts and accumulated in the salivary gland of Prx-KO-infected mosquitoes by 21 days pf were decreased to 10–20% and 3–10%, respectively, of those values in WT-infected mosquitoes. A higher frequency of DNA strand breaks was detected in Prx-KO oocysts than in WT oocysts. Sporozoites carrying the targeted disruption had reduced infectivity in mice; however, the knockout did not affect the ability of the sporozoite to reach the liver parenchyma and initiate exo-erythrocytic form (EEF) development. TPx-1 may be involved in development during exponentially multiplying stages, such as sporozoites and EEF.

As *Plasmodium* spp. actively proliferate in erythrocytes of their vertebrate hosts, the parasites are subjected to the toxic effects of reactive oxygen species (ROS) through their asexual development [1,2]. In contrast, the parasites fertilize and multiply in the digestive tract (midgut) of the vector mosquito and subsequently mature intracellularly in the salivary grand. In these environments, the parasites are also likely to be subject to oxidative stress [3,4]. Because *Plasmodium* spp. are highly susceptible to such oxidative stress, their antioxidant defenses are considered to play essential roles in survival throughout the lifecycle and thus may be potential targets for malaria chemotherapies [5].

Superoxide dismutase, catalase, glutathione (GSH) peroxidase, and peroxiredoxin (Prx) are the four major cellular antioxidant enzymes in aerobes [6,7]. An interesting feature of antioxidant system of malaria parasites is that the parasites do not possess genes that encode catalase or genuine GSH peroxidase, but they are equipped with a 1-Cys Prx, two typical 2-Cys Prxs, a 1-Cys antioxidant protein (AOP) and a GSH peroxidase-like thioredoxin peroxidase [1,2,8,9]. We recently reported that disruption of the gene encoding cytosolic 2-Cys Prx (PbTPx-1) of the rodent malaria parasite *Plasmodium berghei* did not affect its asexual proliferation in mouse erythrocytes but that the disruption caused a defect in gametocyte development [10]. In the present report, we examined the insect-stage phenotype of parasites carrying a targeted knockout (KO) of the Prx gene (*pbtpx-1*). We also examined the phenotype of Prx-KO sporozoites during the early stage of liver infection.

To investigate the effect of *pbtpx-1* disruption on the mosquito stage of the parasite, *Anopheles stephensi* were fed on BALB/c mice that showed high levels of gametocytemia (Table 1). Prx-WT oocysts developed similarly to those of WT in the midgut, and the final numbers of sporozoites that formed in midgut oocysts and that accumulated in the salivary gland by 21 days post-feeding (pf) were equivalent level to those of WT ($P = 0.4–0.8$ and $P = 0.4–0.5$, respectively). For Prx-KO1-3 populations, the number of midgut oocysts at 14–15 days pf was comparable to those of WT. The normal oocyst formation in Prx-KO suggests that the *pbtpx-1* disruption does not affect the gamete fertilization, ookinete formation or transformation of the ookinetes to oocysts, which requires ookinete invasion of

Abbreviations: DHFR-TS, dihydrofolate reductase-thymidylate synthase; GSH, glutathione; Prx, peroxiredoxin; ROS, reactive oxygen species.

* Corresponding author at: National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, 2-13 Inada-cho, Obihiro, Hokkaido 080-8555, Japan. Tel.: +81 155 495846; fax: +81 155 495643.
E-mail address: skawazu@obihiro.ac.jp (S.-I. Kawazu).
[1] Present address: Department of Immunology and Parasitology, School of Medicine, University of Occupational and Environmental Health, Japan, Yahatanishi-ku, Kitakyusyu 807-8555, Japan.

