Title
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Journal
American Journal of Tropical Medicine and Hygiene, 68(5)

ISSN
0002-9637

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Publication Date
2003-05-01

DOI
10.4269/ajtmh.2003.68.613

Peer reviewed

Am. J. Trop. Med. Hyg., 68(5), 2003, pp. 613–619

GENETIC DIVERSITY AND MULTIPLE INFECTIONS OF *PLASMODIUM VIVAX* MALARIA IN WESTERN THAILAND

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Abstract. Using two polymorphic genetic markers, the merozoite surface protein-3α (MSP-3α) and the circumsporozoite protein (CSP), we investigated the population diversity of *Plasmodium vivax* in Mae Sod, Thailand from April 2000 through June 2001. Genotyping the parasites isolated from 90 malaria patients attending two local clinics for the dimorphic *CSP* gene revealed that the majority of the parasites (77%) were the VK210 type. Genotyping the *MSP3*-α gene indicated that *P. vivax* populations exhibited an equally high level of polymorphism as those from Papua New Guinea, a hyperendemic region. Based on the length of polymerase chain reaction products, three major types of the *MSP-3*α locus were distinguished, with frequencies of 74.8%, 18.7%, and 6.5%, respectively. The 13 alleles distinguished by restriction fragment length polymorphism analysis did not show a significant seasonal variation in frequency. Genotyping the *MSP-3*α and *CSP* genes showed that 19.3% and 25.6% of the patients had multiple infections, respectively, and the combined rate was 35.6%. Comparisons of MSP-3α sequences from nine clones further confirmed the high level of genetic diversity of the parasite and also suggested that geographic isolation may exist. These results strongly indicate that *P. vivax* populations are highly diverse and multiple clonal infections are common in this malaria-hypoendemic region of Thailand.

INTRODUCTION

Plasmodium vivax causes most of the malaria morbidity in endemic regions of Central and South America, North Africa, and Asia.[1] In many parts of Southeast Asia, the incidence of *P. vivax* malaria has increased in recent years. Currently, approximately half of the malaria cases in Thailand are the result of *P. vivax* infections alone and mixed infections by *P. vivax* and *P. falciparum* (Sirichaisinthop J, unpublished data). Such an increasing trend of *P. vivax* malaria and the emergence of drug-resistant strains of the *P. vivax* parasite are a major concern for future malaria control. Scientific communities have made great efforts in developing effective malaria vaccine and antimalarial drugs. Information on parasite genetic diversity of field populations is essential for these tasks.[2,3]

Malaria epidemiology, as the result of the complex interplay among humans, vectors, and hosts, varies considerably in different geographic regions. Results from extensive research on *P. falciparum* population structure in the last decade have demonstrated that the parasite populations are highly heterogenetic in both hyperendemic regions and areas where malaria transmission is seasonal. This knowledge has contributed greatly to understanding the dynamics of the disease transmission, which in turn guides our decisions in drug applications and disease control.[2,3] The majority of publications on *Plasmodium* genetic structure focus on *P. falciparum,* using polymorphic markers such as the merozoite surface protein-1 (MSP-1), MSP-2, glutamate-rich protein, and microsatellites. For *P. vivax*, only the dimorphic circumsporozoite protein (CSP) gene and the *MSP-1* gene have been widely used for genotyping. For example, *P. vivax MSP-1* has been used to determine whether a *P. vivax* infection is the result of a new infection or a relapse.[4] Specifically, the *P. vivax CSP* gene has a central repeat domain that is different in sequence and number of the repeat units.[5–8] Two major types, VK210 and VK247, have a worldwide distribution, with VK210 as the major genotype. Antibodies to *P. vivax* CSP protein are readily detected in patients from Thailand and neighboring countries.[9] For the *P. vivax MSP-1* gene, the variable region between interspecies conserved blocks 5 and 6 has been used to genotype *P. vivax* isolates from many different geographic regions, where at least five types have been discovered.[10–15] These studies demonstrate the high levels of heterogeneity of *P. vivax* field populations.

