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Limited genetic polymorphism of the *Plasmodium vivax* low molecular weight rhoptry protein complex in the Colombian population☆

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ABSTRACT

Proteins involved in parasite adhesion and invasion are considered the best candidates for the development of asexual blood-stage antimalarial vaccines. Such vaccine candidates should be accessible by the immune system and have limited diversity. Considering the promising results obtained in previous trials by immunizing monkeys with the rhoptry-associated proteins 1 and 2 (RAP-1 and RAP-2), here we assessed the genetic variability of the *Plasmodium vivax rap-1* and *rap-2* genes isolated from Colombian parasite populations. Limited sequence diversity was found in these genes, possibly as a result of a functional/structural restriction. The presence of several haplotypes at relatively low frequencies and the excess of singleton mutations suggests that a demographic process might be affecting the loci. Our results support the inclusion of PvRAP-1 and PvRAP-2 in the design of an antimalarial subunit-based vaccine against *P. vivax*, which would avoid inducing allele-specific immunity.

1. Introduction

Plasmodium vivax is the second most important and widely distributed human malaria parasite species. About 72–80 million cases of *P. vivax* malaria are estimated per year (Mendis et al., 2001), however, recent studies highlight that this number might be considerably underestimated (Price et al., 2007). It has been calculated that 71–81% of all malaria cases being reported in Central and South America are produced by this species.

Strategies for the control of *P. vivax* malaria include the design of an antimalarial vaccine, but such an attempt has been hampered by the parasite's complex biology (e.g. multiple life-cycle stages, immune evasion strategies) (Genton and Reed, 2007). Among the different approaches followed in the design of antimalarial vaccines, surface proteins from the parasite's asexual stage are considered potential good candidates, unfortunately, a large number of these are polymorphic (Richie and Saul, 2002; Genton and Reed, 2007). It has been also shown that several variants of these antigens might be required to provide protection (Richie and Saul, 2002). Other promising vaccine candidates comprise proteins involved in adhesion and invasion processes which are not necessarily exposed on the parasite membrane but are recognized by the host's immune system.

Several proteins contained inside the parasite apical organelles appear to be crucial for the parasite entry into the red blood cell (RBC). Two families of rhoptry proteins have been described in *P. falciparum*: the high molecular weight proteins (RhopH) and the low molecular weight proteins (RAP). This latter family forms a proteic complex (Howard and Reese, 1990) which is suggested to interact with RBC receptors (Sterkers et al., 2007). Two members of the RAP family have been identified in *P. vivax* (Patarroyo et al., 2005; Perez-Leal et al., 2006), denoted as *P. vivax* rhoptry-associated protein 1 (PvRAP-1) and *P. vivax* rhoptry-associated protein 2 (PvRAP-2). Both proteins are encoded by single-copy genes; *Pvrap1*, which is a 2447-bp gene comprising two exons encoding an 85.6 kDa protein that contains a serine-rich region (Perez-Leal et al., 2006); and *Pvrap-2*, which consists of a single exon of about 1203 bp encoding a 46.6 kDa protein (Patarroyo et al., 2005).

☆ *Note*: Nucleotide sequence data reported in this paper are available in GenBank under the accession numbers GQ480333–GQ480357.

Abbreviations: PvRAP-1, *Plasmodium vivax* rhoptry-associated protein 1; PvRAP-2, *Plasmodium vivax* rhoptry-associated protein 2; *Pvmsp-1*, *Plasmodium vivax* merozoite surface protein 1 encoding gene; Ps, number of parsimony-informative sites; Ss, number of segregating sites; *S*, number of singleton sites; SNP, single-nucleotide polymorphism; *H*, number of haplotypes; Hd, haplotype diversity; d_N, non-synonymous substitutions; d_S, synonymous substitutions; LD, linkage disequilibrium; RFLP, restriction fragment length polymorphisms; RBC, red blood cell; π, nucleotide diversity; θ^W, Watterson estimator.

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Immunization studies with a vaccine containing PfRAP-1 and PfRAP-2 in new world monkeys suggest that these proteins can stimulate the immune system (Collins et al., 2000). Other studies focusing on antibody and T cell responses to recombinant PfRAP-1 and PfRAP-2 in humans have shown that antigens are recognized by the human immune system (Jacobson et al., 1998). Altogether, RAP-2 has been shown to have larger potential as vaccine candidate since PfRAP2 was the major active component in the vaccination mixture (Collins et al., 2000) and immunization trials with recombinant PvRAP-2 in *Aotus* spp. monkeys showed induction of antibodies recognizing the recombinant protein and induction of protection against experimental challenge (Rojas-Caraballo et al., 2009).