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Table 1
Development of Prx-KO *Plasmodium berghei* in *Anopheles stephensi*

	Parasites	14–15 days post-feeding (pf)			21 days pf no. of sporozoites/mosquito[a]		
		Total oocyst number	No. of mosquitoes[b]	Matured oocyst (%)	Midgut	Salivary gland	No. of mosquitoes[b]
Experiment 1	WT	1760		32.1	22000 ± 2740	8600 ± 990	
	Prx WT	1920	28	27.2	25200 ± 2850	7600 ± 610	20
	KO1	1540		4.2	2870 ± 120[c]	270 ± 60[c]	
Experiment 2	WT	1330		18.6	50400 ± 510	9950 ± 1700	
	Prx WT	1040	20	17.9	49100 ± 7700	9600 ± 600	30
	KO1	870		3.1	5470 ± 800[c]	330 ± 70[c]	
Experiment 3	WT	1020		14.5	30000 ± 3280	7070 ± 510	
	Prx WT	1460	20	11.9	28910 ± 3240	7920 ± 1180	25
	KO1	1310		3.7	4720 ± 1130[c]	800 ± 160[c]	
Experiment 4	WT	700		19.7	14100 ± 1370	6430 ± 740	
	KO2	940	20	1.2	3070 ± 930[c]	550 ± 150[c]	30
	KO3	1220		1.8	1700 ± 360[c]	270 ± 90[c]	
Experiment 5	WT	1590		17.9	44400 ± 800	13800 ± 530	
	KO4	1020	25	4.1	4280 ± 970[c]	920 ± 460[c]	25
	KO5	760		3.7	4940 ± 420[c]	660 ± 160[c]	

The *P. berghei* ANKA strain was obtained from the Armed Forces Research Institute of Medical Sciences, Thailand. The Prx knockout (Prx-KO) parasite, which carries a targeted disruption of *pbtpx-1* (PlasmoDB, PB000037.01.0), was established by double-crossover homologous recombination with a selectable marker, the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene with a pyrimethamine-resistance mutation [10]. Five Prx-KO populations (Prx-KO1-3 and Prx-KO4-5 were obtained by two independent electroporation experiments), one wild-type parasite population with pyrimethamine resistance (*dhfr-ts/mt* at *dhfr-ts* locus) and intact *pbtpx-1* (Prx-WT) and the parent strain (WT) were used to infect the animals. After parasite infection, the numbers of gametocytes in the peripheral blood were monitored, and *A. stephensi* mosquitoes were fed on five-week-old BALB/c mice (Clea Japan, Japan) when the number of gametocytes reached 20–30 per 1×10^5 erythrocytes. At 14–15 days pf, the mosquitoes were dissected, and oocyst numbers in the midgut were counted [17]. An oocyst filled with needle-shaped sporozoites was counted as a mature oocyst. One week later (21 days pf), the remaining mosquitoes were dissected, and sporozoite numbers in the midgut oocysts, hemolymph and salivary glands were examined [18]. The animal experiments in this study were carried out in compliance with the Guide for Animal Experimentation at either Ehime University School of Medicine or at the International Medical Center of Japan. Differences were evaluated with Student's *t*-test. $P < 0.05$ was considered statistically significant.

[a] Mean ± S.D.

[b] No. of mosquitoes dissected.

[c] The difference between WT and KO population was significant ($P < 0.01$).

epithelial cells and attachment to the basal lamina of the mosquito's midgut [11]. The lower number of oocysts in Prx-KO4 and 5 populations when compared to that of WT may be attributed to the mosquito's blood feeding, which varies between each experiment. *P. berghei* develops asynchronously in the infected mosquitoes, and the midgut contains both young and mature oocysts. In our experiments, in WT-infected mosquitoes, 15–32% of the oocysts were mature oocysts, which contained needle-shaped sporozoites, at 14–15 days pf. However, the percentages of mature oocysts in Prx-KO-infected mosquitoes at the same time points were lower (1–4%) than those of WT-infected mosquitoes. This phenotype was observed in all Prx-KO populations. Electron microscopic observation of the midguts at 14–15 days pf revealed that there was damaged oocyst with irregular sporogonic development (Fig. 1A), supports the idea that sporogony is abnormal in Prx-KO. This finding strongly suggests that the immature oocysts in Prx-KO populations failed to develop further. Consequently, the final numbers of sporozoites that formed in midgut oocysts and that accumulated in the salivary gland by 21 days pf were significantly decreased to 10–20% and 3–10%, respectively, in Prx-KO-infected mosquitoes than in WT-infected mosquitoes (Table 1). The ratio of salivary gland sporozoites to midgut sporozoites was significantly lower ($P < 0.05$) in Prx-KO-infected mosquitoes (0.06–0.21) than in WT-infected mosquitoes (0.20–0.46). Thus, the reduction in the number of salivary gland sporozoites in Prx-KO populations may not be due solely to the reduced number of sporozoites in the midgut oocysts. Because the ratio of hemolymph sporozoites to midgut sporozoites was comparable between Prx-KO-infected mosquitoes and WT-infected mosquitoes (data not shown), *pbtpx-1* disruption may not affect sporozoite entry to the salivary gland, but it may affect parasite survival in salivary gland after the entry. Sporozoite invasion of the salivary gland requires multiple steps [12], including entry into the secretory cavity, where the parasite has direct contact with saliva. NADPH oxidase activity which produces ROS was detected in anopheline salivary homogenate [13]. Disruption of *pbtpx-1* may affect the parasite survival in the mosquito saliva, but the phenotype requires further investigation to clarify this.