Due to the dimorphic nature of the central repeat region of the *P. vivax CSP* gene, classifying field isolates into either VK210 or VK247 genotype does not provide a good estimate of the genetic diversity of *P. vivax* populations. Genotyping the extremely polymorphic *P. vivax MSP-1* gene relies almost entirely on sequencing. Therefore, there is a need for the development of other molecular markers that are more suitable for rapid genotyping. One such marker is the *MSP-3*α gene because its polymorphism can be readily evaluated by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis.[16,17] The *MSP-3*α gene encodes a merozoite surface protein with an alanine-rich central domain that is predicted to form a coiled-coil tertiary structure.[18] Polymerase chain reaction amplification of this gene from field isolates demonstrates product length polymorphism, and digestion of the PCR products with restriction endonucleases indicates the presence of great numbers of alleles.[16] In this study, we used the dimorphic *P. vivax CSP* and *MSP-3*α markers to evaluate parasite genetic diversity in field *P. vivax* populations in a hypoendemic area in western Thailand. Our study showed that despite low transmission intensity, the parasites displayed a high level of genetic complexity. In addition, at least 35% of patients had multiple infections by different *P. vivax* genotypes. Comparisons of *MSP-3*α sequences from Thai and South American *P. vivax* isolates suggest substantial geographic variations among parasite isolates.

MATERIALS AND METHODS

Study area and sample collection. The study area was in the Mae Sod district, Tak Province, Thailand. Malaria parasite samples used for genotyping were collected from patients at-

tending the clinics from April 2000 through June 2001. Sample collection was approved by the Ethical Review Committee of the Armed Forces Research Institute of Medical Sciences under the auspices of the Ministry of Public Health in Thailand. Approximately 200 μL of blood was spotted and dried on filter paper. Extraction of parasite DNA from filter paper samples was carried out following the method using saponin-Chelex solution.[19]

Malaria incidences and association with climatic factors. Data on malaria monthly incidences were collected in two malaria clinics in Mae Sod for the year 2000 and 2001. All the patients exhibiting febrile symptoms were diagnosed with malaria by light microscopy of Giemsa-stained thick blood smears. Three malaria species, *P. falciparum*, *P. vivax*, and *P. malariae*, were distinguished based on morphology by an experienced microscopist. Mixed species infections were further confirmed by a nested PCR using species-specific primer sets designed from ssrRNA genes of the four human malaria species.[20] To determine the association between *P. vivax* incidences with climatic variables, daily temperature and rainfall were obtained from the Surface Data database of the World Meteorological Organization (http://www.ncdc.noaa.gov/oa/climate/climatedata.html). Rainy (April 20–October 31) and dry (November 1–April 19) seasons were defined based on the daily precipitation data.

Genotyping of *P. vivax* isolates at the *CSP* locus. The repeat region of the *CSP* gene was genotyped for the two major types VK210 and VK247 by PCR and molecular hybridization.[5] The PCR amplification of the *CSP* repeat region was performed as previously described with the following modifications.[5] Specimens were denatured for two minutes at 95°C, amplified through 45 cycles for 30 seconds at 95°C, 30 seconds at 45°C, and two minutes at 72°C, and finally incubated for two minutes at 72°C. The *CSP* genotypes were determined by slot-blot hybridization of the PCR products using the fluorescein-labeled probes derived from the repetitive sequences of either VK210 or VK247 type as previously reported.[21]

Genotyping of *P. vivax* isolates at the *MSP-3α* locus. Allelic diversity of the *MSP-3α* gene was studied using the PCR-RFLP method described by Bruce and others.[16] The *MSP-3α* gene was amplified by a nested PCR and 4 μL of the PCR product was isolated by electrophoresis on a 1.0% agarose gel. For RFLP analysis, only *Hha* I was used for digestion because *Alu* I did not produce clear restriction patterns in some samples during preliminary analysis. Approximately 5 μL of the PCR product was digested with *Hha* I and analyzed by electrophoresis on a 1.8% agarose gel. Multiple infections were distinguished when the summed size of the DNA fragments resulting from *Hha* I digestion exceeded the size of the uncut PCR product.[16,17] Major alleles were classified based on the differences in restriction banding patterns. For multiple infections, genotypes of the parasite were assigned to individual allele groups by comparing the RFLP patterns with those of single infections.[17]

Analysis of *MSP-3α* gene sequences. A total of nine PCR products were cloned into the pZero vector (Invitrogen, Carlsbad, CA) and sequenced using the BigDye terminator kit (Applied Biosystems, Foster City, CA). Each PCR product was sequenced in both directions for accuracy. The sequences were assembled and aligned using PILEUP of the Genetics Computer Group (GCG) software, version 10.1 (University of Wisconsin, Madison, WI). The *MSP-3α* sequence of the Belem strain was used as the reference sequence (accession number AF093584).[18] A phylogenetic tree was derived from the aligned nucleotide and deduced amino acid sequences using neighbor-joining method of the Phylogenetic Analysis Using Parsimony (PAUP) program, version 4.0 (University of Washington, Seattle, WA).

