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Evidence for negative selection on the gene encoding rho-try - associated protein 1 (RAP-1) in *Plasmodium* spp.

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Abstract

Assessing how natural selection, negative or positive, operates on genes with low polymorphism is challenging. We investigated the genetic diversity of orthologous genes encoding the rho-try - associated protein 1 (RAP-1), a low polymorphic protein of malarial parasites that is involved in erythrocyte invasion. We applied evolutionary genetic methods to study the polymorphism in RAP-1 from *Plasmodium falciparum* (n= 32) and *P. vivax* (n= 6), the two parasites responsible for most human malaria morbidity and mortality, as well as RAP-1 orthologous in closely related malarial species found in non-human primates (NHPs). Overall, genes encoding RAP-1 are highly conserved in all *Plasmodium* spp. included in this investigation. We found no evidence for natural selection, positive or negative, acting on the gene encoding RAP-1 in *P. falciparum* or *P. vivax*. However, we found evidence that the orthologous genes in non-human primate parasites (*P. cynomolgi*, *P. inui*, and *P. knowlesi*) are under purifying (negative) selection. We discuss the importance of considering negative selection while studying genes encoding proteins with low polymorphism and how selective pressures may differ among orthologous genes in closely related malarial parasites species.

Keywords

Genetic diversity; Malaria; Merozoite; *Plasmodium*; Rho-try; RAP-1; Positive Selection; Negative selection

1. Introduction

Worldwide, malaria is one of the most important causes of disease and death in humans, the causal agents are four species of parasitic protozoa belonging to the genus *Plasmodium*. Despite considerable biological differences among human malarial parasites, all of them invade

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erythrocytes. This process involves the orchestrated action of a variety of proteins, several of them considered prime targets for anti-malarial vaccines (Richards and Beeson, 2009). Given its importance, highly polymorphic proteins involved in this process have been the subject of several evolutionary genetic studies. The overall approach has been to determine whether the polymorphism observed at a specific gene is subject to positive-balancing selection, and if so, it is considered to be involved in the onset of natural acquired immunity (Escalante et al., 2004; Conway 2007; Tetteh et al., 2009). Less attention, however, has been given to genes encoding proteins that exhibit low polymorphism.

Among these low polymorphic proteins, there are several associated with pear-shaped membrane-bound vesicles located at the apical end of the merozoite called rhoptries (Preiser et al., 2000), such as the rhoptry-associated protein 1 (RAP-1). RAP-1 in *P. falciparum* (*Pf*RAP-1) shows minimal genetic polymorphism with few amino acid substitutions identified (Ridley et al., 1990a; Howard and Peterson, 1996; Escalante et al., 1998b; Tetteh et al., 2009). Thus, given that the limited polymorphism observed is not under balancing selection, RAP-1 seems to be immunologically irrelevant. However, several lines of evidence indicate that RAP-1 is recognized by the host immune system. Monoclonal antibodies directed against rhoptries-associated proteins *Pf*RAP-1 and *Pf*RAP-2 have inhibited the erythrocyte invasion *in vitro* (Schofield et al., 1986; Harnyuttanakorn et al., 1992; Howard et al., 1998) and immunizations of *Saimiri sciureus* and *S. boliviensis* monkeys with the *Pf*RAP-1 and *Pf*RAP-2 complex demonstrated partial protection against *P. falciparum* (Perrin et al., 1985; Ridley et al., 1990b; Collins et al., 2000). In addition, naturally exposed individuals have shown antibodies against *Pf*RAP-1 conserved linear epitopes and recombinant proteins (Jakobsen et al., 1993; 1997; Stowers et al., 1997; Fonjongo et al., 1998; Moreno et al., 2001; Curtidor et al., 2004). Yet, unlike surface antigens expressed in the merozoite (the parasite stage that invades the erythrocyte), RAP-1 immunogenicity does not translate into the maintenance of observable polymorphism. It could be possible that the dynamics of anti-RAP-1 immunity is more complex than previously thought.

