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# *Plasmodium falciparum* populations from northeastern Myanmar display high levels of genetic diversity at multiple antigenic loci

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ABSTRACT

Levels of genetic diversity of the malaria parasites and multiclonal infections are correlated with transmission intensity. In order to monitor the effect of strengthened malaria control efforts in recent years at the China–Myanmar border area, we followed the temporal dynamics of genetic diversity of three polymorphic antigenic markers *msp*1, *msp*2, and *glurp* in the *Plasmodium falciparum* populations. Despite reduced malaria prevalence in the region, parasite populations exhibited high levels of genetic diversity. Genotyping 258 clinical samples collected in four years detected a total of 22 PCR size alleles. Multiclonal infections were detected in 45.7% of the patient samples, giving a minimum multiplicity of infection of 1.41. The majority of alleles experienced significant temporal fluctuations through the years. Haplotype diversity based on the three-locus genotypes ranged from the lowest in 2009 at 0.33 to the highest in 2010 at 0.80. Sequencing of *msp*1 fragments from 36 random samples of five allele size groups detected 13 different sequences, revealing an additional layer of genetic complexity. This study suggests that despite reduced prevalence of malaria infections in this region, the parasite population size and transmission intensity remained high enough to allow effective genetic recombination of the parasites and continued maintenance of genetic diversity.



## 1. Introduction

Malaria continues to be a major public health problem in the developing world. Globally, there are estimated 225 million malaria infections each year, resulting in around a million deaths (WHO, 2011). While most of malaria burden is in sub-Saharan Africa, Southeast (SE) Asia accounts for 10% of global malaria morbidity and 5% of global mortality from 2008. Within the SE Asian region, the Greater Mekong Subregion (GMS) has been one of the most serious foci of malaria, with immense geographical heterogeneity in disease endemicity, complex vector systems, and the co-existence of *Plasmodium falciparum* and *Plasmodium vivax* (Cui et al., 2012a). Furthermore, the GMS is also a hotspot of multidrug resistant *P. falciparum*, and recent detection of artemisinin resistance in this area has raised considerable global concern (Dondorp et al., 2009; Noedl et al., 2008). Another notable feature of malaria epidemiology in the GMS is "border malaria", with most of the malaria cases concentrated along international borders. Cross-border human migration, which is difficult to monitor and responsible for malaria reintroduction, is an important challenge for malaria control. Therefore, knowledge of accurate malaria epidemiology at both macro and micro scales is needed for targeted malaria control, especially during the elimination phase.

Globally, malaria parasite populations exhibit great genotypic and phenotypic diversity (Kemp et al., 1990), which allows the parasites to overcome antimalarial drugs, vaccines and vector control strategies. Allelic polymorphism and sexual reproduction, which is responsible for both classical meiotic recombination and nonreciprocal recombination events, contribute to the generation of genetic diversity. Many cross-sectional studies have established

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a correlation between transmission intensity and genetic diversity (Hoffmann et al., 2001; Paul et al., 1998). In hyperendemic areas, patients tend to harbor more than one parasite isolate, which favors genetic recombination and generates higher genetic diversity. Conversely, in areas of low endemicity, parasite populations experience significant levels of inbreeding. In addition to transmission intensity, other factors such as selective sweeps, resulting from vaccines and drugs, can greatly influence genetic diversity. Such variability in transmission intensity and genetic diversity may have important consequences with regards to the effectiveness of control measures (such as vaccine deployment) as well as the spread of antimalarial resistance.

Historically, the China–Myanmar border areas have been considered malaria hyperendemic, exhibiting the characteristic "border malaria" phenomenon (Cui et al., 2012a). This region has also had quite different antimalarial drug policies in the past, and drug selection has resulted in parasite genotypes that are drastically different from other parts of the GMS (Meng et al., 2010; Yang et al., 2011). In recent years, with increased funding to control malaria from governments and the Global Fund to Fight against AIDS, Tuberculosis and Malaria, malaria control efforts in this region have intensified. Among the control measures are insecticide-treated bednets and artemisinin-based combination therapy (ACT) for *P. falciparum* malaria. As a result, malaria incidence has been significantly curtailed (Cui et al., 2012b). Whereas accurate malaria epidemiology data in the neighboring Myanmar regions are not available, malaria situation in this region is well reflected in the reduction in malaria cases in the border counties of Yunnan province in recent years (Clements et al., 2009). In addition, China is mobilizing toward malaria elimination in the near future. It is therefore critical to evaluate the impact of the control on malaria epidemiology in this region.