To investigate the role of TPx-1 in oocyst maturation, the Prx-KO and WT populations were subjected to *in vitro* oocyst culture (Fig. 1B). Ookinetes derived from WT population could efficiently transform into oocysts, and they conspicuously enlarged in size after the 5th day of culture. The number of oocysts, those that grew more than 6 μm in diameter with normal morphology, was 11146 ± 678 (28 ± 2% of the total number of ookinetes initially added to the culture) on the 7th day of culture. Ookinetes from the Prx-KO population transformed into oocysts like those of the WT population (data not shown). However, their growth after transformation was less pronounced than that observed by WT oocysts. The maximum number of oocysts observed on the 7th day of culture was 6740 ± 898 (17 ± 2% of the total number of ookinetes initially added to the culture), which was significantly lower than that of the WT population. These results indicated that the reduced oocyst development observed in Prx-KO-infected mosquitoes could be reproduced *in vitro*.

To investigate whether the gene disruption can promote DNA damage during the insect stage, oocysts harvested on the 8th day of culture were subjected to comet assay. We found that the comet-tail length of Prx-KO oocysts was significantly longer than that of WT oocysts ($P < 0.05$) (Fig. 1C). Although comet-tail length is considered to reflect breaks in the cellular DNA [14], it is not clear whether the DNA alterations are causal or just a consequence of degrading parasites. However, our findings from immunoelectron microscopic observation also suggested that there was an increase in formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, in Prx-KO oocyst nuclei during early developmental stage (Fig. 1D). Taken together, our data suggest that disruption of *pbtpx-1* induces DNA damage in oocyst nuclei. How malaria parasites deal with the accumulation of oxidative DNA damage during

