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Genetic complexity of *Plasmodium vivax* infections in Sri Lanka, as reflected at the merozoite-surface-protein-3α locus

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The presence of genetically different strains of malarial parasites in cases of human malaria is a severe drawback in the successful control of the disease. In Sri Lanka, although this species accounts for 60%–80% of all of the cases of clinical malaria that occur each year, the genetic complexity of *Plasmodium vivax* on the island remains to be elucidated. In recent studies based on PCR–RFLP and the parasites' merozoite-surface-protein-3α locus, the genetic structure of 201 clinical isolates of *P. vivax*, from two malaria-endemic areas and a non-endemic area of the island, was investigated. Although the PCR only produced amplicons of three sizes [1900 (72.6%), 1500 (25.9%) and 1200 (1.5%) bp], the RFLP analysis based on *Hha*I or *Alu*I digestion yielded 22 and 26 restriction patterns, respectively, with 51 combined patterns recorded. The distribution of the prominent PCR–RFLP haplotypes was area-specific. The probability that an investigated case had a multiple-clone infection (MCI) was higher among the cases from the endemic areas (20.0%) than among those from the non-endemic area (13.8%) but this difference was not statistically significant. Since 17 single-clone isolates produced only 11 different PCR–RFLP haplotypes but (after sequencing) 13 distinct nucleotide haplotypes, it is clear that the results of the PCR–RFLP were not revealing all of the diversity that existed at the nucleotide level. Four mass blood surveys in a malaria-endemic area demonstrated that seasonal changes in the prevalences of human infection with *P. vivax* may influence the occurrence of MCI.

A host may carry a genetically complex or homogeneous infection with a single species of parasite. Exploration of the genetic diversity of parasite populations is a prerequisite for understanding the relevant molecular epidemiology, developing effective vaccines and drugs and planning vaccination and therapeutic strategies (Cui *et al.*, 2003).

Malarial parasites are haploid except during a brief period of sexual recombination in the mosquito vector. The infection of a single mosquito with multiple strains of *Plasmodium* results from the mosquito feeding either on a single host that carries more than one parasite genotype or, as the result of interrupted feeding, on multiple hosts within one gonotrophic cycle (Druilhe *et al.*, 1998). In humans, multiple-clone infections (MCI) with malarial parasites may result not only from a single bite of a mosquito that has a multiple-clone infection itself but also from relapse infections and super infections in the human hosts (Havryliuk and Ferreira, 2009). The degree of genetic heterogeneity seen in both *P. vivax* and *P. falciparum* infections in humans has been

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associated with the prevailing pattern and intensity of transmission in the study area (Bruce *et al.*, 1999, 2000; Engelbrecht *et al.*, 2000; Magesa *et al.*, 2002). In areas where the entomological inoculation rate (EIR) is below one infectious bite/person-year, only 30%–60% of the human infections have been found to be MCI (Suwanabun *et al.*, 1994; Joshi *et al.*, 1997; Cui *et al.*, 2003). In areas of intense transmission, such as many parts of Papua New Guinea, however, >65% of the human malarial infections investigated have been identified as MCI (Kolakovich *et al.*, 1996; Bruce *et al.*, 2000; Cole-Tobian *et al.*, 2005).

The genotyping of *P. vivax* infections has been based on several polymorphic antigens (Premawansa *et al.*, 1993; Kolakovich *et al.*, 1996; Bruce *et al.*, 1999, 2000; Cui *et al.*, 2003; Cole-Tobian *et al.*, 2005). The gene coding for the parasite's merozoite surface protein-3α (*msp*-3α) appears to be a particularly reliable marker for the molecular epidemiology of *P. vivax* in various geographical areas, having a central polymorphic region that is readily evaluated by use of a combination of PCR and RFLP (Bruce *et al.*, 1999, 2000; Galinski *et al.*, 1999; Cui *et al.*, 2003; Cole-Tobian *et al.*, 2005; Véron *et al.*, 2009).

In Sri Lanka, human malaria is considered to be unstable, with generally low levels of transmission (Rajendram and Jayawickreme, 1951). Almost the entire dry zone of the country is considered to be endemic for *P. vivax* and this species accounts for 60%–80% of the total annual incidence of malaria in the country (Anon., 2000), inclusive of relapse infections (Fonseka and Mendis, 1987). The complexity of the *P. vivax* infections in Sri Lanka has previously been demonstrated in a few small-scale studies based on various markers and tools (Langsley *et al.*, 1988; Udagama *et al.*, 1990; Premawansa *et al.*, 1993; Gunesekera *et al.*, 2007; Karunaweera *et al.*, 2008; Manamperi *et al.*, 2008). In the present, relatively large-scale investigation, the genetic complexity of *P. vivax*, in clinical isolates from Sri Lanka, was explored via the *msp*-3α marker.

SUBJECTS AND METHODS

Ethics, Sample Collection and DNA Extraction

The study protocol was approved by the Ethics Review Committee of the University of Colombo, Sri Lanka. Blood samples were only collected with informed consent.

Between December 1998 and March 2000, pre-treatment blood samples were collected, in three areas (Fig. 1), from 217 adults (aged >15 years) with microscopically confirmed *P. vivax* infections. The subjects either came from the *P. vivax*-endemic areas of Anuradhapura (8°22′N, 80°20′E; $N=62$) and Kataragama–Buttala (6°25′N, 81°20′E; $N=91$) or lived in non-endemic Colombo (7°55′N, 79°50′E; $N=64$) but had been infected during visits to regions of the island with *P. vivax* transmission (Fonseka and Mendis, 1987). Age, gender, number of malarial attacks experienced, time symptomatic prior to treatment, and time since previous malarial attack were recorded for each subject.

Genomic parasite DNA was extracted from 1-ml samples of venous blood (Gunasekera *et al.*, 2007). Briefly, plasma and white blood cells were removed from the whole-blood samples, by centrifugation and filtration through a CF11 column, respectively. The erythrocytes eluted from the column were lysed with 0.015% saponin in NET buffer (150 mM NaCl, 10 mM EDTA and 50 mM Tris; pH 7.5), pelleted by centrifugation, and then treated with 1% (w/v) *N*-lauroyl-sarcosine, RNase A (100 μg/ml) and proteinase K (200 μg/ml). Parasite DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25 : 24 : 1, by vol.) and precipitated with 0.3 M sodium acetate and absolute ethanol. The DNA pellets were air dried, resuspended in Tris buffer (10 mM Tris and 1 mM EDTA) and stored at −20°C until further use.