Statistical analysis. Association between monthly malaria incidences and climatic variables was analyzed by cross-correlogram. The Chi square test was used to compare the frequencies of individual alleles between the rainy and dry seasons.[22]

RESULTS

Malaria prevalence in northwest Thailand. We have analyzed malaria case data from the two clinics in Mae Sod, located near the Thailand-Myanmar border. As one of the major factors of morbidity, malaria in this area is perennial with the peak transmission season in May–August, following the patterns of rainfall (Figure 1). In addition to the apparent association between the peaks in monthly rainfall and malaria incidence in July–September, the cross-correlation function analysis indicated a significant one-month lagged correlation between precipitation and malaria incidence ($R = 0.55$, $P < 0.01$). A total of 7,089 and 6,670 cases were recorded for the years 2000 and 2001, respectively. All four human malaria species were detected in this area with *P. falciparum* and *P. vivax* as the predominant species (99%). In Mae Sod, the 2000–2001 data indicated that *P. vivax* had become equally prevalent as *P. falciparum* (>46%), consistent with the trend in the whole country.

The prevalence of the CSP variants VK210 and VK247. *Plasmodium vivax* parasites from 90 febrile patients were successfully genotyped for the two major CSP alleles. A total of 113 parasite genotypes could be recognized, from which the frequencies of the VK210 and VK247 were 77% and 23%, respectively (Table 1). The majority (96.7%) of VK247 genotypes occurred as multiple infections, consistent with the result of an earlier study conducted in different regions of Thailand.[23] The proportion of VK210 was slightly higher in

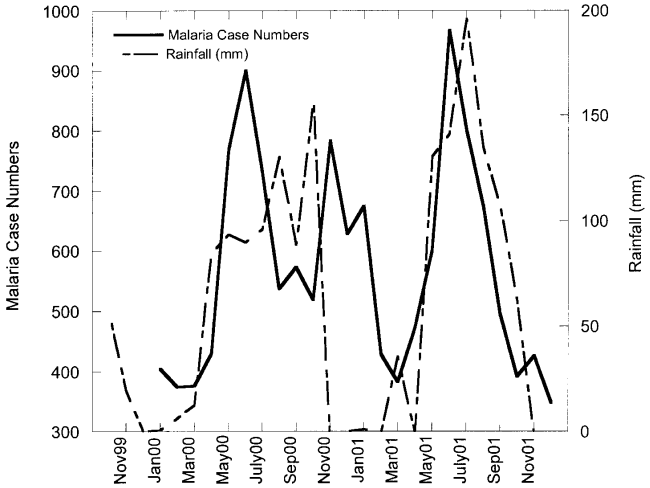

FIGURE 1. Dynamics of monthly rainfall and malaria case numbers in 2000 and 2001 at two malarial clinics in Mae Sod, Thailand.

TABLE 1

Seasonal frequency (%) of the two *Plasmodium vivax CSP* types and 13 *MSP-3*α alleles distinguished by PCR-RFLP analysis using *Hha* I*

Alleles	Total (%)	Rainy seasons (%)	Dry seasons (%)
CSP			
VK210	77.0	83.0	71.7
VK247	23.0	17.0	28.3
*MSP-3*α			
A1	16	12.5	19.2
A2	4	6.2	1.9
A3	4	6.2	1.9
A4	11	8.3	13.5
A5	7	10.4	3.8
A6	18	16.7	19.2
A7	6	8.3	3.8
A8	3	4.2	1.9
A9	3	0	5.8
A10	2	4.2	0
B1	5	4.2	5.8
B2	1	2.1	0
C	20	16.7	23.1