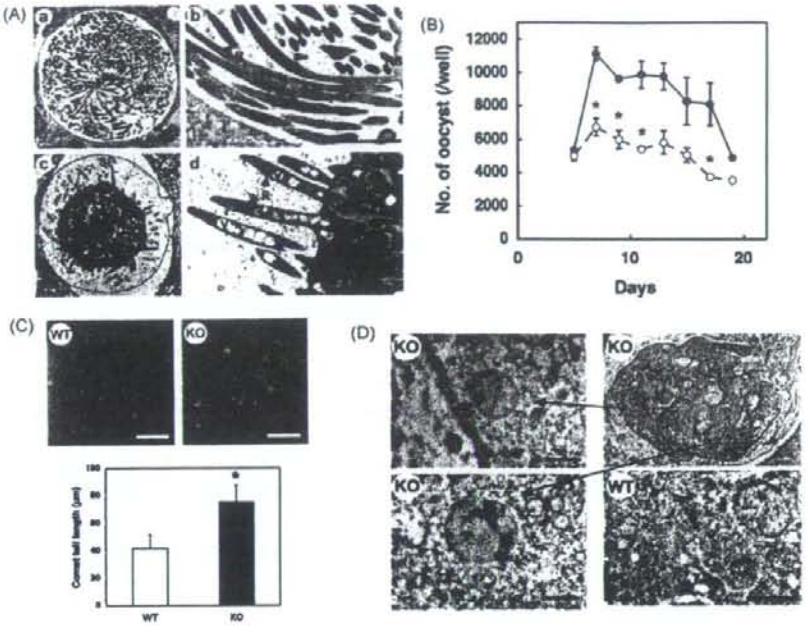

Fig. 1. (A) Electron microscopy image of oocyst and sporozoite in midguts of mosquitos infected with WT (a and b) and Prx-KO (c and d). Electron microscopy was performed as described previously [18] with a transmission electron microscope (Hitachi H-7000, Japan). The midguts were dissected at 15 days pf. Matured oocyst in WT is packed with fully formed sporozoites, and the sporoblast decreases in size to become one residual body (a). The soprozoites show normal structure with a firm nucleus and rhoptries (b). Oocyst in KO contains one large sporoblast, which does not form multiple islands (c). The sporoblast and the sporozoites have deformed nuclei, swollen rhoptries and abnormal cytoplasmic vacuoles (d). Eighty-two oocysts, 39 of which had begun sporozoite budding, from three midgut samples were observed for Prx-KO, and none of the oocysts contained normal sporozoites. Such abnormal sporogony was rarely seen in WT-infected mosquitoes. N, SB, SP, R, RB and V indicate nucleus, sporoblast, sporozoite, rhoptries, residual body and vacuole, respectively. Bars indicate 5 μm. (B) Effect of *pbtpx-1* disruption on oocyst development *in vitro*. Oocyst culture was basically carried out as described previously [19]. Ookinetes (4×10^4) derived from WT (●) and Prx-KO (○) populations were initially added to the culture well. The number of oocysts (those that grew more than 6 μm in diameter with normal morphology) was counted on every other day from 5th to 19th day of culture. Data are mean ± S.D. of the oocyst number in triplicate cultures. *The difference between the WT and KO populations was significant ($P < 0.05$). (C) Effect of *pbtpx-1* disruption on DNA damage in oocysts. DNA strand breaks in oocysts harvested on the 8th day of culture were evaluated by comet assay. Comet assay was performed according to the manufacturer's instructions (CometAssay™, Trevigen, Inc., USA). Quantitative analysis was done by measuring the tail length of the degraded DNA from each oocyst cell spot in WT and Prx-KO (KO) populations with a confocal laser scanning microscope (LSM510, Carl Zeiss, Germany). Bars indicate 100 μm. Data are mean ± S.D. of the comet-tail length (μm) of 75 oocysts from WT (□) and Prx-KO populations (■). *$P < 0.05$. (D) *pbtpx-1* disruption promotes 8-OHdG formation in oocyst nuclei. 8-OHdG formation in nuclei of oocysts at an early developmental stage (young oocyst developed in the mosquito's midgut at 15 days pf) was detected by immunoelectron microscopy with anti-8-OHdG antibody (Japan Institute for the Control of Aging, Japan) and secondary antibody conjugated with gold particle. Immunoelectron microscopy was performed as described previously [18]. The presence of gold particles in the nuclei of Prx-KO oocysts (arrowheads in panels labeled KO) indicates formation of 8-OHdG, which was rarely observed in nuclei of WT oocysts. Arrowheads in panel labeled WT showed traceable deposition of these particles in the cytosol. Bars represent 1 μm.

the lifecycle is of interest. Because oocyst maturation was reduced in Prx-KO *in vitro*, host factors may not contribute to the accumulation of oxidative DNA damage during the mosquito stage. Recent data from microarray analyses of oocysts cultured in *P. berghei* revealed that expression of genes encoding thioredoxin and 2-Cys peroxiredoxin (PbTPx-1) are upregulated during the early stage of oocyst development [15]. This finding suggests that ROS are present during the early stage of oocyst development (young oocyst), when oxygen metabolism in the cell may be elevated. There is a consistent data indicating that genes encoding enzymes in the mitochondrial electron transport chain, which produces ROS, are upregulated in young oocysts [15].

To evaluate infectivity of Prx-KO populations in mice, salivary-gland sporozoites were injected intravenously into BALB/c mice, and the infection rate and the period required for the parasite to develop 0.5% parasitemia of erythrocytic stage (pre-patent period) were compared to those of WT. Parasitemia of the animals was monitored every 12 h. After inoculation of 100, 1000 and 10000 sporozoites from Prx-KO2, Prx-KO3 and WT populations, all animals developed erythrocytic-stage infection. Therefore, the infectivity of the Prx-KO population was assessed based on pre-patent period (**Supplementary Table 2**). The pre-patent period recorded for WT infections increased according to decreasing numbers of sporozoites in the inoculum, the mean pre-patent period for animals ($n = 5$) inoculated with 10000, 1000, and 100 sporozoites were 4.6, 5.3 and 6.2 days, respectively. The mean pre-patent period for animals ($n = 5$) inoculated with 1000 Prx-KO sporozoites were 6.1 (KO2) and 6.3 (KO3) days, which was equivalent to that recorded in animals inoculated with 100 WT sporozoites. These data indicate that the infectivity of Prx-KO populations in mice was reduced to 1/10 of WT.