Besides being recognized by the immune system, vaccine candidates should display low genetic variability. Orthologous proteins from other *Plasmodium* species (Saul et al., 1992; Howard and Peterson, 1996; Escalante et al., 1998) as well as from species belonging to other Apicomplexa (Suarez et al., 1994) show low polymorphism; nevertheless, these reports are limited to a reduced number of isolates. The present study describes sequence polymorphisms in both *P. vivax rap-1* and *rap-2* genes among Colombian isolates, showing that these two genes display low polymorphism.

2. Materials and methods

2.1. Parasites

Peripheral blood samples from patients being diagnosed with *P. vivax* malaria by microscopic examination were collected into EDTA-containing vacutainer tubes. Previous to blood withdrawal, patients were informed about the purpose of the study and all gave their informed consent. Samples were collected in five different geographical regions of Colombia (Fig. 1A): Caribbean region ($n = 11$), Pacific region ($n = 10$), Andean region ($n = 9$), Orinoco region ($n = 4$) and Amazon region ($n = 5$). Blood samples were processed and stored from 2007 until 2009. DNA was obtained using the Wizard Genomic DNA Purification kit (Promega, Madison, WI) following manufacturer's instructions and used as template in PCR assays. All procedures carried out in this study were approved by our institute's ethics committee.

2.2. Genotyping of P. vivax samples

All parasite samples were genotyped by PCR–RFLP of the *Pvmsp-1* gene's segment 2 (blocks 6–8) as previously described (Imwong et al., 2005). Samples showing infection by a single *P.*