* *CSP* = circumsporozoite protein; *MSP-3*α = merozoite surface protein-3α; PCR-RFLP = polymerase chain reaction–restriction fragment length polymorphism. A total of 90 and 88 samples were used for *CSP* and *MSP-3*α genotyping, respectively. Chi-square analysis did not detect significant seasonal differences between the two *CSP* types ($\chi^2 = 3.07$, degrees of freedom [df] = 1, $P > 0.05$) and major *MSP-3*α alleles (A1, A4, A6, and C) ($\chi^2 = 2.69$, df = 5, $P > 0.05$).

the rainy season (83.0%) than in the dry season (71.6%) ($\chi^2 = 3.07$, degrees of freedom [df] = 1, $P > 0.05$). Genotyping of the *CSP* gene showed that 23 of 90 patients (25.6%) had multiple infections with different *P. vivax* genotypes.

Diversity of the *MSP-3*α gene. Eighty-eight of 90 malaria samples (97.8%) were successfully amplified for the *MSP-3*α gene. Based on the size of the PCR products, three major types, A (1.9 kilobases [kb], B (1.5 kb), and C (1.1 kb), were identified (Figure 2). A total of 107 parasite genotypes could be recognized from the 88 samples by PCR analysis of the *MSP-3*α gene because some patients had infections by more than one parasite genotype. Type A, which corresponded to the expected size of the published sequence of the Belem laboratory strain, was the most predominant (74.8%). The other two types, B and C, accounted for 6.5% and 18.7% of the parasite genotypes, respectively. The frequencies of the three *MSP-3*α types were consistent with those (70.5%, 6.7%, and 22.8%) found in Papua New Guinea.[17]

Major alleles of the *MSP-3*α gene and their frequencies in field parasite populations. The PCR-RFLP analysis of the *MSP-3*α gene further demonstrated that *P. vivax* parasites in western Thailand were highly diverse. The clear restriction patterns produced from digestion with *Hha* I from the majority of the samples makes this enzyme our prime choice in this study (Figure 2). The PCR-RFLP patterns of all samples showed a band of ~1.0 kb. Although this band is also slightly polymorphic, it was not included for distinguishing different *MSP-3*α alleles because the size difference was not easily resolved with our electrophoresis conditions. Smaller bands ranging from 190 to 500 basepairs (bp) were used for RFLP analysis, from which at least 13 allele groups (A1–A10, B1, B2, and C) could be identified (Figure 3). Alleles A1, A4, A6, and C were most abundant, accounting for 65% of the samples (Table 1). By comparison, we found that at least seven alleles were common to those from the previous studies,[16,17] suggesting that some parasite genotypes may have a worldwide distribution. To test whether there was seasonal fluctuations in allele frequency, we compared the frequencies of different alleles between the rainy and dry seasons. The chi square test did not detect significant difference in allele frequencies between the two seasons ($\chi^2 = 2.69$, df = 5, $P > 0.05$). With this genotyping method, 19.3% of the patients were found to have multiple infections by different *P. vivax* genotypes, including nine multiple infections not identified by typing of only the *P. vivax CSP* gene. We must admit that although it was easy to identify multiple infections by this method, it was not possible to always unambiguously assign the multiple isolates to specific allele groups. When genotyping results at these two genetic loci are combined, the multiple infection rate increased to 35.6%.