To investigate the influence of Prx-KO on sporozoite invasion to the liver parenchyma, parasite burden in the liver shortly after the sporozoite inoculation, which represents the number of sporozoites that could cross the sinusoidal cell layer, was compared between WT, Prx-WT, and Prx-KO1 by TaqMan® fluorescent quantitative RT-PCR. For this purpose, mice were inoculated intravenously with 2000 sporozoites, and their livers were perfused with PBS 1 h after the inoculation and then removed. The parasite burden in the liver sample, which represents number of sporozoites in Kupffer cells, the space of Disse, and hepatocytes, was assessed as the ratio of the amount of parasite 18S rRNA to the amount

of mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Supplementary Table 3). The parasite rRNA levels were measured with the copy number-based standard curve ($R > 0.99$), and they were at least 100000-fold less than the mouse GAPDH mRNA levels. Parasite 18S rRNA was detected in all liver samples examined, except for the sample from the animal inoculated with formalin-inactivated sporozoites (data not shown). There was no significant difference in the liver parasite burden, which was quantified for C-type (sporozoite-type) 18S rRNA, between groups of mice infected with WT ($5.40 \pm 2.55 \times 10^{-5}$, $n = 4$) and Prx-WT ($5.03 \pm 1.22 \times 10^{-5}$, $n = 5$) ($P = 0.89$) and with WT and Prx-KO1 ($4.81 \pm 1.98 \times 10^{-5}$, $n = 6$) ($P = 0.86$). There was also no significant difference in the liver parasite burden, which was quantified for A-type (asexual-type) 18S rRNA, between groups of mice infected with WT ($2.24 \pm 0.97 \times 10^{-5}$, $n = 4$) and Prx-WT ($1.58 \pm 0.44 \times 10^{-5}$, $n = 5$) ($P = 0.52$) and with WT and Prx-KO1 ($1.86 \pm 0.85 \times 10^{-5}$, $n = 6$) ($P = 0.78$). The results of our animal infection experiments suggested that the Prx-KO population requires a longer pre-patent period to appear in mouse erythrocytes than the WT population. This finding indicates that Prx-KO has a defect in liver-stage infection because this population can multiply in erythrocytes with efficiency similar to that of WT [10]. However, the results of quantitative RT-PCR experiments, which targeted the parasite C-type 18S rRNA, suggested that Prx-KO does not affect sporozoite invasion into the mouse liver parenchyma. Zhu et al. [16] suggested that invasion of liver cells by sporozoites and transformation to the exo-erythrocytic form (EEF) can trigger the ribosome switch. In the present study, the ribosome switch from C to A was detected in all liver samples examined (data not shown). However, there was also no significant difference in the liver parasite burden, which was quantified for A-type 18S rRNA, between groups of mice infected with WT and Prx-KO (Supplementary Table 3). These results suggested that Prx-KO sporozoites could invade liver parenchyma and initiate EEF development with similar efficiency to WT during the early stage of liver infection. This finding suggests that the Prx-KO population can produce a few but intact sporozoites with normal activity to initiate the liver stage development. If this is the case, it is presumed that Prx-KO parasites have a defect in EEF development after the early stage of liver infection. *pbtpx-1* may be involved in development during exponentially multiplying parasites stages, such as sporozoites and EEF. The 2nd generation Prx-KO parasites have the same defect in gametocytes production as that of the 1st generation [10] (data not shown). The activity of its sporozoite production should be confirmed.

Although the specific mechanism by which disruption of *pbtpx-1* leads to DNA damage and reduces maturation of oocysts remains to be elucidated, the present findings suggest that the parasite antioxidant system contributes to sporozoite development during the insect stage. Further studies to clarify the role of TPx-1 in sporozoite development will provide further insights into the contribution of this antioxidant protein to the insect-stage development of malaria parasites and may provide novel transmission-blocking strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2008.03.002.