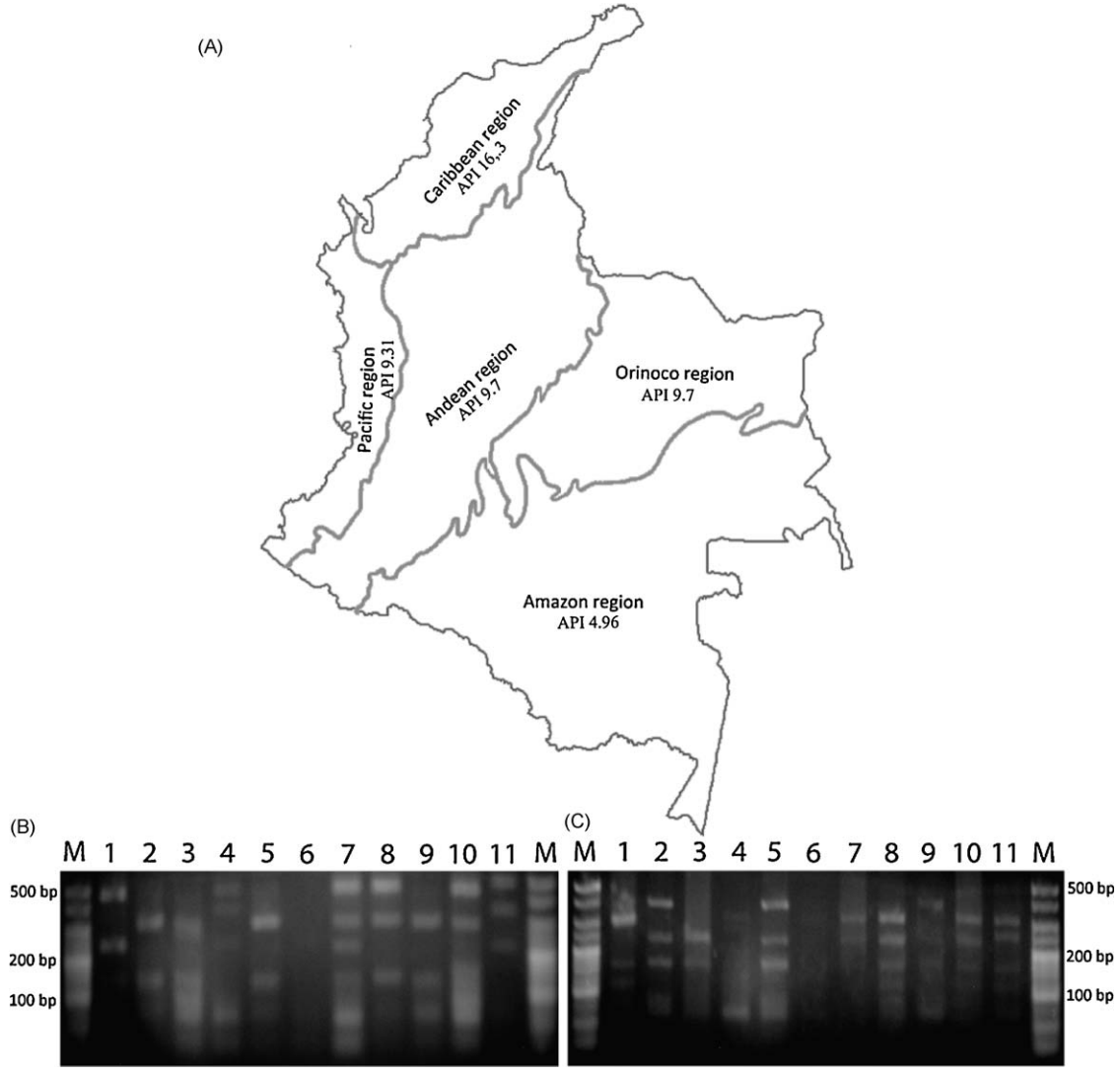

Fig. 1. (A) Geographical localization and annual parasitemia index (API) in each region of Colombia (INS, 2006). Genotyping analysis of the *Pvmsp1* locus by PCR–RFLPs using Alu I (B) and Mnl I (C). RFLP patterns of *P. vivax* isolates confirm the presence of different genotypes. M: Molecular weight marker, lanes 1–11 correspond to different isolates used in this study.

vivax isolate were selected and PCR–RFLP patterns were obtained to confirm the presence of different haplotypes from a highly polymorphic locus.

2.3. PCR and gene isolation

Primers were designed to amplify *Pvrap-1* and *Pvrap-2* DNA fragments based on the GenBank sequences DQ311677 and DQ130066, respectively. Two sets of primers were designed to amplify and clone *Pvrap-1*, whose sequences were: Pvrap1-1fwd 5′-TTACGCACTCTACCAAATG-3′; Pvrap1-1rev 5′-GGGCAGGTG-TAAAGAATG-3′; Pvrap1-2fwd 5′-GAGGGACTTAGGCATTTC-3′ and Pvrap1-2rev 5′-TACTCCAATCGCTTGTAGA-3′. Primers designed to amplify *Pvrap-2* were: Pvrap2fwd 5′-CAATAATGGTGTAGATGGAC-3′and Pvrap2rev 5′-TTATGACTCCATACCTTTCTC-3′. Each reaction mixture contained 0.5× Green GoTaq® Flexi Buffer (Promega); 2 mM $MgCl_2$; 0.2 mM of each dNTP; 0.4 μM of each primer, 0.06 U/μl of GoTaq® DNA polymerase (Promega) and 10–40 ng of DNA template in a final 25 μl volume. Thermal conditions were set as follows: one cycle of 3 min at 95 °C; 35 cycles of 1 min at 58/56/56 °C (Pvrap1-1fwd/rev; Pvrap1-2fwd/rev and Pvrap2fwd/rev primers, respectively), 1.3 min at 72 °C, 1 min at 95 °C and a final cycle of 1 min at each primer's corresponding annealing temperature followed by 5 min final extension at 72 °C. PCR products were purified using the Wizard PCR preps (Promega), ligated into pGEMT-Easy Vector System (Promega) and cloned into *E. coli* JM109 cells. Positive clones were selected using an ampicillin positive selection and α complementation of *lacZ* gene. Plasmid DNA was extracted by using the Wizard plus Minipreps (Promega). DNA sequencing was performed in both directions using T7 and SP6 primers with the BigDye™ Terminator cycling conditions (MACROGEN, Seoul, South Korea). Four clones obtained from independent PCR amplifications were sequenced per isolate to discard errors.

2.4. Polymorphism analysis

Colombian *P. vivax rap-1* and *rap-2* sequences were compared and analyzed against the previously described Sal-I strain sequences (XM_001616799 and XM_001613127, respectively). Electropherograms were checked and assembled using CLC DNA workbench 5 (CLC bio, Cambridge, MA, USA). A preliminary alignment and sequence comparison were performed using Clustal W software (Thompson et al., 1994) and minor editions were carried out using GeneDoc software (Nicholas and Nicholas, 1997).