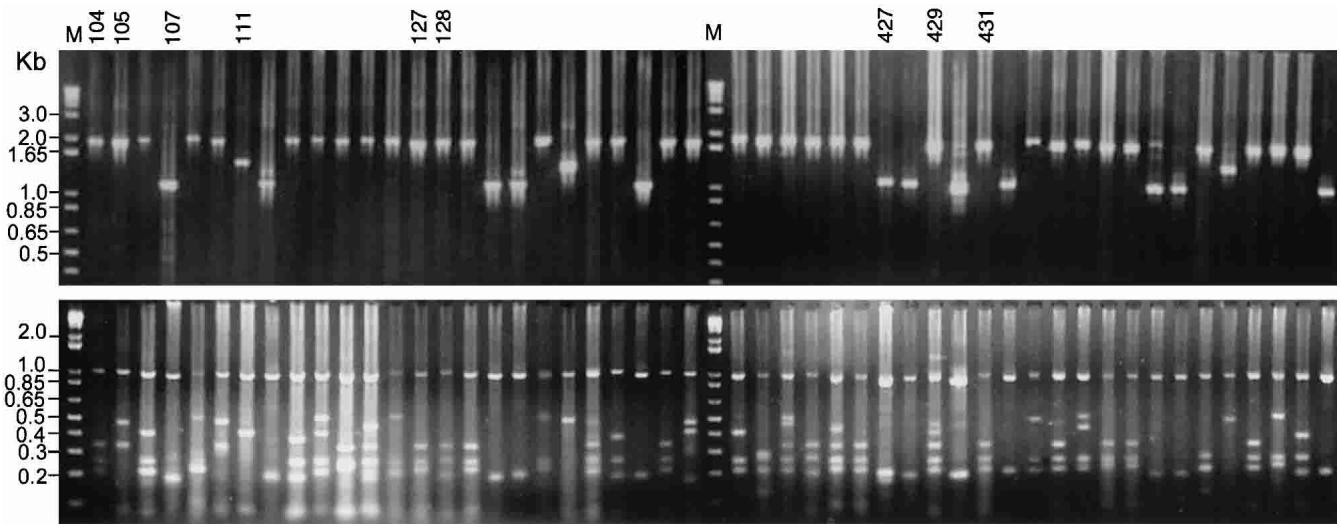

FIGURE 2. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) typing of the *Plasmodium vivax* isolates using the merozoite surface protein-3 α (*MSP-3*α) gene. **Upper panel**, undigested PCR products amplified using primers N1 and N2 (Figure 4). **Lower panel,** PCR products digested with *Hha* I. Lane M = 1 kilobase (Kb) DNA marker with the fragment sizes indicated (Invitrogen). The nine parasite isolates from which the *MSP-3*α gene was sequenced are indicated.

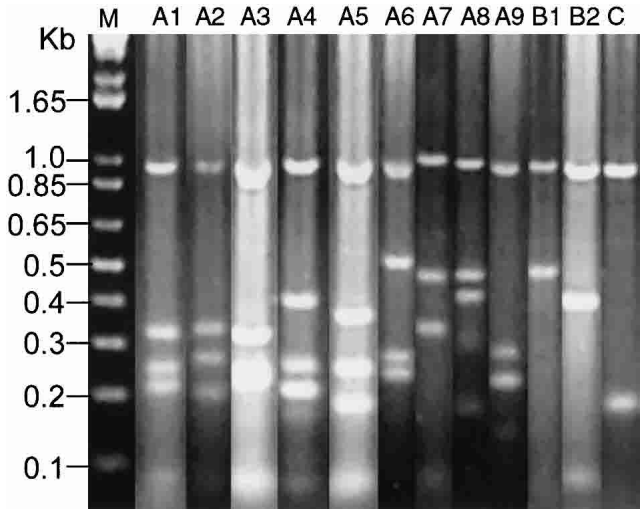

FIGURE 3. Major merozoite surface protein-3α alleles identified by a polymerase chain reaction and digestion with *Hha* I in the *Plasmodium vivax* population from Mae Sod, Thailand. Lane M = 1 kilobase (Kb) DNA marker. The nine alleles of type A, two alleles of type B, and one allele of type C are labeled with the suffixes A, B, and C, respectively. Frequencies of these alleles during dry and rainy seasons are presented in Table 1.

Analysis of *MSP-3α* gene sequences. Nine PCR products (6 Type A, 2 Type B, and 1 Type C) corresponding to nucleotides 205-2100 of *P. vivax MSP-3α* gene of the Belem strain[18] were sequenced (Figure 4). This region covers the complete central alanine-rich domain.[18] Of the 6 Type A clones, two clones (104 and 105) had the same length as the Belem strain (1,908 bp), whereas the other four clones (127, 128, 429, and 431) were 1,887, 1,893, 1,920, and 1,896 bp, respectively. The only Type B clone sequenced (111) was 1,449 bp, and was missing a 458-bp fragment corresponding to nucleotide sequence 399-856 of the Belem strain. The two Type C clones (107 and 427) were 1,137 and 1,149 bp, respectively. Both sequences had a 747-bp deletion corresponding to nucleotide sequence 475-1221 of the Belem strain. Sequence alignment demonstrated great variations of the *MSP-3α* gene among individual parasite genotypes in the Mae Sod district. Higher levels of sequence polymorphism were observed in sequences closer to the 5′ end of the central alanine-rich domain. Particularly, sequence polymorphism was clustered at nucleotides 298-618, 801-922, 1075-1160, and 1727-1749. Except for the polymorphic region at 1727-1749, sequences after the deletion of the Type C genotypes were highly conserved. It is noteworthy that most nucleotide substitutions at 1727-1749 were at the second codon positions, causing amino acid changes. As a result of these nonsynonymous substitutions, the amino acid sequences of this region were dimorphic with either KEATAAKL or TAANVVKD. Analysis of nine *MSP-3α* gene sequences suggested that the two variant sequences were present equally in the *P. vivax* field populations from western Thailand.