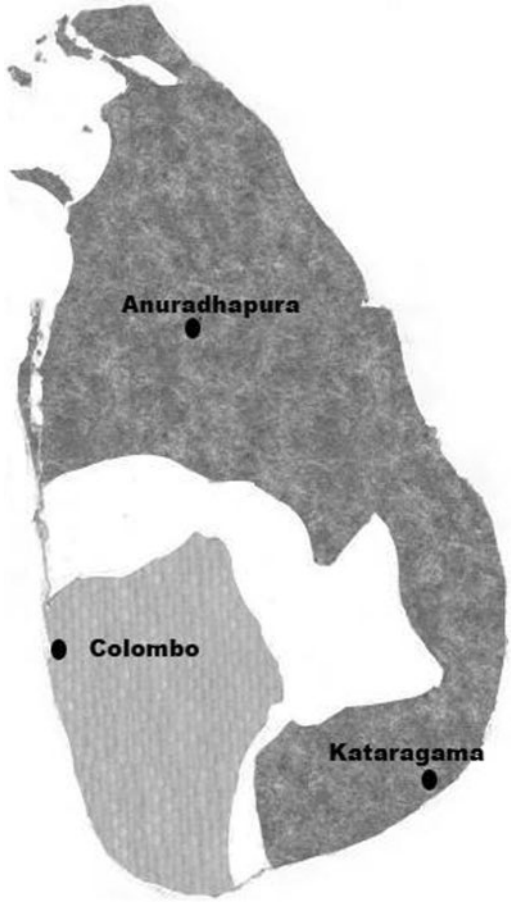

FIG. 1. Map of Sri Lanka showing the extent of the dry, malaria-endemic zone (■) the wet, non-endemic zone (■) and the intermediate zone (□), as well as the locations of the three sample-collection sites.

Parasite Genotyping and Identification of Single-clone Infections

The clonality of each *P. vivax* infection was investigated using PCR–RFLP analysis of alleles at the polymorphic *msp*-3α locus (Fig. 2) and a slight modification of the method described by Bruce *et al.* (1999). Nested PCR amplified a fragment (of approximately 1900 bp) of the polymorphic region of the *msp*-3α locus. For this, the P1 (5′-CAG CAG ACA CCA TTT AAG G-3′) and P2 (5′-CCG TTT GTT GAT TAG TTG C-3′) primers were used for the outer PCR while N1 (5′-GAC CAG TGT GAT ACC ATT AAC C-3′) and N2 (5′-ATA CTG GTT CTT CGT CTT CAG G-3′) were used for the nested reaction. Each amplification was carried out in a 20-μl reaction mixture containing either 1 μl of DNA extract (primary round) or 0.5 μl of the products of the primary reaction (nested round). The final amplicons were subjected to digestion with 2.5 U *Hha*I (New England BioLabs, Ipswich, MA) or 5 U *Alu*I (New England BioLabs), in a reaction volume of 20 μl. Whichever enzyme was used, the reaction mixtures were incubated for 4–5 h at 37°C and then 20 min at 65°C.

After the digestion fragments produced in each reaction were separated by electrophoresis, the lengths of the DNA fragments (in bp) were estimated. Any MCI were identified either directly from the PCR, when more than one band was obtained from the sample, or from the RFLP analysis, when the sum of the lengths of the fragments produced from a digestion with *Hha*I or *Alu*I was greater than the size of the uncut PCR product. Isolates identified as single clones were chosen for further analysis.

DNA Sequencing and Alignment Analysis

The amplicons from the PCR were resolved on agarose gels and purified using a commercial kit (Promega, Madison, WI) before direct sequencing. Each amplicon was sequenced commercially three times, in both the forward and reverse directions, using the N1 and N2 primers again, a forward (5′-CAG TAG TGG CAA AGG AGG AAG-3′) and reverse (5′-AAA TGC AGC AGA GTC AGC CA-3′) internal primer, and BigDye® termination chemistry (Macrogen, Seoul), achieving six-fold sequence coverage. A single continuous sequence of approximately 1200–1900 nucleotides was derived from the isolates from 17 subjects with single-clone infections (GenBank accessions GU175269–GU175285). These sequences were aligned and verified using the Seqman II software package (DNAStar, Madison, WI) while consensus sequences were aligned using the ClustalW program from version 4.1 of the

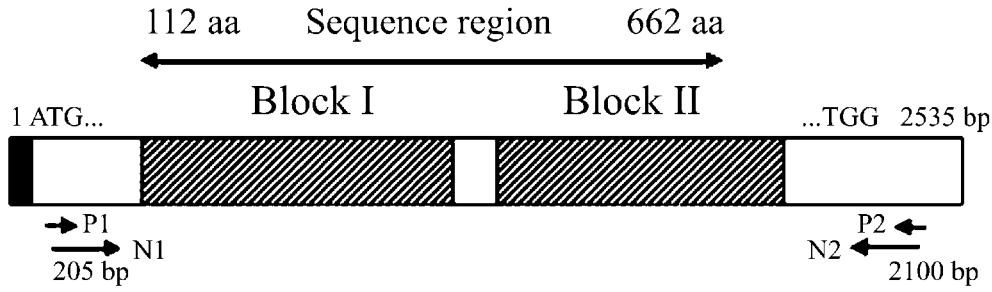

FIG. 2. Schematic representation of the *msp*-3α structure, showing the signal peptides (■) and the two blocks of coiled-coil structural domain in the polymorphic region (▨). The positions of the primary (P1 and P2) and nested (N1 and N2) oligonucleotide primers used in the PCR are indicated by arrows. Numbers refer to the nucleotide base pairs (bp) within the corresponding sequence of the Belem strain of *Plasmodium vivax* or the amino-acid (aa) positions in the readable sequence of the *msp*-3α gene in Sri Lankan isolates.

MEGA software package (Tamura *et al.*, 2007; www.megasoftware.net).

Various indices of genetic diversity — π (nucleotide diversity), k (mean number of nucleotide differences), S (number of polymorphic sites), H (number of haplotypes) and Hd (haplotype diversity) — were calculated using version 4.5 of the DnaSP software package (Rozas *et al.*, 2003), while synonymous and non-synonymous nucleotide diversities were evaluated using version 4.1 of the MEGA software package and the method of Nei and Gojobori, with the Jukes–Cantor correction (Tamura *et al.*, 2007). The MEGA software package was also used to produce a phylogenetic tree from the aligned nucleotide sequences, employing the neighbour-joining (NJ) method with 1000 bootstrap replicates and the Kimura two-parameter distance model.