Recent investigations have shown that anti-*Pf*RAP-1 immune responses (*falciparum* malaria) may be associated with clinical manifestations of disease such as anemia, a major cause of sickness and death among children with *P. falciparum* malaria in sub-Saharan Africa (Sterkers et al., 2007; Awah et al., 2009) and also one of the clinical manifestations of severe malaria by *P. vivax*. Specifically, it has been reported that non-protective levels of immunity against *Pf*RAP-1 tagged erythrocytes may trigger their destruction (Sterkers et al., 2007; Awah et al., 2009). If partial anti-RAP-1 immunity could actually be detrimental to the host, then polymorphism could be selected against, leading to low variation at the protein level. Unfortunately, assessing how natural selection, negative or positive, operates on genes with low polymorphism is extremely difficult. As an example, alternatively to negative selection, low polymorphism could be simply the result of demographic processes such as bottle-necks, a phenomenon expected in malarial parasites due to their origins, a result of host switches that lead to recent population expansions (Escalante et al., 2005; Krif et al., 2010). Consequently, there is no evidence so far indicating that negative natural selection could be acting on the limited polymorphism observed in *Pf*RAP-1 (Escalante et al., 1998; Tetteh et al., 2009) or any other ortholog genes in the genus *Plasmodium*.

We explored the mechanisms that shaped the genetic diversity observed in the orthologous genes encoding RAP-1 in several primate malarias, including the two major human malarial parasites: *P. falciparum* and *P. vivax*. While no compelling evidence was found for natural selection, negative or positive, acting on RAP-1 in the two human parasites using these samples, all of the RAP-1 orthologs from non-human primate malarial parasites show patterns consistent with negative selection. Thus far, this study provides the first evidence indicating that negative selection may be acting on a gene encoding a protein involved in the erythrocyte invasion in

any *Plasmodium* species. These results suggest that some genes with low diversity may be indeed under negative selection and such a possibility should be carefully explored.

2. Materials and methods

2.1 Parasite strains

All strains and field isolates used in our study were provided by the Centers for Disease Control and Prevention. We have taken an approach to obtain a limited set of sequences from different geographic locations, which increases the probability of sampling the most divergent alleles in order to infer the history and processes involved in the evolution of the observed polymorphism (Kliman and Hey, 1993). In the case of *P. falciparum*, we sequenced the RAP-1 gene in 28 field isolates: nine from India (Delhi collected in 2000, see Escalante et al., 2001), seven from Thailand (hospital based samples from Bangkok, year 2000, see Escalante et al., 2001), four from Kenya (Asembo Bay, 1996–1997), three from Cameroon (Yuonde, Cameroon, 2001), four from Venezuela (Tumeremo, Bolivar State, 2002), and the strain Honduras I from Central America. In addition, we included in our investigation four published sequences of the PfRAP-1 under the following accession numbers: strain FVO from Vietnam (AF205284), FCC1/HN from China (AF206631), FC27 (U20985), strain K1 (M32853), and the RAP-1 from *P. reichenowi* (U20986) in order to compare the RAP-1 sequences that have been reported.

In the case of *P. vivax*, in addition to the Salvador I strain available in the gene bank (XM_001616799), we sequenced RAP-1 in five laboratory isolates (Chesson from New Guinea, Indonesia I, Mauritania I, Sumatra I, and Vietnam II). These sequences were compared with four sequences from different isolates of *P. cynomolgi* (strains Berok, Gombok, PT1, and PT2 all from Malaysia, see Coatney et al., 1971) and five sequences from isolates of *P. inui* (Leaf Monkey II, Leucosphyrus, OS from Malaysia; Taiwan I and II from Taiwan, see Coatney et al., 1971). We also included in our studies two strains of *P. fieldi* (ABI and N-3 from Malaysia), one sequence from *P. simiovale*, one sequence from *P. coatneyi*, one sequence from *P. hylobati* (a gibbon parasite), and five strains of *P. knowlesi* (H, Hackeri, Malayan strains from Malaysia; the Nuri strain from India, and the Philippine strain; see Coatney et al., 1971). The sequences reported in this investigation are deposited in the GenBank under the accession numbers GQ281604 to GQ281655. In order to estimate the RAP-1 phylogeny, we also included the orthologs from two rodent malarial parasites, *P. yoelii* (PY00622) and *P. chabaudi* (PCAS_103190).