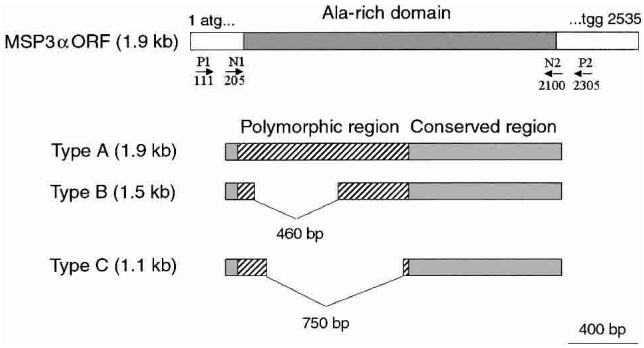

FIGURE 4. Schematic representation of the sequence variations and deletions of the merozoite surface protein-3 α (*MSP-3α*) gene of *Plasmodium vivax* among field isolates. The 1.9-kilobase (kb) nested polymerase chain reaction product covering the entire alanine (Ala)–rich domain of the *MSP-3α* gene is indicated by the **shaded** region at the top. Positions of the outer (P1 and P2) and the inner (N1 and N2) primers are labeled below the *MSP-3α* open reading frame (ORF). The polymorphic region (**hatched**) and the conserved region (**shadowed**) are indicated. The positions of the deletions present in types B and C are depicted. bp = basepairs.

Both the aligned nucleotide and deduced amino acid sequences were used for phylogenetic analysis. An unrooted tree was drawn using the neighbor-joining method of the PAUP program (Figure 5). It is noteworthy that the deletions were removed from the sequences used for phylogenetic analysis. Both DNA and protein sequences predicted the same tree. Four sequences (127, 427, 429, and 431) were not assigned to individual branches in the tree. The three major

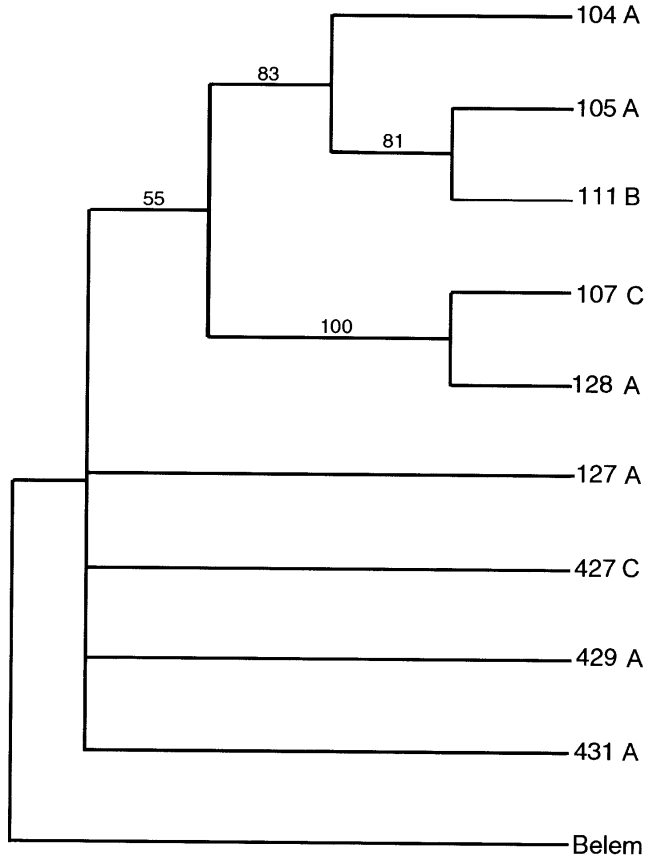

FIGURE 5. Phylogenetic relationships among the 10 haplotypes of the *Plasmodium vivax* merozoite surface protein-3α (MSP-3α) gene. Nine sequences of the *MSP-3α* central region (corresponding to the polymerase chain reaction product using primers N1 and N2) of the Thai isolates were compared with that of the Belem strain.[18] Bootstrap support (>50%) is shown as the percentage from 1,000 pseudoreplications.