Seasonal Malaria Prevalence and MCI in the Endemic Kataragama–Buttala Area

The Kataragama–Buttala area of the Monaragala district of Sri Lanka's Uva province has been endemic for *P. vivax* malaria for many years (Briët *et al.*, 2003). Although malaria is reported throughout the year in this area, the incidence of the disease peaks between November and late February, during the north–eastern monsoon rains. At the same time as the sample collection for the PCR–RFLP analysis, four mass blood surveys were carried out in three adjacent villages in this area, at approximately 3-month intervals, between December 1998 and December 1999. The protocol for these surveys was also approved by the Ethics Review Committee of the University of Colombo, Sri Lanka. Stained thick and thin bloodsmears were used for the microscopic detection of malarial infection in the villagers. Attempts were then made to establish association(s) between the (rainfall-related) seasonal variation in the prevalence of *P. vivax* infection in the villagers and the occurrence of MCI in the Kataragama–Buttala area.

Statistical Analysis

The collected data were analysed using version 11 of the SPSS for Windows software package (SPSS Inc, Chicago, IL) and version 6 of the Epi Info package (Centers for Disease Control and Prevention, Atlanta, GA). Proportions of independent samples were compared using χ^2 test while means were compared using Student's *t*-tests and analysis of variance, as appropriate. A *P*-value of <0.05 was considered indicative of a statistically significant difference.

RESULTS

Characteristics of the Test Populations

Although >90% of the subjects from the endemic areas presented (for malaria

diagnosis and treatment) within 4 days of the onset of their symptoms, only 50% of the subjects from non-endemic Colombo had sought treatment within 6 days of becoming symptomatic. The mean ages of the subjects from the three study areas were similar ($P>0.05$ for each comparison) but the subjects from Kataragama–Buttala reported a significantly higher number of previous malarial attacks than the subjects from Anuradhapura or Colombo, with means of eight, three and two, respectively ($P<0.05$). The subjects from Anuradhapura, Kataragama–Buttala and Colombo presented with means of 0.10%, 0.07% and 0.14% of their erythrocytes infected, respectively, the values for Kataragama–Buttala and Colombo being significantly different ($P<0.05$). Although half of the subjects from Anuradhapura had had a malarial attack in the previous 5 months and half of those from Kataragama had had such an attack in the previous 3 months, most of the subjects from Colombo were suffering from their first attack of malaria when investigated in the present study.

Prevalence of Multiple-clone *Plasmodium vivax* Infections

Overall, five of the 201 isolates that gave successful amplification in the PCR produced more than one amplicon each, indicating that they were MCI (see Table). For each of another 32 isolates, summation of the lengths of the RFLP fragments resulted in a significantly greater value than the length of the corresponding undigested product, again indicating the presence of more than one *msp*-3α allele (Table). Of the isolates successfully investigated by PCR–RFLP, 19.2% of those from Anuradhapura, 20.8% of those from Kataragama–Buttala but only 13.8% of those from non-endemic Colombo appeared to come from subjects with MCI (Table; $P>0.05$).

Prevalence of Different Alleles of *msp*-3α

All but 16 of the 217 clinical isolates of *P. vivax* investigated were successfully amplified at the *msp*-3α locus. The nested PCR produced amplicons of 1200, 1500 and/or 1900 bp. The 1900-bp product ($N=146$; 72.6%) was significantly more common than the 1500-bp ($N=52$; 25.9%; $P<0.05$) or 1200-bp ($N=3$; 1.5%; $P<0.01$) products in all three study areas. As indicated previously, five isolates gave more than one amplicon, each giving amplicons of 1500 and 1900 bp (Table).

Although the undigested PCR products fell into just three sizes, the RFLP analysis yielded highly diverse size fragments and banding patterns, even for the isolates that gave only one PCR product each, indicating substantial diversity at the nucleotide level.

With all of the isolates that were successfully investigated, the largest fragments resulting from *Hha*I digestion or *Alu*I

TABLE. *The numbers of samples investigated and multiple-clone infections identified in each of the three study areas in Sri Lanka*

		No. of multiple-clone infections identified by:*				
			PCR–RFLP using:			
Study area	No. of samples	PCR	*Hha*I only	*Alu*I only	Both enzymes	Any method
Anuradhapura (endemic)	52	1	3	4	2	10
Kataragama (endemic)	91	3	8	7	1	19
Colombo (non-endemic)	58	1	1	4	2	8
All three areas	201	5	12	15	5	37

*Overall, 201 clinical isolates were successfully investigated by PCR, and 196 by PCR–RFLP.

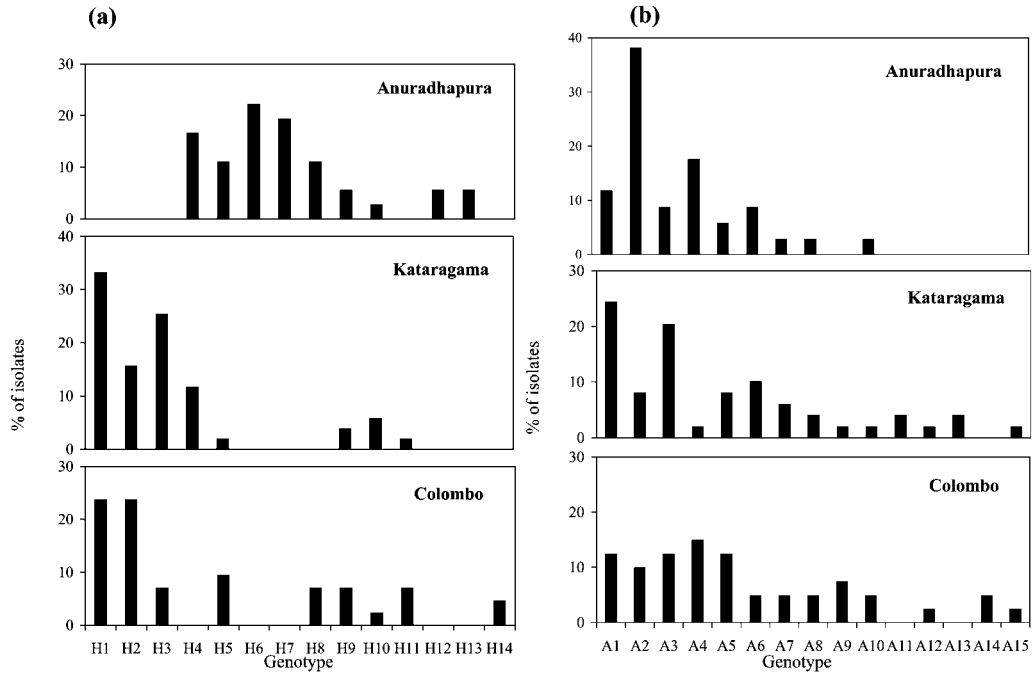

FIG. 3. Some of the results of the PCR–RFLP genotypes, showing the percentages of the single-clone isolates from each of the three sample-collection sites identified as (a) genotypes H1–H14 (after *Hha*I digestion) or (b) genotypes A1–A15 (after *Alu*I digestion). Only the data for genotypes that each represented >1% of the single-clone isolates with 1900-bp alleles are shown.

digestion were of the same sizes (about 1000 and 500 bp, respectively). It was therefore the smaller fragments, of 175–600 bp with *Hha*I and 150–450 bp with *Alu*I, that were used to distinguish genotypes (see below).