DnaSP v.5 software (Librado and Rozas, 2009) was used for calculating the number of segregating sites (Ss), number of singleton sites (S), number of parsimony-informative sites (Ps), number of haplotypes (H), haplotype diversity (Hd), Watterson estimator (θ^W) and nucleotide diversity (π). Natural selection was estimated using the Nei–Gojobori method (Nei and Gojobori, 1986) to calculate the average number of non-synonymous (d_N) and synonymous (d_S) substitutions. Differences between d_N and d_S were assessed by applying the Z-test incorporated in MEGA software (Tamura et al., 2007). A higher d_N than d_S indicated positive selection whereas a higher d_S than d_N indicated purifying selection. Tests to assess departure from the neutral model were applied using Tajima's D (Tajima, 1989), Fu & Li's D^* and F^* (Fu and Li, 1993), and Fu's Fs (Fu, 1997) statistics. The former statistic compares the difference between two estimators of the neutral parameter, the average number of nucleotide differences between sequence pairs and the total number of segregating sites. Fu & Li test calculates D^* and F^* statistics; D^* is based on the difference between the number of singletons and the total number of mutations, whereas F^* is based on the difference between the number of singletons and the average number of nucleotide differences between sequence pairs. Positive and negative values from both tests correspond to departures from neutrality. Fu's test which is based on the gene frequency distribution is used to detect population growth and genetic hitchhiking. All tests were applied using DnaSP v.5 software, considering coalescent simulations for obtaining confidence intervals (Librado and Rozas, 2009).

Recombination events in *Pvrap-1* and *Pvrap-2* sequences were assessed by using the RDP3 v.3 software (Recombination Detection Program) (Martin and Rybicki, 2000). This tool searches for evidence of recombination among aligned sequences by examining all possible triplet combinations, applying different recombination detection methods. Additionally, the ZZ statistic was used to detect intragenic recombination. An association between polymorphic sites or linkage disequilibrium (LD) was evaluated by calculating the Z_{nS} (average of r^2) statistic. Confidence intervals for these statistics were obtained by coalescent simulations using DnaSP v.5 software (Librado and Rozas, 2009).

3. Results

3.1. Genotyping of parasite isolates

All parasite isolates stored since 2007 corresponded to single *P. vivax* infections. The analysis of the *Pvmsp-1* locus showed that the gene is polymorphic in Colombian parasite isolates. *Pvmsp-1* genotyping (Fig. 1B and C) found twenty-nine samples that represented a different allelic variant, which confirmed the presence of dissimilar genotypes.

3.2. Polymorphisms in Pvrap-1

A 2413-bp fragment of the *Pvrap-1* gene spanning the two exons and the intron was amplified by PCR and sequenced in twenty nine samples, independently of the *msp-1* genotype present in each of the isolates. Thirteen haplotypes were found (Fig. 2A). The H1 haplotype was found to be predominant (34%), followed by H2 (17%) and H3 (13%) whereas the remaining haplotypes corresponded to low frequency variants (rare alleles). Two haplotypes were distinguishable within the intronic region, which differed by a 25 nucleotide deletion (data not shown). Of the total nucleotide sequence length being analyzed, 2400 sites were monomorphic and 13 were polymorphic, being 10 parsimony-informative sites and 3 singleton sites. Only one single-nucleotide polymorphism (SNP) was identified in exon I nucleotide 172–F69L- (according to the *P. vivax* VCG-I strain numbering, GenBank accession ABC41135), whereas exon II contained 12 segregating sites consisting of 3 singleton and 9 parsimony-informative sites (Table 1). The H2 and H5 haplotypes had a distinguishable insertion of the SGSKY sequence, while nucleotides 952–954 (amino acid 318) were deleted in haplotype H11 (Fig. 2A) (according to the *P. vivax* VCG-I strain numbering, GenBank accession DQ311677).

The nucleotide diversity of *Pvrap-1* ($\pi = 0.00088$) was lower than the one previously reported for *Pfrap-1* ($\pi = 0.0019$) (Escalante et al., 1998); however, it should be taken into account that the number of samples in such study was smaller. Nucleotide diversity differed between the two *Pvrap-1* exons, being higher for exon II than for exon I (Table 1). Identity values between DNA sequences and deduced protein sequences were 97.76% and 98.09%, respectively, which places *Pvrap-1* as one of the most conserved *P. vivax* vaccine candidates reported up to the moment (Table 2). Regarding *Pvrap-1* diversity among the different regions of Colombia, the gene was most conserved in Orinoco and Amazon (data not shown). Nevertheless, this might be also influenced by the small number of sequences being analyzed for these regions in our study.