types based on PCR length polymorphism were mixed instead of falling into separate groups, suggesting that the deletion mutations may have arisen in multiple parasite lines. Although geographic structure in the parasite populations in Mae Sod can not be tested here, the Thai parasite genotypes in this study were more distantly related to the South American Belem strain (Figure 5).

DISCUSSION

In western Thailand, malaria is hypoendemic and characterized by the presence of all four parasite species. In recent years, *P. vivax* infections have become more prevalent than *P. falciparum* infections in some regions of Thailand. Mixed infections by two parasite species have been found in more than 20% of malaria cases,[19] which is comparable to the situation in Papua New Guinea.[17] Using a combination of two molecular markers, the *CSP* gene and the *MSP-3*α gene, we investigated the genetic diversity of *P. vivax* populations from an area in western Thailand with low malaria endemicity. These results demonstrated that even in areas of low transmission intensities, *P. vivax* displayed high levels of genetic diversity. With two molecular markers, slight, but not significant, seasonal fluctuations of major parasite genotypes were observed. Moreover, the presence of common alleles in different geographic areas indicated a global distribution of certain parasite genotypes, which may have been maintained through positive selection by hosts. In spite of this, sequence analysis of the *MSP-3*α gene suggests that some variations might be regionally biased.

To determine the distribution of the two *P. vivax CSP* genotypes VK210 and VK247, multiple surveys have been carried out using both a PCR for genotyping parasites in human blood and an enzyme-linked immunosorbent assay (ELISA) for detecting parasite CSP variants in mosquitoes.[23–26] The results from these surveys have demonstrated a global distribution of the two *P. vivax* CSP genotypes in many malaria-endemic regions, although the prevalence of individual genotypes varies geographically. Our previous study using an ELISA on sporozoites produced by feeding mosquitoes on patients infected with *P. vivax* in Thailand indicates an overall predominance of VK210 (70.5%),[23] and the present study by genotyping parasites in human blood showed a similar result, with 78% of the parasites as VK210 type. These results are in sharp contrast to the result of an earlier study in Thailand, in which VK247 was estimated to be the predominant type (83%).[25] This difference may be due to sampling biases or temporal fluctuations of individual genotypes. Such a significant decrease over time in the proportions of VK247 has also been observed in a two-year investigation performed in a malaria-hyperendemic area of Papua New Guinea in both the mosquito and human populations.[24] This phenomenon may be attributed to selection by host immune pressure on a particular genotype, and/or the preferential production of sporozoites carrying a specific CSP variant in a mosquito species. In a recent study on *P. vivax* malaria in Mexico, different anopheline mosquitoes were found to be differentially susceptible to infections by VK210 and VK247 variants.[27] This explains very well the correlation between the prevalence of two CSP phenotypes with the distribution of different vector species.[28] An earlier longitudinal survey over a five-year period[23] and this study spanning two seasons both showed seasonal variations in the prevalence of VK210/VK247, although the tendency of this fluctuation was different. Seasonal population dynamics of different mosquito vectors, which are not equally susceptible to infections by VK210 or VK247 parasite isolates, may be one of the factors contributing to this seasonal fluctuation.