Genotypes Identified by PCR–RFLP

The RFLP banding patterns of 196 single-clone samples indicated 22 different genotypes with *Hha*I digestion, and 26 with *Alu*I. The combined *Hha*I and *Alu*I patterns resulted in 51 different genotype combinations (results not shown). The diverse genotypes were mostly confined to the 1900-bp allele. Just four genotypes from each digestion (recorded as H1–H4 for *Hha*I and A1–A4 for *Alu*I) represented >50% of the infections. Genotypes H1, H2 and H3 were only detected in the samples from Kataragama–Buttala, while another three genotypes (H6, H7 and H8) were exclusively found in the samples from Anuradhapura [Fig. 3(a)]. Although these six genotypes were therefore 'area-restricted', they also accounted for the majority of the sampled infections in the areas where they did occur (86.3% of the single-clone isolates from Kataragama–Buttala being H1, H2 and H3 and 80.5% of such isolates from Anuradhapura being H6, H7 or H8). The A1–A4 genotypes also showed uneven distribution between the two endemic areas [Fig. 3(b)].

Genetic Diversity of *Plasmodium vivax msp*-3α in Sri Lanka

Direct sequencing of the 1900- (N=8), 1500- (N=8) or 1200-bp (N=1) amplicons, produced from 17 single-clone infections, resulted in sequences that were readable only between amino-acid positions 112 and 662 (Fig. 2) of the corresponding (*msp*-3α) sequence for the Belem strain of *P. vivax* (Rayner *et al.*, 2002). These sequences corresponded to the polymorphic alanine-rich coiled coils of domain I and the coiled coils of the heptad repeats of domain II

(Galinski *et al.*, 1999; Rayner *et al.*, 2002). The presence of the N-terminal and C-terminal variable regions in the 1900-bp allele, and the deletion of the N-terminal segment of this allele in the 1500- and 1200-bp alleles, were apparent.

While the 196 polymorphic sites identified in the 1900-bp genotype ($N=8$) gave rise to 202 mutations (Fig. 4), the 1500-bp genotype ($N=8$), with only five polymorphic sites identified, resulted in only five mutations (the limited polymorphism in the 1500-bp genotype probably resulting from the deletion within the hyper-variable region of block I). More evidence of the conserved nature of the 1500-bp allele came from the indices of nucleotide diversity (with π-values of 0.001 for this allele and 0.0555 for the 1900-bp allele) and the mean numbers of nucleotide differences (with k-values of only 1.25 for the 1500-bp allele and 88.0 for the 1900-bp).

Deletions and a large number of amino-acid substitutions were found at the *msp*-3α locus. Interestingly, the 1900-bp genotype was represented by eight different haplotypes, resulting in high haplotype diversity (Hd=1.000), whereas only five haplotypes were identified in the 1500-bp allele type (Hd=0.786; Fig. 5).

Examination of synonymous and non-synonymous nucleotide substitutions revealed the presence of 121 non-synonymous substitutions in the 1900-bp genotype but only one such substitution (Asp/Glu) in the 1500-bp genotype. The more frequent NS substitutions within the 1900-bp genotype again indicate the presence of high variability.

Although the PCR–RFLP data for the 17 isolates investigated by sequencing indicated only 11 different haplotypes, the corresponding sequence data resulted in 13 distinct haplotypes, indicating that there was more diversity at the nucleotide level than revealed by PCR–RFLP alone.

In the phylogenetic analysis, the isolates with the 1900-bp allele were not clustered into a specific clade but were distributed throughout the tree, forming three clades (Fig. 5). The 1500-bp isolates, in contrast, were robustly clustered into a single subgroup of a clade (Fig. 5).

Relationship between MCI and Seasonal Malaria Prevalence

During three of the four mass blood surveys carried out to evaluate the point prevalence of malarial infection, >75% of the combined populations of the three study villages were screened. The recorded prevalences and corresponding rainfall pattern are presented in Figure 6. The prevalence of *P. vivax* infections detected during the surveys ranged from 0.38% to 1.72% (Fig. 6). The corresponding range for *P. falciparum* was 0.58%–3.02%.

Comparison of the proportions of investigated clinical isolates from Kataragama–Buttala identified as multiclonal — 18 (22.5%) of the 80 isolates collected in the high-prevalence or high-transmission season (September–March) and none (0%) of the 11 isolates collected in the low-prevalence/low-transmission season (April–August) — indicated that seasonal changes in *P. vivax* prevalence may influence the occurrence of MCI.

DISCUSSION

The close association between the genetic complexity of the local malarial infections, the prevalence of MCI and transmission intensity is well established (Babiker *et al.*, 1997; Anderson *et al.*, 2000; Cristiano *et al.*, 2008). Globally, between 10.6% and 65% of the *P. vivax* infections investigated, in humans living in endemic areas, have been found to be multiclonal (Suwanabun *et al.*, 1994; Kolakovich *et al.*, 1996; Joshi *et al.*, 1997; Bruce *et al.*, 2000; Cole-Tobian *et al.*, 2002; Cui *et al.*, 2003; Kim *et al.*, 2006; Cristiano *et al.*, 2008; Moon *et al.*, 2009). Unfortunately, differences in the sampling and genotyping methods used in these investigations limit the significance of the apparent geographical variation observed.