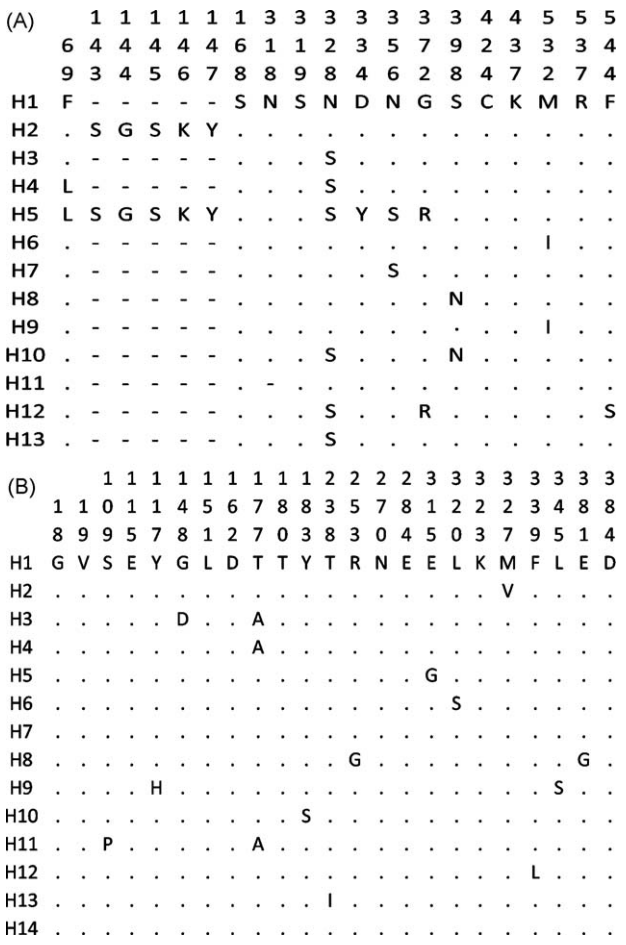

Fig. 2. (A) Alignment of non-conserved residues for PvRAP-1 showing the 13 haplotypes found in the Colombian parasite population. Gaps represent insertions/deletions in several haplotypes. (B) Alignment of non-conserved residues for PvRAP-2 showing the 14 haplotypes found in Colombian isolates. Dots indicate conserved residues. Amino acid positions are indicated according to the *P. vivax* VCG-I strain numbering (GenBank accessions ABC41135 and AAZ68036 for PvRAP-1 and PvRAP-2, respectively).

3.3. Selection in the Pvrap-1 locus

Values yielded by the neutral evolutionary tests and comparisons of the mean number of synonymous substitutions per synonymous sites (d_S) and the number of non-synonymous substitutions per non-synonymous sites (d_N) of the entire *Pvrap-1* nucleotide sequence and its exons showed no statistically significant differences (Table 1). This could be suggesting lack of selective pressure on this locus. Even though there does not seem to be a clear selective process affecting this locus, statistically significant Fs values suggest that a demographic process could be taking place (Table 1).

3.4. Polymorphisms in Pvrap-2

A 1161-bp fragment of the *Pvrap-2* gene was amplified by PCR and sequenced. Fourteen different haplotypes were established for this gene among the 35 isolates (Fig. 2B); being haplotype H1 the most predominant (60%), whereas the remaining haplotypes corresponded to rare alleles. Of the total nucleotide sequence, 1138 sites were monomorphic whereas all remaining sites were polymorphic sites.

Pvrap-2 has a low nucleotide diversity (π = 0.00141) (Table 1), which is reflected both in DNA alignments (98.02% identity) as well as in alignments of deduced protein sequences (96.63% identity),

Table 1
Estimates of DNA polymorphisms, selection and neutrality test for the *Pvrap-1* (n = 29) and *Pvrap-2* (n = 35) loci.