2.2 PCR amplification, cloning, and sequencing

Amplification reactions were carried out in a 50 µl volume and included 20 ng/µl of total genomic DNA, 2.5 mM MgCl₂, 1× PCR buffer, 1.25 mM of each deoxynucleoside triphosphate, 0.4 mM of each primer, and 0.03U/µM AmpliTaq® Gold polymerase (Applied Biosystems, Roche-USA). Specific primers were designed in order to amplify the RAP-1 gene by polymerase chain reaction (PCR) in all isolates from *Plasmodium* spp. The primers forward 5' TAT AAT GAG TTT CTA TTT GGG TAG 3' and reverse 5' CCT TCA AGA GAT TAG ATT AAG AAT A 3' were used to amplify the complete gene in *P. falciparum* isolates. The PCR conditions for amplifying were: a partial denaturation at 94 °C for 1 min and 30 cycles of 1 min at 94 °C, 1 min at 52 °C, and 3 min extension at 72 °C, and a final extension of 10 min was added in the last cycle. In the specific cases of *P. vivax*, *P. cynomolgi*, *P. inui*, *P. fieldi*, *P. fragile*, *P. simiovale*, *P. knowlesi*, and *P. coatneyi*, we used the primers forward 5' ATG ATA ACK TRC GYA AGT TC 3' and reverse 5' CCA ATC KCT TGT AGA GCA AAT 3' to amplify their RAP-1 genes. We also obtained partial sequences of *P. hylobati* (2235 bp) and *P. fragile* (2223 bp) using primers forward 5' GCA CTS TAC CAA AAT GTT TCC 3' and reverse 5' ATA ATC ATY RCG CAT TTC C 3'. The PCR conditions were: a partial

denaturation at 94 °C for 3 min and 35 cycles with 1 min at 94 °C, 1 min at 50–56 °C, and 2 min extension at 72 °C, and a final extension of 10 min was added in the last cycle.

The amplified product was purified, cloned using the pGEM®-T Easy Vector Systems I from Promega (USA), and sequenced. Both strands were sequenced from at least two clones. Two independent alignments were made using ClustalX Version 1.83 with manual editing: one alignment included all *Plasmodium* species, and another for *P. vivax* and related species found in non-human primates from Southeast Asia.

2.3 Evolutionary genetic analyses

We performed phylogenetic analyses on both protein and nucleotide sequences on two alignments: one with all known RAP-1 orthologous from malarial parasites species found in mammals and, separately, *P. vivax* and closely related species found in non-human primates from Southeast Asia. The rationale was that the distances among the major mammalian *Plasmodium* species may reduce the resolution of the phylogeny within the group of species that includes *P. vivax*, given problems aligning highly divergent proteins. The gene phylogenies were first determined by using the Neighbor-Joining method (Saitou and Nei, 1987) with the Tamura-Nei model in the case of nucleotides and the Dayhoff (PAM) models for proteins as implemented in MEGA. The reliability of the nodes in the NJ trees was assessed by bootstrap method with 1000 pseudo-replications (Nei and Kumar, 2000). Additionally, Bayesian phylogenetic analyses were performed as implemented in MrBayes (Ronquist and Huelsenbeck, 2003). In the case of nucleotide sequences we used a General Time Reversible (GTR) + I + Γ_4 ; we used the GTR model for estimating phylogenies from protein sequences. In all Bayesian analyses, proteins or DNA, we used 2,000,000 Markov Chain Monte Carlo (MCMC) steps and discarded the first 250,000 (25%) as a burn-in. Sampling was performed every 100 generations. Mixing of the chains and convergence was properly checked after runs.

The genetic polymorphism within each *Plasmodium* species was estimated using the parameter π , which estimates the average number of substitutions between any two sequences. In addition, the average number of synonymous (Ds) and non-synonymous (Dn) substitutions between a pair of sequences was investigated to explore the effect of natural selection. The average number of Ds and Dn was estimated using Nei and Gojobori's method (Nei and Kumar, 1986) with the Jukes and Cantor correction as implemented in the MEGA program (Kumar et al., 2001). We estimated the difference between Ds and Dn, and the standard deviation was calculated using bootstrap with 1000 pseudo-replications for Ds and Dn, as well as a two tail Z-test on the difference between Ds and Dn (Nei and Kumar, 2000). The null hypothesis is that $Ds=Dn$; thus, we assumed as null hypothesis that the observed polymorphism was neutral.