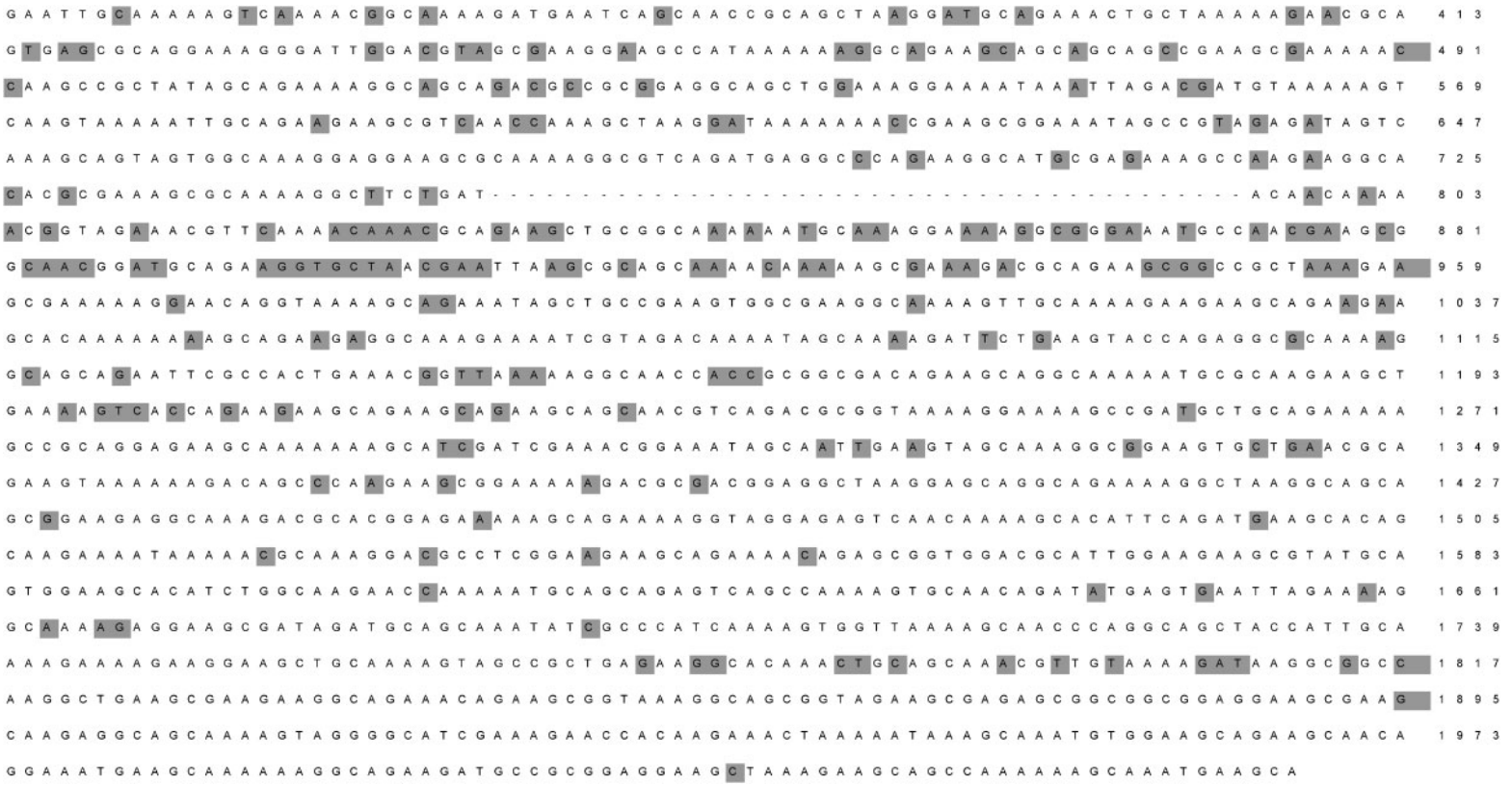

FIG. 4. Nucleotide polymorphisms in blocks I and II of the *msp*-3α genes of 17 Sri Lankan isolates. The consensus sequence was obtained from aligning the nucleotide sequences of the 17 *msp*-3α sequences, the polymorphic sites being highlighted here by grey shading.

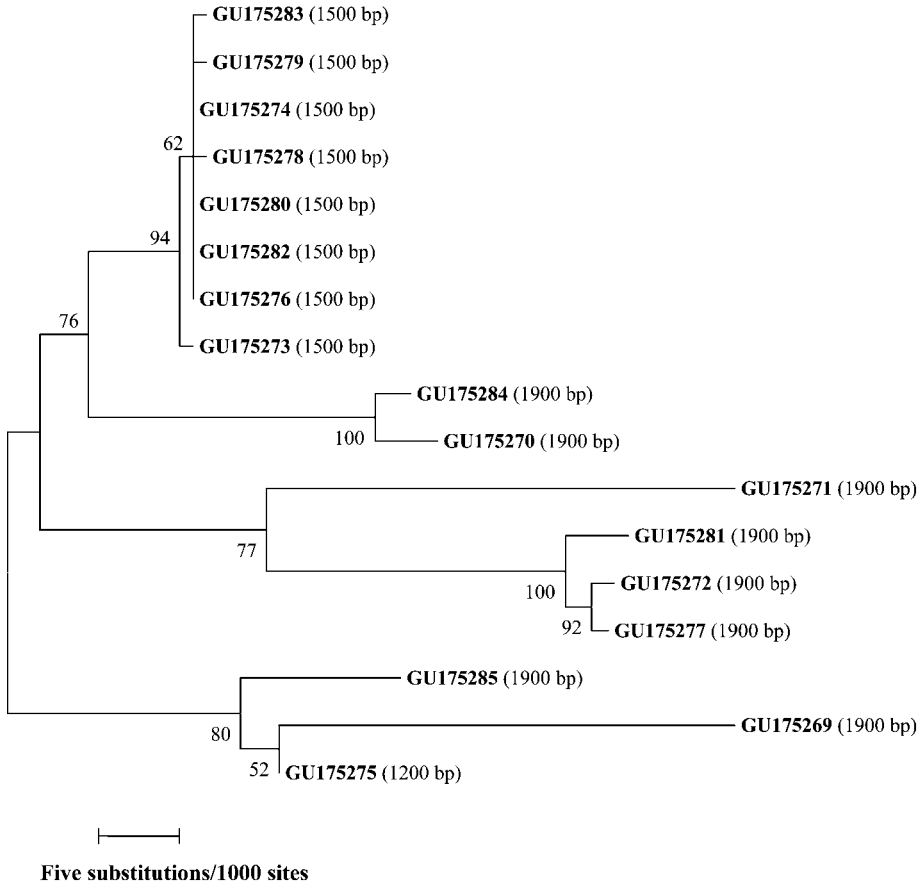

FIG. 5. Phylogenetic tree based on the *msp*-3α genes of 17 Sri Lankan isolates of *Plasmodium vivax*. The tree was constructed using the neighbour-joining method. The *msp*-3α alleles investigated were represented by 1900-, 1500- or 1200-bp amplicons in the PCR. Each isolate number on the phylogenetic tree is the relevant GenBank accession number.

The present study appears to be the first large-scale investigation on the genetic complexity of *P. vivax* infections in Sri Lanka. The proportion of infections found to be multiclonal (20%) and the size polymorphism and diverse genotypes identified in studies at the *msp*-3α locus indicate a high degree of genetic complexity in the local *P. vivax* infections, on an island with generally low and unstable malarial transmission (Rajendram and Jayewickreme, 1951; Mendis *et al.*, 1990).