The *P. vivax MSP-3*α gene has been developed as a molecular marker for epidemiologic studies of *P. vivax* malaria.[16] In evaluating genetic diversity and dynamics of the parasite population in asymptomatic patients in Papua New Guinea, Bruce and others[17] identified 24 alleles of the *MSP-3*α gene in *P. vivax* based on PCR-RFLP analysis using two restriction enzymes. In this study, we identified at least 13 alleles based on *Hha* I digestion alone, similar to the number of alleles identified by *Hha* I digestion in the study by Bruce and others.[17] This suggests that *P. vivax* populations in Thailand are equally diverse in spite of the low endemicity of the disease. This seems to contradict the results from studies on *P. falciparum*, in which a relationship between parasite diversity and transmission intensity has been established.[29,30] Many factors may explain such a high level of genetic diversity of the *P. vivax* parasite. First, some intrinsic biologic properties of the *P. vivax* malaria such as relapse and early gametocytemia favor the cross-fertilization and meiotic recombination of distinct parasite genotypes within vector mosquitoes. Second, the migration of parasite-carrying foreign workers from Myanmar also adds to the gene pool of the parasite. Third, drug resistance might further affect the duration of infection and enhance genetic recombination in mosquitoes, although there is no clear indication of drug resistance for *P. vivax* parasite in the study area. Based on the analysis of *P. falciparum* populations in the same hypoendemic region of Thailand, Paul and others[31] argued that the relationship between the genetic structure of the malaria parasite populations and transmission intensity might be nonlinear. The results of our present study further support this argument.

Sequence analysis of the *MSP-3*α clones identified the regions of deletions for the Type B and C variants. Both variants have deletions close to the N-terminus of the central alanine-rich domain. Inasmuch as both variant types have been found in isolates from other malarial regions, it is reasonable to assume that these deletions are not essential for the survival of the parasite. However, it may have reduced their fitness because the two variants are present in less than 30% of parasite genotypes. Comparing with Type C, Type B has an additional deletion of 26 amino acids upstream of the type C deletion. This portion may be more important than the region of Type C deletion, since less than 6% of parasite genotypes belong to this type. It is noteworthy that the regions with deletions are the most polymorphic, which suggests that this region of the molecule might be selected against by the host immune systems. At present, the limited numbers of *MSP-3*α gene sequences available would not allow us to perform a detailed phylogenetic comparison of geographically separated clones. However, the fact that a South American clone is more distantly related to the Thai clones suggests that certain degrees of geographic isolation may exist. This preliminary analysis will serve as the foundation for future detailed studies of population structure of *P. vivax* in different geographic regions.

The multiplicity of malaria infections, i.e., one person simultaneously infected by more than one parasite genotype, is

common in endemic areas and particularly high in holoendemic areas. This can arise from co-infection, super-infection, or even somatic mutation during the course of infection. In many African regions with high perennial malaria transmission, the multiplicity of *P. falciparum* infections can reach very high levels.[32] The proportions of multiple *P. vivax* infections have been estimated in Papua New Guinea, India, Brazil, and Thailand, but it is difficult to compare these values due to differences in sampling and genotyping methods.[14,17,23,33,34] However, it could be high in particular cases, since as many as six parasite genotypes have been found in a single host in Papua New Guinea.[17] As concluded from this and several earlier studies, using several markers allows the detection of more multiple infections than using a single marker.[14] In Papua New Guinea, the combination of MSP-1, CSP, and Duffy antigen binding protein identified multiple infection in 65% of the samples.[14] In Thailand, our study using two molecular markers identified 35.6% multiple infections. The difference in multiple infection rates in different malaria-endemic regions may reflect the difference in malaria transmission intensity.[29] In Thailand, our current study found that the overall percentage of multiple infections (35.6%) was close to that from our previous study (29.5%),[23] but less than the 58% found in an earlier study.[25] Furthermore, our result is in sharp contrast to the scarcity of multiple infections found in similarly hypoendemic areas of Sri Lanka using a panel of monoclonal antibodies,[35] suggesting that multiplicity of infection is influenced by many factors that differ geographically. Nevertheless, the high levels of parasite population diversity and lack of significant seasonal variations of major parasite alleles suggest that the parasite population structure might be maintained in this hypoendemic area. Therefore, the causes of high parasite heterogeneity and detailed knowledge of malaria epidemiology in this hypoendemic area of Thailand deserve further study.

Received November 21, 2002. Accepted for publication January 9, 2003.

Acknowledgments: We thank Dr. Kuijun Zhao, Christy Pepple, and Jennifer Sommer for their assistance in sequencing some MSP-3α clones.

Financial support: This study was partially supported by a grant R01 AI-46472-02S1 from the Research Supplement for Underrepresented Minorities Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health to Liwang Cui, and grants R01 AI-50243 and D43 TW01505 to Guiyun Yan.

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