In this study, we assessed the genetic diversity of the *P. falciparum* parasites from the China–Myanmar border area near Laiza, Myanmar. Using longitudinal clinical samples, we studied the allelic diversity of genes encoding the polymorphic antigens, including merozoite surface protein (*msp*)-1 and -2, as well as glutamate-rich protein (*glurp*), and determined the temporal dynamics of their prevalence in this region.

## 2. Materials and methods

### 2.1. Collection of clinical parasite samples

*P. falciparum* samples were collected in 2007–2010 from patients presenting with uncomplicated malaria infection at two malaria clinics in Laiza township at the China–Myanmar border area. These malaria clinics, located ~0.8 km apart, provide malaria diagnosis and treatment to patients in a catchment area of ~20 km radius. In this area, malaria transmission occurs perennially but is most intense during the rainy season from April to October. Samples from these two clinics were pooled for analysis. A total of 284 *P. falciparum* clinical isolates from patients aged 3–66 were collected. The samples collected in different years were not from the same patients. Only patients with clinical symptoms and microscopically confirmed *P. falciparum* infection were enrolled in this study. Written informed consent was obtained from the participants or guardians. The human subjects protocol for this study was approved by the Institutional Review Board of Kunming Medical University. Malaria infection was diagnosed by microscopic examination of Giemsa-stained thick and thin blood films. If *P. falciparum* infection was confirmed, 0.2 ml of finger-prick blood was spotted on a piece of Whatman 3MM filter paper and used for molecular studies.

### 2.2. Allelic typing of P. falciparum msp1 and msp2, and glurp genes

For genotyping, parasite genomic DNA was extracted from the filter papers by using a QIAamp DNA microkit (Qiagen, Germany) following the manufacturer's instructions. DNA was eluted in 80 μl of elution buffer. DNA was genotyped at *msp*1 (block 2), *msp*2, and *glurp* using nested PCR by previously described methods (Kaneko et al., 1997; Viriyakosol et al., 1995). The primary PCR products were used in nested reactions with allele family-specific primers for K1, MAD20, and RO33 of *msp*1, and for FC27 and 3D7 of *msp*2. Final PCR products were separated in 2% agorose gel, stained with ethidium bromide and visualized under UV light. The lengths of the PCR products were estimated based on their mobility relative to the DNA size standard (TaKaRa, Japan). DNA fragment sizes were binned into different classes of ~50 bp ranges with each bin assigned as an allele.

### 2.3. Estimation of parasite clone numbers per sample

The minimum number of parasite clones per sample, or the multiplicity of infection (MOI), was defined as the largest number of alleles at any one locus detected in the sample (Paganotti et al., 2004b). For this study, alleles were identified based only on type and fragment size of the amplified PCR products. If an isolate had one allele at each of the three loci, this sample was considered to have monoclonal infection. This method is conservative and may underestimate the number of clones likely to be present in the samples. Yet, this may best reflect the level of endemicity in this area. The tri-allelic haplotypes and their relative annual frequencies were assessed only from infections with no more than one locus showing multiclonal infections.

### 2.4. Sequence analysis of genetic polymorphism of msp1 block 2

In order to further examine the genetic diversity of the parasite population, we sequenced *msp*1 block 2 DNA fragments from PCR products showing similar molecular sizes in agarose gels. The PCR products were purified from the gel using a Gel extraction mini kit (Watson Biotechnologies, Inc.) and prepared for sequencing. Partial gene sequences were obtained from each end of the PCR product using the same primers for the nested PCR reactions. DNA sequences were assembled and BLAST searched in GenBank to identify identical or similar sequences.

### 2.5. Statistical analysis

We compared counts of samples by MOI status, genotype, year using Pearson's $\chi^2$ test and Fisher's exact test. The Student–Newman–Keuls test was used for a post hoc analysis of the MOI data across the study years.

## 3. Results

### 3.1. Size polymorphisms of msp1, msp2 and glurp

Of the 284 patient samples collected, 3.2% (*msp*1), 4.9% (*msp*2), and 1.4% (*glurp*) failed to produce PCR results, while 258 were successfully genotyped at all three loci. The PCR fragment sizes for *msp*1, *msp*2 and *glurp* were 150–300 bp, 250–550 bp, and 500–1000 bp, respectively (Fig. 1). Both *msp*1 and *msp*2 showed eight size classes, whereas *glurp* had six size classes. For *msp*1 and *msp*2, all allele types identified earlier (K1, MAD20, and RO33 for *msp*1, and IC/3D7 and FC27 for *msp*2) were amplified, with each further subdivided into multiple size alleles. For *msp*1, the overall frequencies of the K1, MAD20 and RO33 allele types were