In earlier studies based on serology (Udagama *et al.*, 1990) or sequence analysis (Premawansa *et al.*, 1993), only about 10% of *P. vivax* infections in Sri Lankans were identified as multiclonal. As the serological screening (Udagama *et al.*, 1990) was mostly performed on isolates that differed from those used to produce the monoclonal antibodies employed, certain genotypes may not have been detected. Surprisingly, in a recently published genotyping study using microsatellites, the proportion of the investigated *P. vivax* isolates identified as MCI varied from 9.1% in 2003 to 60.0% in 2005, although the sample sizes (22 and 20, respectively) were fairly small (Karunaweera *et al.*, 2008).

In the present study, the proportion of investigated isolates found to be multiclonal (20%) was higher than the values recorded

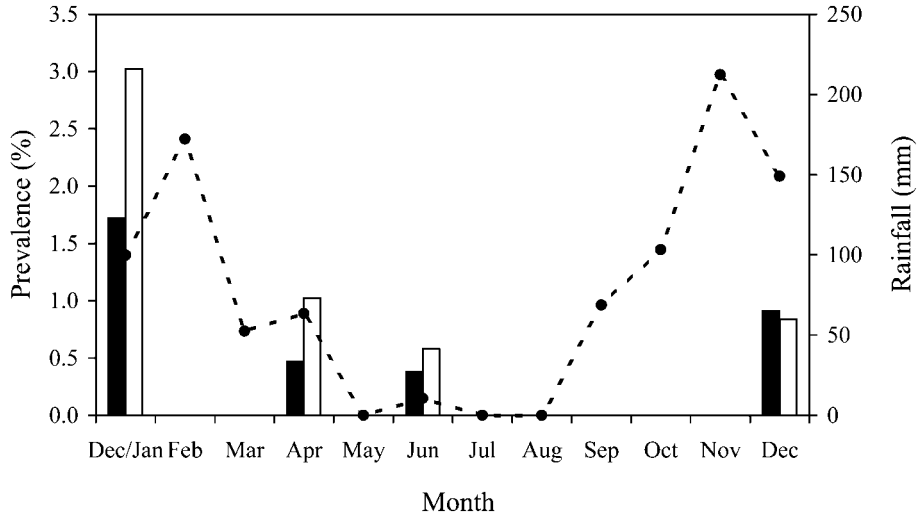

FIG. 6. The point prevalences of *Plasmodium vivax* (■) and *P. falciparum* (□) infection in humans living in the malaria-endemic Kataragama–Buttala area, as measured in four mass blood surveys (in December 1998–January 1999, April 1999, June 1999 and December 1999), and the corresponding rainfall (●) in the same area.

in Sri Lanka prior to 2005. As this value is based on a relatively large sample and the genotyping of the *msp*-3α locus (which is generally considered to be a reliable molecular epidemiological marker; Kim *et al.*, 2006; Zakeri *et al.*, 2006; Cristiano *et al.*, 2008; Véron *et al.*, 2009) by PCR–RFLP, it may be the best representation of the local situation yet produced. The 20% frequency recorded in the present study is similar to the value recorded in other regions with hypo-endemic *P. vivax* malaria, including Thailand and Venezuela (Bruce *et al.*, 1999; Cui *et al.*, 2003; Ord *et al.*, 2005). The number of MCI detected in the present study may, however, under-estimate the number represented by the investigated samples, since low parasitaemias may hinder the PCR-based amplification of a subpatent secondary strain (De Roode *et al.*, 2005; Havryliuk *et al.*, 2008).

The distribution of human malaria across Sri Lanka is mainly determined by climatic factors that are essential to the development of the vector mosquitoes, with the island's dry zone traditionally being endemic. Those Sri Lankans who live in non-endemic areas, such as Colombo, only become infected with *P. vivax* when they visit endemic areas. Three basic factors may be responsible for a multiple-clone *Plasmodium* infection in a human (Cui *et al.*, 2003): the super-infection of the human host, the simultaneous co-infection of the human host, or the somatic mutation of a single clone of parasites in the human host. When Sri Lankans are infected in endemic areas, the local residents and visitors from non-endemic areas are, presumably, equally likely to be infected by the bite of a mosquito carrying a multiple-clone infection. Although the risk of super-infection may clearly be greater among the infected residents of endemic areas than among the infected residents of non-endemic areas, the general level of transmission in Sri Lanka is so low that super-infection must be a rare event even in the endemic areas. In Kataragama, for example, the chance of an individual being bitten by an infectious mosquito on a single night has been estimated to be only one in 500 (Mendis *et al.*, 1990). This may explain why, in the present study, among the human *P. vivax* infections investigated in each area, MCI were only slightly (and not significantly) less common in non-endemic Colombo than in Anuradhapura or Kataragama–Buttala. In

an earlier study in Sri Lanka, MCI were also found to be rarer among isolates from non-endemic Colombo than among those from endemic settings (Premawansa *et al.*, 1993). The vast majority of MCI that are detected in non-endemic areas, in people who have, in general, only paid brief visits to areas with malarial transmission, are presumably the results of bites by mosquitoes that have MCI themselves (Druilhe *et al.*, 1998).

Few residents of Kataragama (or, presumably, other endemic villages in Sri Lanka) have acquired clinical immunity to *P. vivax* infection and few seek prompt treatment for such infection (Gunawardena *et al.*, 1994). In Sri Lanka, relapses may occur 8–24 weeks after primary infections with *P. vivax* (Fonseka and Mendis, 1987), and a general reluctance to complete the recommended primaquine regimen presumably allows relapse infections to develop (Havryliuk and Ferreira, 2009). Thus, multiclonal infections may result when relapse infections develop at the same time as single-clone re-infections.

Although Fonseka and Mendis (1987) attributed 18% of clinical *P. vivax* infections from Colombo to relapses, four (50%) of the MCI from the city identified in the present study were first infections and the rest were probably the result of re-infection (as all four cases had recently visited malarious areas). The contribution of relapse infections to MCI in this urban setting therefore seems to be slight.

The geographical variation seen, in the present study, in the distributions of some of the *P. vivax* genotypes (probably resulting from genetic drift) has not been reported previously in Sri Lanka. Given the generally low transmission intensity, it is not surprising that some genotypes common in Anuradhapura were rare or undetected 250 km away, in Kataragama–Buttala, and vice versa. With little transmission occurring and only 20% of *P. vivax* infections in the local human populations being multiclonal, unrelated *P. vivax* parasites rarely co-exist in the same mosquito bloodmeal. The resultant parasite inbreeding, which occurs in other areas with low levels of transmission, such as South America (Anderson *et al.*, 2000), helps to restrict the geographical spread of particular genotypes. In contrast, parasite outbreeding is more usual in areas with high levels of transmission, such as much of Africa, and tends to reduce the geographical variation in the predominant genotypes (Anderson *et al.*, 2000).