The assumption of neutrality was also tested in all *Plasmodium* species by using the McDonald and Kreitman test (McDonald and Kreitman, 1991), which compares the intra- and inter-specific number of synonymous and nonsynonymous sites. Significance was assessed by using a Fishers exact test for the 2×2 contingency table as implemented in the programs DnaSP Version 4.0 (Rozas et al., 2003). In this analysis, we compared the following species: *P. falciparum* with *P. reichenowi*; *P. vivax* with *P. cynomolgi*, *P. inui*, *P. fieldi*, and *P. knowlesi*; *P. cynomolgi* with *P. inui*, *P. fieldi*, and *P. knowlesi*; *P. inui* with *P. fieldi* and *P. knowlesi*; and *P. fieldi* with *P. knowlesi*. While other neutrality tests have been widely used in *Plasmodium* spp. studies using laboratory isolates (e.g. Tetteh et al., 2009), their *ad hoc* use for seeking evidence for positive selection (e.g. Putaporntip et al., 2006) was not considered necessary in the context of this investigation.

3. Results

3.1 Description of RAP-1 orthologs in *Plasmodium* spp.

Figure 1 depicts a description of the RAP-1 protein on rodent and primate malarial parasites. The RAP-1 orthologs in malarial parasites from macaques, like previously reported for *PvRAP-1*, are encoded by two exons while their homologues in *P. falciparum* and the rodent species are encoded by a single exon (Figure 1). We identified the 22 residue signal peptide in all the species of *Plasmodium* studied; however, we could not identify the serine-rich region, considered characteristic of RAP-1, in the rodent parasites orthologs. As a result, the two rodent parasites have a smaller protein, 608–663 aa compared with more than 700 aa in the primate malarias. Two additional gaps (10–20 residues) are also indicated in the alignment (Figure 1).

The serine-rich region, between residues 59 and 183, and the eight cysteine residues distributed between amino acids 353 and 616 are conserved between *P. falciparum* and *P. reichenowi*. The octapeptide repeat sequence p82 (KSSSPSSTKSSSPSNV, residues 143–158), a potentially antigenic region (Ridley et al., 1990a; Howard et al., 1993; Jakobsen et al., 1993), is highly conserved among the 28 alleles of *PfRAP-1* and *P. reichenowi*. Indeed, we found only a single residue change (Ser versus Ile or Val) in nine of the 28 alleles included in this sample. The epitope TLTPLEELYPT (residues 201–211) is conserved in all the *PfRAP-1* alleles and in *P. reichenowi*. The only exception is one allele from Cameroon where we found (TLTPLEQLYPT). This epitope is in part of the protein that has been identified as erythrocyte specific-binding region (Curtidor et al., 2004). The other erythrocyte specific-binding region previously identified (Curtidor et al., 2004) between residues 461 and 540 has a similar pattern.

In the case of *P. vivax* and the closest related species of non-human primate parasites, all the alleles have a serine-rich region between residues 47 and 272, one cysteine residue in the signal peptide, and six cysteine residues conserved and distributed between amino acids 420 and 683. Although *PvRAP-1* does not have the KSSSPS repeat observed in *PfRAP-1*, we could identify a KSGS repeat in residue 233, using the sequence of Salvador I as reference (Figure 2). This repeat varies in number between two and four of the six alleles. However, this motif appears only once in *P. inui*, *P. hylobati*, and *P. knowlesi*. Additionally, RAP-1 of *P. knowlesi* has a different repeat (KSGGS) close to KSGS repeat (Figure 2). Both repeats are located within the RAP-1 serine-rich region. In contrast, *P. cynomolgi* does not have a repetitive region.

3.2 Diversity in the RAP-1 gene and phylogenetic analyses

Tables 1 and 2 show the genetic variation found in RAP-1 in *Plasmodium* spp. The *PfRAP-1* alleles are 2346bp long (782 aa, Table 1), except for one allele from Kenya that has an insertion in residue 385 of the protein. In the case of the six *PvRAP-1* alleles, the gene length varied between 2422 and 2458bp (740–748 aa, Table 1). The same pattern was found in the other species of non-human primate parasites included in this investigation (Table 1). The length of the *PvRAP-1* intron also varied between 187 and 212bp (Table 1).

The genetic diversity of the RAP-1 gene of *P. falciparum* is twice that observed in *P. vivax* (π of 0.0041 versus 0.0026). The *PfRAP-1* polymorphism reported in this investigation is higher than previously reported ($\pi = 0.0020$, $N=3$ in Escalante et al., 1998, and the same value in 17 laboratory isolates Tetteh et al., 2009). An obvious explanation for this difference is that this investigation used a more comprehensive sampling that included several field isolates from different malaria endemic areas. More important than the overall diversity is that the reported epitopes are indeed conserved among all field isolates. Like in *P. falciparum*, orthologs in *P. vivax* and non-human primate malarias also show low genetic diversity when compared to proteins expressed at the surface of the merozoite. It is worth noting that when compared to RAP-1 orthologs from non-human primate malarias, the genetic diversity observed in

PvRAP-1 was an order of magnitude lower. The RAP-1 genes in *P. cynomolgi* and *P. inui* have a higher diversity than *P. vivax* (π of 0.0395 and 0.0222 versus 0.0026 respectively, Table 1). Overall, the RAP-1 orthologs of the two macaque malarial parasites, *P. cynomolgi* and *P. inui*, are twice as polymorphic than *P. vivax*. The same pattern was found when we compared the genetic diversity of the CDS, exons 1 and 2 separately (Table 2).