Gene	Sites	Ss	S	Ps	H	Hd	θ^W	π	Tajima	Fu & Li		Fu	d_S (se)	d_N (se)	Z_{nS}	ZZ	
									D	D^*	F^*	Fs					
Pvrap-1	2413	13	3	10	13	0.727	0.00140	0.00088	−1.2225	0.1868	−0.2931	−5.371[†]	0.0016 (0.0008)	0.0007 (0.0003)			
Pvrap-1 Exon 1	243	1	0	1	2	0.133	0.00105	0.00055	−0.7529	0.5985	0.2606	−0.410	0	0.0007 (0.0007)	0.094	0.030	
Pvrap-1 Exon 2	1958	12	3	9	13	0.727	0.00157	0.00101	−1.1779	0.0764	−0.3640	−5.784[†]	0.0020 (0.0011)	0.0008 (0.0003)			
Pvrap-2	1161	23	18	5	14	0.677	0.00481	0.00141	−2.4307[‡]	−3.5989[§]	−3.7937[§]	−9.200[‡]	0.0030 (0.0010)[§]	0.0010 (0.0003)	0.060	−0.030	

Ss: number of segregating sites, S: number of singleton sites, Ps: number of parsimony-informative sites, H: number of haplotypes, Hd: haplotype diversity, θ^W: Watterson estimator, π: nucleotide diversity, d_S: synonymous substitutions, d_N: non-synonymous substitutions, se: standard error.

[†] p-Values < 0.05.
[‡] p-Values < 0.01.
[§] p-Values < 0.02.

Table 2
Nucleotide diversity for *P. vivax* antigens.

	Gene	*n*	Sites	π	Reference
P. vivax	*msp-3α*	35	2577	0.0496	Mascorro et al. (2005)
	msp-1	175	417	0.0451	Figtree et al. (2000)
	msp-5	22	1524	0.0375	Gomez et al. (2006)
	ama-1	72	1000	0.0130	Figtree et al. (2000)
	dbp	23	1388	0.0122	Martinez et al. (2004)
	trap	39	1470	0.0059	Putaporntip et al. (2001)
	rap-2	35	1161	0.0013	This paper
	msp-4	30	846	0.0011	Martinez et al. (2005)
	rap-1	29	2413	0.0008	This paper

n: number of isolates, π: nucleotide diversity.

being *Pvrap-2* together with *Pvrap-1* among the most conserved vaccine candidates reported up to the moment (Table 2). Colombian regions with the lowest variability in this locus were the Caribbean and Andean regions, while the Pacific region was the most polymorphic one (data not shown).

3.5. Selection in the Pvrap-2 locus

Comparisons of d_S and d_N values obtained for *Pvrap-2* in the Colombian parasite population showed that the evolution of this locus is possibly driven by purifying selection since d_S was higher than d_N (Table 1). However, although a higher d_S than d_N was observed within regions, these values were not significant (data not shown). The *Pvrap-2* gene showed significant values below 0 in the tests of neutral evolution (Tajima and Fu & Li and Fu) (Table 1 and Fig. 3B). These values indicate an excess of low frequency alleles caused by some sort of evolutionary process such as directional selection, selective sweep or a population growth.

3.6. Linkage disequilibrium (LD) and recombination

Random associations were observed between *Pvrap-1* and *Pvrap-2* haplotypes, therefore suggesting their independent segregation. LD analyses measured by r^2 between nucleotide variants and by Z_{nS} for whole data measured (average of r^2) showed no association between polymorphic sites in *Pvrap-1* and neither between polymorphic sites in *Pvrap-2*. The relationship of r^2 (linkage disequilibrium) with physical distance by the regression analysis showed that LD does not decline as nucleotide distance increase neither in *Pvrap-1* nor in *Pvrap*-2, which indicates that recombination events are not taking place in this gene. Recombination analysis of both *Pvrap-1* and *Pvrap*-2 using the RDP v.3 software showed no signs of recombination in these genes. On the