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Evolution and phylogeny of the heterogeneous cytosolic SSU rRNA genes in the genus *Plasmodium* ☆

Yuriko Nishimoto [a,b], Nobuko Arisue [c], Satoru Kawai [d], Ananias A. Escalante [e], Toshihiro Horii [c], Kazuyuki Tanabe [f], Tetsuo Hashimoto [a,g,*]

[a] *The Institute of Statistical Mathematics, Minami-Azabu, Minato-ku, Tokyo 106-8569, Japan*
[b] *Department of Biosystems Science, Graduate University for Advanced Studies (Sokendai), Hayama, Kanagawa 240-0193, Japan*
[c] *Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan*
[d] *Department of Tropical Medicine & Parasitology, Dokkyo Medical University School of Medicine, Mibu, Shimotsuga, Tochigi 321-0293, Japan*
[e] *School of Life Sciences, Arizona State University, P.O. Box 874501, Tempe, AZ 85287, USA*
[f] *Laboratory of Malariology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan*
[g] *The Institute of Biological Sciences, University of Tsukuba, Tsukuba 305-8572, Japan*

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Abstract

Unlike other eukaryotes, malaria parasites in the genus *Plasmodium* have structurally and functionally different paralogous copies of the cytosolic (cyto-) SSU rRNA (18S rRNA) gene that are expressed at different developmental stages. In *P. falciparum*, *P. vivax*, and *P. berghei*, A-type cyto-SSU rRNA is expressed in asexual stage, while S-type in sporozoite stage. A third type (O-type) has been described in *P. vivax*. It is expressed only in oocyst stage in the mosquito. Recently, it has been shown that the maintenance of heterogeneous cyto-SSU rRNAs in *Plasmodium* can be modeled as a birth-and-death process under strong purifying selection [Rooney, A.P., 2004. Mechanisms underlying the evolution and maintenance of functionally heterogeneous 18S rRNA genes in Apicomplexans. Mol. Biol. Evol. 21, 1704–1711]. In this study, we performed detailed phylogenetic analyses of *Plasmodium* cyto-SSU rRNAs with special emphasis on the evolution of multi-copy genes in simian *Plasmodium* species. We sequenced paralogous copies of the cyto-SSU rRNA genes from an African simian *Plasmodium* species, *P. gonderi*, and Asian simian *Plasmodium* species, *P. fragile*, *P. coatneyi*, *P. inui*, *P. hylobati*, *P. fieldi*, *P. simiovale*, and *P. cynomolgi*. Interestingly, all Asian simian *Plasmodium* species have a single S-type-like gene and several A-type-like genes. Alignment analysis demonstrated for the first time that an approximately 50-residue insertion in the V7 variable region near the stem 43 is shared exclusively by the S-type-like sequences of the Asian simian *Plasmodium* species and the S- and O-type sequences of *P. vivax*. We comprehensively analyzed all cyto-SSU rRNA sequences of the genus *Plasmodium* currently available in the database. Phylogenetic analyses of all publicly available cyto-SSU rRNA sequences for the genus *Plasmodium* clearly demonstrated that gene duplication events giving rise to A- and S-type-like sequences took place independently at least three times in the *Plasmodium* evolution, supporting the hypothesis that these genes evolve according to a birth-and-death model.

Keywords: *Plasmodium*; SSU rRNA; Molecular phylogeny; Simian malaria parasites; Multi-copy genes

1. Introduction

The genus *Plasmodium* comprises unicellular malaria parasites that infect various vertebrate hosts including primates, rodents, reptiles, and birds. Five *Plasmodium* species, *P. falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi*, and *P. ovale*, are known as human parasites. To better understand the evolution of the genus *Plasmodium* and to iden-

☆ Sequences: The sequences reported in this paper have been submitted to GenBank, EMBL, and DDBJ databases under Accession Nos., AB265789–AB265791 and AB287269–AB287290.

* Corresponding author. Address: The Institute of Biological Sciences, University of Tsukuba, Tsukuba 305-8572, Japan. Fax: +81 29 853 6614.
E-mail address: hashi@biol.tsukuba.ac.jp (T. Hashimoto).

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