As a first approximation for assessing whether the observed polymorphism was under natural selection, we explored the diversity in RAP-1 by comparing the number of synonymous (Ds) and non-synonymous (Dn) substitutions in each species (Table 2). There were no significant differences in the synonymous and non-synonymous substitution rates among the alleles of *P. falciparum* and *P. vivax* when we used the Z-test (Table 2, Nei and Kumar, 2000). However, the RAP-1 orthologs in macaque parasites showed a significant excess of synonymous versus non-synonymous polymorphism (Table 2). Such excess of synonymous substitutions is indicative of negative (purifying) selection.

The phylogeny of the RAP-1 gene is depicted in Figure 3 using protein sequences. As expected, *P. vivax* and closely related non-human malarial parasites found in Southeast Asia conform a monophyletic group (Escalante et al., 1998a;2005). Overall, the RAP-1 phylogeny is consistent with previous phylogenies (Escalante et al., 1998a;2005); however, the clade of *P. inui*-*P. hylobati* appears as sister taxa of *P. vivax* rather than *P. cynomolgi*. Phylogenetic analyses using nucleotide sequences in all *Plasmodium* species as well as one focusing only on *P. vivax* and non-human malarial parasites from Southeast Asia yield similar results (not included). In all these analyses, *P. cynomolgi*, *P. simiovale*, and *P. fieldi* RAP-1 orthologs are closely related to *P. vivax*; however, there is not support that these species form a clade that is a sister group of *P. vivax*. Such inconsistencies with other phylogenetic studies are expected when we study single gene phylogenies. *P. coatneyi* appears in another clade sharing a recent common ancestor with *P. knowlesi*. Given that both *P. cynomolgi* and *P. inui* share recent common ancestors with *P. vivax*, they are useful for evolutionary genetic analyses using comparative approaches.

The assumption of neutrality was tested in all *Plasmodium* RAP-1 orthologs by using the McDonald and Kreitman test (McDonald and Kreitman, 1991). When we compared the genetic diversity of RAP-1 in *P. falciparum* (PfRAP-1) with *P. reichenowi*, we found no evidence indicating that natural selection is acting on PfRAP-1. However, there was an overall excess of non-synonymous over synonymous substitutions in the *P. vivax* polymorphism when compared with *P. cynomolgi*, *P. inui*, and *P. fieldi* ($p < 0.05$ using a Fisher's exact test, Table 3). Such patterns usually are interpreted as evidence for positive selection.

4. Discussion

A first conclusion derived from our results is that, overall, RAP-1 orthologs are less polymorphic than antigens expressed on the surface of the merozoite across *Plasmodium* spp., a pattern that has been well documented in *P. falciparum* (Volkman et al., 2007). Although there are limited studies on the genetic polymorphism of genes encoding merozoite surface antigens in these non-human primate parasites (Pacheco et al., 2007; Tanabe et al., 2007), the fact that all the malarial parasites included in this investigation shared their most recent common ancestor in a distant past (Escalante et al., 1998a; Perkins and Schall, 2002) allows us to infer that this pattern has prevailed during the evolutionary history of primate malarias.

While antigenic and polymorphic proteins are usually under balancing selection (Escalante et al., 1998b, 2004; Conway and Polley, 2002; Tetteh et al., 2009), low polymorphic genes may undergo processes that need to be investigated. Low polymorphism could be easily attributed to the demographic histories of human malarial parasites, where founder effects and populations expansions appear to be common after they originated from non-human hosts (Joy

et al., 2003; Cornejo and Escalante, 2006; Escalante et al., 2005; Krief et al 2010). However, other possibilities are difficult to explore in such genes with low polymorphism. Limited amino-acid polymorphism could be, for example, the result of negative (purifying) selection. In the case of genes involved in the erythrocyte invasion, such findings are important because they could indicate functional constraints or that only few variants can evade the host immunity. Thus, if a protective immune response can be elicited by a vaccine construct against those few dominant alleles, such proteins could be excellent targets. An alternative that also will generate patterns consistent with negative selection is that those polymorphisms are indeed negatively selected because variants are detrimental to the host. Partial non-protective immunity, as has been reported in *PfRAP-1* (Sterkers et al., 2007; Awah et al., 2009), could enhance disease severity contributing in part to a pattern consistent with negative selection. Differentiating among all these scenarios requires several lines of evidence.