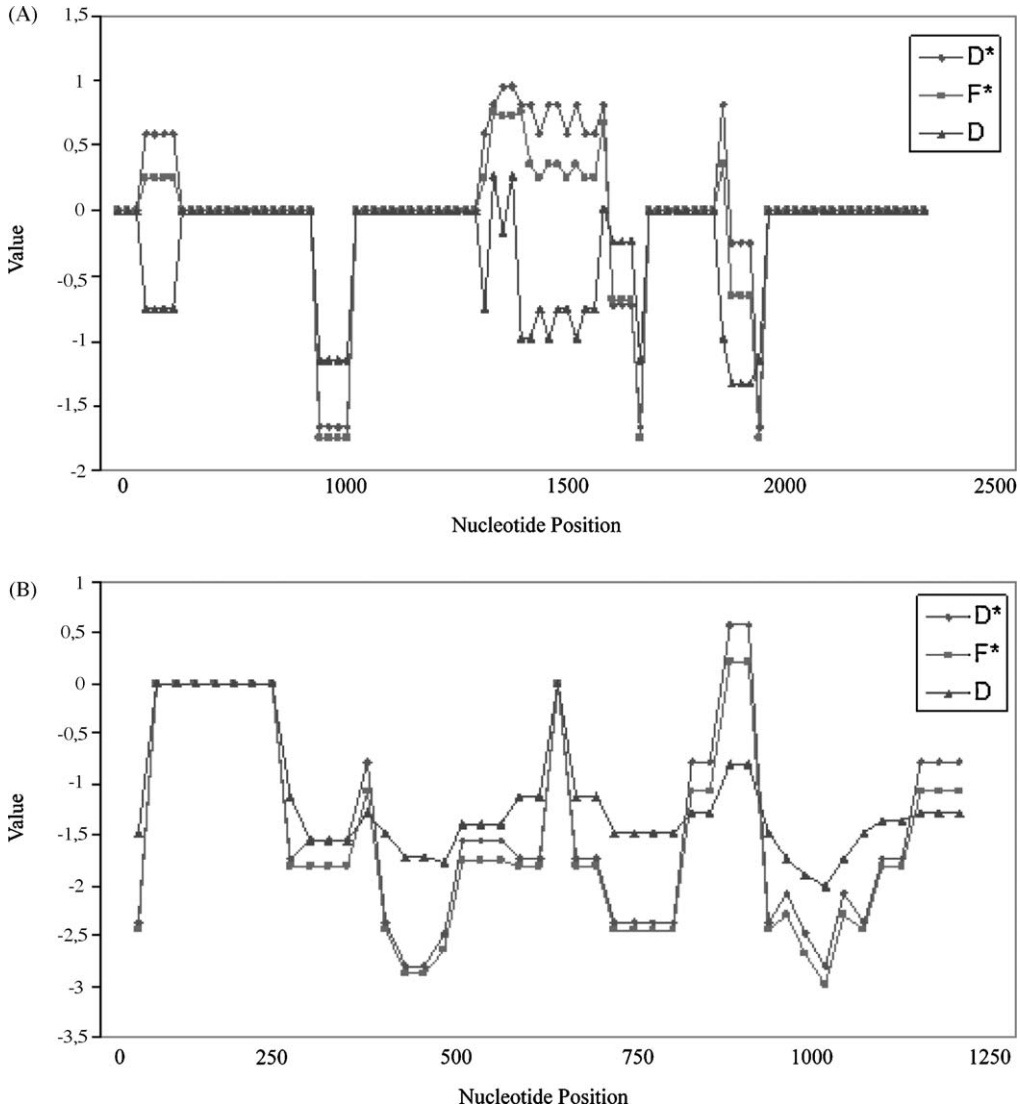

Fig. 3. Sliding window analysis of Tajima and Fu & Li test for *Pvrap-1* (A) and *Pvrap-2* (B). A pattern of neutrality is found in *Pvrap*-1 whereas the pattern in *Pvrap-2* suggests purifying selection or a demographic process.

other hand, the ZZ statistic used for detecting intragenic recombination showed no significant values (Table 1). All these data suggest that recombination processes are not taking place in *Pvrap-1* nor in *Pvrap-2*.

4. Discussion

Based on the promising protection-inducing results obtained with RAP-1 and RAP-2 in previous immunization trials (Jacobson et al., 1998; Collins et al., 2000; Rojas-Caraballo et al., 2009), we analyzed the genetic variability of *Pvrap-1* and *Pvrap-2* among the Colombian *P. vivax* population. Previous studies suggested a limited number of polymorphism for the *Pfrap-1* (Howard and Peterson, 1996; Escalante et al., 1998) and *Pfrap*-2 (Saul et al., 1992) loci, however the number of isolates was not larger than 6 in such studies. Our study indicates that both *Pvrap-1* and *Pvrap-2* had a low genetic diversity and we observed no correlation between nucleotide diversity and *P. vivax* endemicity across geographic regions (data not show).