Knowledge of the genotypes that predominate in a particular area may allow the geographical origin of a human infection to be identified. In the present study, for example, it was sometimes possible to identify which of the Colombo subjects had probably been infected in the Kataragama–Buttala area and which in the Anuradhapura area, from the *P. vivax* genotypes that these subjects harboured. In each case, the identified 'probable source of infection' matched the travel history of the subject involved (data not shown).

The results of the four mass blood surveys that were carried out in the Kataragama–Buttala area provide a longitudinal view of the prevalence of malarial infection throughout a year. The apparent absence of MCI during the months of June and July, when rainfall and *P. vivax* point prevalence were both at their seasonal minima, indicates that there might be a significant association between malaria transmission intensity and the occurrence of MCI, at least in the Kataragama–Buttala area.

When pair-wise indices of nucleotide diversity (π) were obtained using various *P. vivax* merozoite proteins but the same clinical isolates as investigated in the current study (P. V. Udagama-Randeniya, unpubl. obs.), the value for *msp*-3α ($\pi=0.0590$) was found to be higher than that for *dbp*II (0.0098), *ama*-1 (0.0095) or *msp*-1p_{42} (0.0231). Relatively high levels of diversity in *msp*-3α were also reported by Mascorro *et al.* (2005). The overall haplotype diversity (Hd) recorded, in the present study, for the 1900-bp fragment indicates very high levels of genetic diversity, at the *msp*-3α locus,

within Sri Lankan *P. vivax*. Together, the high values recorded, in the present study, for pair-wise nucleotide diversity, haplotype diversity (for the 1900-bp amplicon) and prevalence of the 1900-bp allele provide further evidence for the suitability of the *msp*-3α gene as a genetic marker in the evaluation of the genetic complexity of *P. vivax* infections, at least in Sri Lanka.

The observation that, in the phylogenetic analysis (Fig. 5), the 1900-bp allele formed three clades that were distributed throughout the tree whereas the 1500-bp allele was restricted to a single subgroup of one clade may indicate that the 1900-bp version is the ancestral form and has had far longer to diverge (the 1500- and 1200-bp being derived forms that evolved more recently).

In conclusion, the present results indicate that about 20% of the *P. vivax* infections in the humans in two malaria-endemic areas of Sri Lanka (with low and unstable malaria transmission) are multiclonal. Super infections (presumably more common in endemic areas than in non-endemic) and relapses contribute to multiple-clone infections. The prevailing genetic diversity, the frequency of MCI and the association of particular genotypes with particular endemic areas highlight the complexity of the *P. vivax* infections in Sri Lanka.

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REFERENCES

Anderson, T. J. C., Haubold, B., Williams, J. T., Estrada-Franco, J. G., Richardson, L., Mollinedo, R., Bocharie, M., Mokili, J., Mharakurwa, S., French, N., Whitworth, J., Velez, I. D., Brockman, A. H., Nosten, F., Ferreira, M. U. & Day, K. (2000). Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Molecular Biology and Evolution*, **17**, 1467–1482.

Anon. (2000). *Annual Health Bulletin 2000*. Colombo, Sri Lanka: Ministry of Health.

Babiker, H. A., Lines, J., Hill, W. G. & Walliker, D. (1997). Population structure of *Plasmodium falciparum* in villages with different malaria endemicity in East Africa. *American Journal of Tropical Medicine and Hygiene*, **56**, 141–147.

Briët, O. J. T., Gunawardena, D. M., van der Hoek, W. & Amerasinghe, F. P. (2003). Sri Lanka malaria maps. *Malaria Journal*, **2**, 22.

Bruce, M. C., Galinski, M. R., Barnwell, J. W., Snounou, G. & Day, K. P. (1999). Polymorphism at the merozoite surface protein-3α locus of *Plasmodium vivax*: global and local diversity. *American Journal of Tropical Medicine and Hygiene*, **61**, 518–525.

Bruce, M. C., Galinski, M. R., Barnwell, J. W., Donnelly, C. A., Walmsley, M., Alpers, M. P., Walliker, D. & Day, K. P. (2000). Genetic diversity and dynamics of *Plasmodium falciparum* and *P. vivax* populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea. *Parasitology*, **121**, 257–272.

Cole-Tobian, J., Cortes, A., Baisor, M., Kastens, W., Xainli, J., Bockarie, M., Adams, J. & King, C. (2002). Age-acquired immunity to a *Plasmodium vivax* invasion ligand, the Duffy binding protein. *Journal of Infectious Diseases*, **186**, 531–539.

Cole-Tobian, J. L., Biasor, M. & King, C. L. (2005). High complexity of *Plasmodium vivax* infections in Papua New Guinean children. *American Journal of Tropical Medicine and Hygiene*, **73**, 626–633.

Cristiano, F. A., Pérez, M. A., Nicholls, R. S. & Guerra, A. P. (2008). Polymorphism in the

Plasmodium vivax msp 3α gene in field samples from Tierralta, Colombia. *Memórias do Instituto Oswaldo Cruz*, **103**, 493–496.

Cui, L., Mascorro, C. N., Fan, Q., Rzomp, K. A., Khuntirat, B., Zhou, G., Chen, H., Yan, G. & Sattabongkot, J. (2003). Genetic diversity and multiple infections of *Plasmodium vivax* malaria in western Thailand. *American Journal of Tropical Medicine and Hygiene*, **68**, 613–619.

De Roode, J. C., Helinski, M. E. H., Anwar, M. A. & Read, A. F. (2005). Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *American Naturalist*, **166**, 531–542.

Druilhe, P., Daubersies, P., Patarapotikul, J., Gentil, C., Chene, L., Chongsuphajaisiddhi, T., Mellouk, S. & Langsley, G. (1998). A primary malaria infection is composed of a very wide range of genetically diverse but related parasites. *Journal of Clinical Investigation*, **101**, 2008–2016.

Engelbrecht, F., Tögel, E., Beck, H. P., Enwezor, F., Oettli, A. & Felger, I. (2000). Analysis of *Plasmodium falciparum* infections in a village community in northern Nigeria: determination of *msp2* genotypes and parasite-specific IgG responses. *Acta Tropica*, **74**, 63–71.

Fonseka, J. & Mendis, K. N. (1987). A metropolitan hospital in a non-endemic area provides a sampling pool for epidemiological studies on vivax malaria in Sri Lanka. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **81**, 360–364.

Galinski, M. R., Corredor-Medina, C., Povoa, M., Crosby, J., Ingravallo, P. & Barnwell, J. W. (1999). *Plasmodium vivax* merozoite surface protein-3 contains coiled-coil motifs in an alanine-rich central domain. *Molecular and Biochemical Parasitology*, **101**, 131–147.