We could not find evidence consistent with selection acting on the polymorphism of *PfRAP-1* and *PvRAP-1* (negative or positive). However, orthologous genes encoding RAP-1 in non-human primate malarias closely related to *P. vivax*, such as *P. cynomolgi*, *P. inui*, *P. fieldi*, and *P. knowlesi* indicate that RAP-1 orthologs in these non-human primate malarial parasites are under negative or purifying selection. Although there is no information regarding the immunologic role played by RAP-1 in non-human primate malarial parasites, this is the first time that evidence of negative selection has been reported in a putative malaria antigen, especially one with low polymorphism. Whether this is a process taking place in *P. vivax* or *P. falciparum* exceeds the power of this study. However, if non-human primate malarias are a good proxy to the biology of human malarias and their pathogenesis (Davison et al., 2005; Dutta et al., 2005), our observations require further investigations. It may be possible that we are failing to detect such a pattern in human malarias simply because, given the low polymorphism, it is hard to detect evidence of negative selection because it is masked by complex demographic processes. Alternatively, this could be simply a phenomenon restricted to macaque malarias. Regardless of how these results could be generalized in terms of RAP-1, this observation may also have practical consequences for malaria comparative genomics based on few genomes that ignore the polymorphism within species (e.g Carlton et al., 2008; Tetteh et al., 2009). As an example, when we compared *P. vivax* with *P. cynomolgi*, *P. inui*, and *P. fieldi* samples, these analyses led to significant departure from neutrality using the MK test. In all these comparisons, the significance of the MK test was explained by an excess of amino acid replacements in the polymorphism of *PvRAP-1*. While in other studies on *Plasmodium* antigens, such results were usually interpreted as evidence of positive (balancing) selection, in this particular case, such a conclusion is incorrect because the orthologous genes in non-human primate malarias are under negative (or purifying) selection. Thus, the MK test very likely indicated a pattern compatible with relaxation of negative selection due to differences between the human and the non-human primate immune systems or other functional differences (Hughes, 2007). Alternatively, such a pattern could be the result of the bottleneck that took place when *P. vivax* originated as a result of a host switch from a non-human primate host (Escalante et al., 2005) that allows the fixation of semi-deleterious mutations (e.g. Parsch et al., 2009). While such patterns affecting the MK test have been observed elsewhere (Hughes 2007; Parsch et al., 2009), they are usually ignored in comparative studies of malarial parasites (and other pathogenic organisms) simply because "positive selection by the host immune system" is commonly assumed. In this specific case, our results in *P. vivax* and related species are a reminder that signatures of balancing selection inferred solely on differences of non-synonymous versus synonymous substitutions against one reference genome could be misleading if we ignore the polymorphism of the reference species. We cannot explore these processes in *P. falciparum* simply because there is only one isolate of *P. reichenowi*, so we have no information about the polymorphism of the chimpanzee parasite.

In summary, RAP-1 orthologs are less polymorphic than genes encoding antigens expressed on the surface of the merozoite in *Plasmodium* spp. We found evidence for negative natural selection acting on the gene encoding RAP-1 in non-human primate malarias. This observation suggests that negative selection could be one process behind the low genetic polymorphism in a protein that otherwise elicits an immune response. However, no evidence of negative selection was found in the two human malarial parasites. Overall, our capacity of properly interpreting evolutionary comparative approaches increases if the polymorphism in the focal species, in this case human malarial parasites, can be contrasted with the polymorphism of a closely related species.

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Fig. 1. Diagram of the protein alignment for the RAP-1 in *Plasmodium spp*; the length of the protein is indicated for each species. The putative signal peptide sequences, the serine-rich regions, and the cysteines are shown. The two exons are indicated for *P. vivax* and related non-human primate parasites. Gaps in the protein alignment bigger than 10 residues are also reported. The serine-rich region is not found in rodent malarial parasites.