Different tests to assess the effect of natural selection in the *P. vivax* low molecular weight rhoptry protein complex were utilized to determine the type of pressure that is acting on these genes. For *Pvrap-1*, there was no evidence of selective pressure; however, according to the neutral model, polymorphism is expected to be larger in the absence of a selective pressure unless the protein is under a functional or structural constraint that, in the case of PvRAP-1, might be due to the key role of this protein in parasite survival. Regarding the *Pvrap-2* locus, the analysis suggests that a purifying selective pressure could be driving its evolution. Significantly higher d_S values than d_N values and significant values of D (Tajima), D^* and F^* (Fu & Li) statistics support this notion (Table 1). Nevertheless, significantly negative values obtained for *Pvrap-2* with Tajima and Fu & Li tests could be suggesting a demographic process.

It is worth noting that there was a low genetic diversity for both *Pvrap-1* as well as for *Pvrap-2*, which could be the result of (i) a selective sweep or (ii) directional selection. Statistically significant values of D, D^*, F^* and Fs statistics obtained for *Pvrap-2* and significant Fs values obtained for *Pvrap1* would support the idea of selective sweep. However, hypothesis of selective sweep can be evaluated by correlating any of these statistics with high linkage disequilibrium (Schlenke and Begun, 2004). Z_{nS} and r^2 values found for both genes are not significant (Table 1), which would turn down such hypothesis. Yet, an analysis of the variability in regions adjacent to these genes would be needed in order to confirm whether there is selective sweep or not. The directional selection hypothesis would be supported since *Pvrap-2* appears to be under the action of purifying selection (higher d_S than d_N in the Colombian parasite population). On the other hand, a possible functional or structural constraint should be also considered since this constraint added to a purifying selection might be maintaining variability in the *Pvrap-2* locus low.

Interestingly, both loci showed a relatively high number of haplotypes with rare alleles (low frequency variants). This could be explained by (i) selective sweep, (ii) negative directional selection, or (iii) population growth. The first hypothesis would be ruled out same as mentioned before. A negative directional selection would not only reduce genetic variability but also increase the rate of rare alleles. The results obtained in this study suggest that this type of selection would be affecting the low molecular weight rhoptry protein complex. On the other hand, population growth would be supported by the D, D^* and F^* statistics where rare alleles would be recently emerging variants that might be fixed or eliminated from the population; nevertheless, the values of these neutrality tests would support this possibility for *Pvrap-2* but not for *Pvrap-1*. A statistic broadly used for detecting population growth was developed by Fu (1997). This statistic showed significant values for both loci (Table 1), which suggests that there is indeed a population growth and that new variants are the result of recent mutations that have not been yet fixed or eliminated from the population. In fact, higher Hd values and lower π values are expected for populations that are growing, which is the behavior here observed for rhoptry-complex genes. Therefore, evolutionary phenomena such as directional selection and population growth could explain the large number of low frequency haplotypes (in the Colombia population) and the low genetic variability; these events appear to be, together with functional/structural restriction, the more reasonable mechanisms to explain the structure of the *Pvrap-1* and *Pvrap-2* loci.

Different criteria have been considered in the selection of candidates for the design of antimalarial vaccines: (1) vaccine candidates should be accessible to the host's immune system, (2) should induce protection and (3) have limited polymorphism (Richie and Saul, 2002). The two first criteria are supported by reports of protective immune responses induced by PfRAP-1 and Pf/PvRAP-2 peptides (Jacobson et al., 1998; Collins et al., 2000; Rojas-Caraballo et al., 2009). The last criteria is supported by the low polymorphism levels detected in this study, which would give additional support to the use PvRAP-1 and PvRAP-2 in the design of a subunit vaccine against *P. vivax*.

Although there are no data available regarding the genetic variability of these proteins in other regions of the world, and given that the only sequence reported outside the area of study (Sal-I, obtained from Central America) has the same sequence as the most prevalent haplotype in Colombia, added to the low variability of orthologous proteins and we found no correlation between endemicity and genetic variability in this study, PvRAP-1 and PvRAP-2 are probably proteins of low polymorphism worldwide. Therefore, a vaccine that includes rhoptry complex proteins is likely to have larger success worldwide since it would not induce allele-restricted protection. However, it should be taken into account that the RAP1/RAP2 complex is released during RBC invasion, a process that takes no more than a minute, such brief exposure to the immune system would require of a very quick and potent immune response to block parasite entry into RBCs.

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