Gunasekera, A. M., Wickramarachchi, T., Neafsy, D. E., Ganguli, I., Perera, L., Premaratne, P. H., Hartl, D., Handunnetti, S. M., Udagama-Randeniya, P. V. & Wirth, D. F. (2007). Genetic diversity and selection at the *Plasmodium vivax* apical membrane antigen-1 (*Pvama-1*) locus in a Sri Lankan population. *Molecular Biology and Evolution*, **24**, 939–947.

Gunawardena, D. M., Carter, R. & Mendis, K. N. (1994). Patterns of acquired anti-malarial immunity in Sri Lanka. *Memórias do Instituto Oswaldo Cruz*, **89**, 61–63.

Havryliuk, T. & Ferreira, M. U. (2009). A closer look at multiple-clone *Plasmodium vivax* infections: detection methods, prevalence and consequences. *Memórias do Instituto Oswaldo Cruz*, **104**, 67–73.

Havryliuk, T., Orjuela-Sánchez, P. & Ferreira, M. U. (2008). *Plasmodium vivax*: microsatellite analysis of multiple-clone infections. *Experimental Parasitology*, **120**, 330–336.

Joshi, H., Subbarao, S. K., Adak, T., Nanda, N., Ghosh, S. K., Carter, R. & Sharma, V. P. (1997). Genetic structure of *Plasmodium vivax* isolates in India. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **91**, 231–235.

Karunaweera, N. D., Ferreira, M. U., Munasinghe, A., Barnwell, J. W., Collins, W. E., King, C. L., Kawamoto, F., Hartl, D. L. & Wirth, D. F. (2008). Extensive microsatellite diversity in the human malaria parasite *Plasmodium vivax*. *Gene*, **410**, 105–112.

Kim, J. R., Imwong, M., Nandy, A., Chotivanich, K., Nontprasert, A., Tonomsing, N., Maji, A., Addy, M., Day, N. P., White, N. J. & Pukrittayakamee, S. (2006). Genetic diversity of *Plasmodium vivax* in Kolkata, India. *Malaria Journal*, **5**, 71.

Kolakovich, K., Ssengoba, A., Wojcik, K., Tsuboi, T., Al-Yaman, F., Alpers, M. & Adams, J. H. (1996). *Plasmodium vivax*: favored gene frequencies of the merozoite surface protein-1 and the multiplicity of infection in a malaria endemic region. *Experimental Parasitology*, **83**, 11–18.

Langsley, G., Patarapotikul, J., Handunnetti, S., Khoury, E., Mendis, K. N. & David, P. H. (1988). *Plasmodium vivax*: karyotype polymorphism of field isolates. *Experimental Parasitology*, **67**, 301–306.

Magesa, S. M., Mdira, K. Y., Babiker, H. A., Alifrangis, M., Farnert, A., Simonsen, P. E., Bygbjerg, I. C., Walliker, D. & Jakobsen, P. H. (2002). Diversity of *Plasmodium falciparum* clones infecting children living in a holoendemic area in north–eastern Tanzania. *Acta Tropica*, **84**, 83–92.

Manamperi, A., Mahawithanage, S., Fernando, D., Wickremasinghe, R., Bandara, A., Hapuarachchi, C., Abeywickreme, W. & Wickremasinghe, R. (2008). Genotyping of *Plasmodium vivax* infections in Sri Lanka using *Pvmsp-3α* and *Pvcs* genes as markers: a preliminary report. *Tropical Biomedicine*, **25**, 100–106.

Mascorro, C. N., Zhao, K., Khuntirat, B., Sattabongkot, J., Yan, G., Escalante, A. A. & Cui, L. (2005). Molecular evolution and intragenic recombination of the merozoite surface protein MSP-3α from the malaria parasite *Plasmodium vivax* in Thailand. *Parasitology*, **131**, 25–35.

Mendis, C., Gamage-Mendis, A. C., De Zoysa, A. P. K., Abhayawardena, T. A., Carter, R., Herath, P. R. J. & Mendis, K. N. (1990). Characteristics of malaria transmission in Kataragama, Sri Lanka: a focus for immuno–epidemiological studies. *American Journal of Tropical Medicine and Hygiene*, **42**, 298–308.

Moon, S., Lee, H., Kim, J., Na, B., Cho, S., Lin, K., Sohn, W. & Kim, T. (2009). High frequency of genetic diversity of *Plasmodium vivax* field isolates in Myanmar. *Acta Tropica*, **109**, 30–36.

Ord, R., Polley, S., Tami, A. & Sutherland, C. J. (2005). High sequence diversity and evidence of balancing selection in the *Pvmsp3α* gene of

Plasmodium vivax in the Venezuelan Amazon. *Molecular and Biochemical Parasitology*, **144**, 86–93.

Premawansa, S., Snewin, V. A., Khouri, E., Mendis, K. N. & David, P. H. (1993). *Plasmodium vivax*: recombination between potential allelic types in the merozoite surface protein MSP1 in parasites isolated from patients. *Experimental Parasitology*, **76**, 192–199.

Rajendram, S. & Jayawickreme, S. H. (1951). Malaria in Ceylon. *Indian Journal of Malariology*, **5**, 1–2.

Rayner, J. C., Corredor, V., Feldman, D., Ingravallo, P., Iderabdullah, F., Galinski, M. R. & Barnwell, J. W. (2002). Extensive polymorphism in the *Plasmodium vivax* merozoite surface coat protein MSP-3α is limited to specific domains. *Parasitology*, **125**, 393–405.

Rozas, J., Sánchez-Delbarrio, J. C., Messeguer, X. & Rozas, R. (2003). DnaSP 4.50, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19**, 2496–2497.

Suwanabun, N., Sattabongkot, J., Wirtz, R. A. & Rosenberg, R. (1994). The epidemiology of *Plasmodium vivax* circumsporozoite protein polymorphs in Thailand. *American Journal of Tropical Medicine and Hygiene*, **50**, 460–464.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1. *Molecular Biology and Evolution*, **24**, 1596–1599.

Udagama, P. V., Gamage-Mendis, A. C., David, P. H., Peiris, J. S. M., Perera, K. L. R. L., Mendis, K. N. & Carter, R. (1990). Genetic complexity of *Plasmodium vivax* parasites in individual human infections analyzed with monoclonal antibodies against variant epitopes on a single parasite protein. *American Journal of Tropical Medicine and Hygiene*, **42**, 104–110.

Véron, V., Legrand, E., Yrinesi, J., Volney, B., Simon, S. & Carme, B. (2009). Genetic diversity of *msp3* and *msp1_b5* markers of *Plasmodium vivax* in French Guiana. *Malaria Journal*, **8**, 40.

Zakeri, S., Barjesteh, H. & Djadid, N. D. (2006). Merozoite surface protein-3α is a reliable marker for population genetic analysis of *Plasmodium vivax*. *Malaria Journal*, **5**, 53.