Pvi Salvador I	KETGS	>	<	KSSASA	KSGS	KSGS	----	----	KYGGSS	YSDYSAYD
Pvi Indonesia I	KETGS	>	<	KSSASA	KSGS	KSGS	KSGS	----	KYGGSS	YSDYSAYD
Pvi Sumatra	KETGS	>	<	KSSASA	KSGS	KSGS	KSGS	KSGS	KYGGSS	YSDYSAYD
Pvi Chesson	KETGS	>	<	KSSASA	KSGS	KSGS	KSGS	KSGS	KYGGSS	YSDYSAYD
Pvi Vietnam II	KETGS	>	<	KSSASA	KSGS	KSGS	----	----	KYGGSS	YSDYSAYD
Pvi Mauritania	KETGS	>	<	KSSASA	KSGS	KSGS	----	----	KYGGSS	YSDYSAYD
Pin Taiwan II	KEMDS	>	<	KSRYS	KSGS	-----	----	----	KSRGSS	YSDYSAYD
Pin Taiwan I	KEMDS	>	<	KSRYS	KSGS	-----	----	----	KSRGSS	YSDYSTYD
Pin Leucosphyrus	KEMDS	>	<	NSRYS	KSGS	-----	----	----	KSGGSS	YSGYSAYD
Pin Leaf Monkey II	KEMDS	>	<	NSRDS	KSGS	-----	----	----	KSAGSS	YSDYSAYD
Pin OS	KEMGN	>	<	NSRDS	KSGP	-----	----	----	KSGGSL	YSDYSAYD
Phylobati	KDMDT	>	<	KSKHS	KSGS	-----	----	----	KSGESS	YSDYSAYG
Psimiovale	KETGS	>	<	KSSGSG	KSGT	KSVSS	K----	----	-----	HSDYSAYD
Pfie ABI	KDTGS	>	<	RSSSSG	KSGT	KSVSS	K----	----	-----	HSDYSAYD
Pfie N32	KETGS	>	<	KSSNSG	KSGT	KSVSS	K----	----	-----	YSDYSAYD
Pcy Gombok	KETGS	>	<	KSSKSS	KS-N	KSGY	----	----	KSGNSS	YSDYSAYE
Pcy Berok	KETGS	>	<	KSSKSS	KS-N	KSGY	----	----	KSGGSS	YSDYSAYE
Pcy PT2	KETGS	>	<	KSSKSS	KS-N	KSGY	----	----	KSGGSS	YSDYSAYE
Pcy PT1	KETGS	>	<	KSSKSS	KSGH	-----	----	----	KSGGSS	RSEYSAYD
Pkn Malayan	KSGGS	>	<	KSGGSV	KSGS	KSPKS	V----	----	-----	YSDYSAYD
Pkn H	KSGGS	>	<	KSGGSV	KSGS	KSPKS	V----	----	-----	YSDYSAYD
Pkn Nuri	KSGGS	>	<	KSGGSV	KSGS	KSPKS	V----	----	-----	YSDYSAYD
Pkn Hackeri	KSGGS	>	<	KSGGSA	KSGS	KSPKS	V----	----	-----	YSDYSAYD
Pkn Philippine	KSGGS	>	<	KSGGSA	KSGS	KSPKS	V----	----	-----	YSDYSAYD
Pcoatney	KSGGS	>	<	KSGH--	----	KSPAS	I----	----	-----	HSDYTAYD
Pfragile SriLanka	KDSGS	>	<	KPPASG	-SGS	ASG-H	GSVP	----	KSPGSI	HSQYSAYD

Fig. 2.
Repetitive sequences observed in the RAP-1 in *Plasmodium* spp.

Fig. 3. Bayesian phylogenetic tree of the gene encoding RAP-1 using MrBayes. The numbers on nodes of the tree are posterior probabilities presented as percentage based on 2,000,000 Markov Chain Monte Carlo (MCMC) steps; the first 250,000 (25%) were discarded as a burn-in. Sampling was performed every 100 generations.

Table 1

Characterization of the RAP-1 from *Plasmodium* spp.

Species	n	Protein (aa)	Intron (bp)	Gene (bp)	π (SE)
<i>P. falciparum</i>	32	782 – 783	-	2346 – 2349	0.0041 (0.0006)
<i>P. vivax</i>	6	740 – 748	187 – 212	2422 – 2458	0.0026 (0.0008)
<i>P. coatneyi</i>	1	730	256	2449	-
<i>P. cynomolgi</i>	4	736 – 740	237 – 261	2460 – 2484	0.0395 (0.0029)
<i>P. ffieldi</i>	2	736	207 – 256	2418 – 2467	0.0540 (0.0042)
<i>P. inui</i>	5	737	181 – 191	2395 – 2405	0.0222 (0.0021)
<i>P. knowlesi</i>	5	734 – 736	205 – 206	2411 – 2417	0.0098 (0.0014)
<i>P. simiovale</i>	1	736	184	2395	-

Table 2

Polymorphism found in the RAP-1 in *Plasmodium* spp.

	π (SE)	Ds	Dn	Ds-Dn (S.D.)	Z
<i>P. falciparum</i> (n = 32)					
CDS	0.0041 (0.0006)	0.0029	0.0044	-0.0015 (0.0008)	Ds = Dn
<i>P. vivax</i> (n=6)					
CDS	0.0026 (0.0007)	0.0039	0.0023	0.0017 (0.0022)	Ds = Dn
Exon 1	0.0046 (0.0026)	0.0102	0.0031	0.0071 (0.0107)	Ds = Dn
Intron	0.0018 (0.0018)				
Exon 2	0.0023 (0.0007)	0.0030	0.0022	0.0008 (0.0019)	Ds = Dn
<i>P. cynomolgi</i> (n = 4)					
CDS	0.0382 (0.0030)	0.0717	0.0319	0.0398 (0.0111)	Ds > Dn ($P<0.05$)
Exon 1	0.0181 (0.0060)	0.0476	0.0108	0.0368 (0.0263)	Ds = Dn
Intron	0.0514 (0.0110)				
Exon 2	0.0411 (0.0033)	0.0755	0.0350	0.0406 (0.0116)	Ds > Dn ($P<0.05$)
<i>P. inui</i> (n = 5)					
CDS	0.0214 (0.0023)	0.0322	0.0191	0.0131 (0.0061)	Ds > Dn ($P<0.05$)
Exon 1	0.0174 (0.0050)	0.0524	0.0092	0.0432 (0.0220)	Ds = Dn
Intron	0.0320 (0.0082)				
Exon 2	0.0220 (0.0022)	0.0295	0.0206	0.0089 (0.0063)	Ds = Dn
<i>P. knowlesi</i> (n = 5)					
CDS	0.0082 (0.0015)	0.0190	0.0055	0.0135 (0.0047)	Ds > Dn ($P<0.05$)
Exon 1	0.0123 (0.0049)	0.0281	0.0083	0.0198 (0.0189)	Ds = Dn
Intron	0.0268 (0.0083)				
Exon 2	0.0076 (0.0014)	0.0176	0.0051	0.0125 (0.0052)	Ds > Dn ($P<0.05$)
<i>P. feldi</i> (n = 2)					
CDS	0.0500 (0.0042)	0.1031	0.0393	0.0638 (0.0164)	Ds > Dn ($P<0.05$)
Exon 1	0.0290 (0.0094)	0.0910	0.0139	0.0771 (0.0441)	Ds = Dn
Intron	0.0996 (0.0201)				
Exon 2	0.0530 (0.0052)	0.1049	0.0430	0.0619 (0.0181)	Ds > Dn ($P<0.05$)

Table 3

McDonald and Kreitman Test (Neutrality Index)

	Exon 1	Exon 2	Gene
<i>P. falciparum</i> vs. <i>P. reichenowi</i>			1.255 (NS)
<i>P. vivax</i> vs. <i>P. cynomolgi</i>	1.200 (NS)	2.575 ($P<0.05$)	2.437($P<0.05$)
<i>P. vivax</i> vs. <i>P. inui</i>	0.542 (NS)	3.106 ($P<0.05$)	2.458 ($P<0.05$)
<i>P. vivax</i> vs. <i>P. fieldi</i>	0.625 (NS)	2.694 ($P<0.05$)	2.33 ($P<0.05$)
<i>P. cynomolgi</i> vs. <i>P. inui</i>	0.270 (NS)	0.788 (NS)	0.707 (NS)
<i>P. cynomolgi</i> vs. <i>P. fieldi</i>	0.058 ($P<0.05$)	0.766 (NS)	0.619 (NS)
<i>P. inui</i> vs. <i>P. fieldi</i>	0.208 ($P<0.05$)	1.045 (NS)	0.863 (NS)