Malaria in the China-Myanmar border region is still severe; local transmission of both falciparum and vivax malaria persists, and there is a risk of geographically expanding antimalarial resistance. In this research, the pfmdr1, pfcrt, pvmdr1, and K13-propeller genotypes were determined in 26 Plasmodium falciparum and 64 Plasmodium vivax isolates from Yingjiang county of Yunnan province. The pfmdr1 (11.5%), pfcrt (34.6%), and pvmdr1 (3.1%) mutations were prevalent at the China-Myanmar border. The indigenous samples exhibited prevalences of 14.3%, 28.6%, and 14.3% for pfmdr1, respectively, whereas the samples from Myanmar showed prevalences of 10.5%, 21.1%, and 5.3%, respectively. The most prevalent genotypes of pfmdr1 and pfcrt were Y86Y184 and M74N75T76, respectively. No pvmdr1 mutation occurred in the indigenous samples but was observed in two cases coming from Myanmar. In addition, we are the first to report on 10 patients (38.5%) with five different K13 point mutations. The F446I allele is predominant (19.2%), and its prevalence was 28.6% in the indigenous samples of Yingjiang county and 15.8% in samples from Myanmar. The present data might be helpful for enrichment of the molecular surveillance of antimalarial resistance and useful for developing and updating guidance for the use of antimalarials in this region.
of Whatman filter paper (3MM) and air dried. The samples were labeled with study numbers, names, and dates and stored at −20°C until DNA extraction.

Preparation of DNA template from blood samples. Parasite genomic DNA from all blood spot samples collected in microcentrifuge tubes was extracted by use of a QIAamp DNA blood kit (Qiagen, Valencia, CA), following the dried blood spot protocol provided in the kit. The known polymorphisms pfmdr1 and pfcr were assessed. Also, we investigated the mutation of the PF3D7_1343700 kelch propeller domain (PF13_0238, also called K13 propeller), a molecular marker of artemisinin resistance. Sequences were evaluated using nested PCR followed by restriction fragment length polymorphism (RFLP) analysis, as described previously (14, 18). The pfmdr1 single nucleotide polymorphisms (SNPs) at 976 were detected using a DNA template mismatch primer method (19). Polymorphisms were analyzed by Shanghai DNA Biotechnologies Co., Ltd. (Shanghai, China). Sequences were analyzed by the BLAST program (http://blast.ncbi.nlm.nih.gov/). Multiple nucleotide sequence alignments and analysis were performed using the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Data analysis. Data were analyzed using Microsoft Excel 2003 and SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). The map was created by using ArcGIS 10.1 (Environmental Systems Research Institute, Inc.). The Fisher exact test was used to assess the differences in the gene polymorphisms between indigenous cases and cases from Myanmar. The P values were calculated, and results were considered statistically significant when P was <0.05.

Ethical considerations. The study was reviewed and approved by the ethical committee of the Chinese Centre for Disease Control and Prevention (China CDC).

RESULTS
Study samples. A total of 90 malaria cases were included in this study: 64 P. vivax and 26 P. falciparum. The P. vivax cases were composed of 18 indigenous cases and 46 cases from Myanmar, while the P. falciparum cases were composed of 7 indigenous cases and 19 cases from Myanmar (Fig. 1).

Table 1. Selection of P. falciparum and P. vivax polymorphisms

<table>
<thead>
<tr>
<th>SNP</th>
<th>No.</th>
<th>Total</th>
<th>%</th>
<th>No.</th>
<th>Total</th>
<th>%</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfmdr1 N86Y</td>
<td>1</td>
<td>4</td>
<td>28.6</td>
<td>3</td>
<td>17</td>
<td>18.8</td>
<td></td>
</tr>
<tr>
<td>pfcr M741</td>
<td>1</td>
<td>4</td>
<td>28.6</td>
<td>5</td>
<td>24</td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td>pfcr N75E</td>
<td>0</td>
<td>4</td>
<td>28.6</td>
<td>0</td>
<td>24</td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td>pfmdr1 K76T</td>
<td>2</td>
<td>4</td>
<td>28.6</td>
<td>5</td>
<td>24</td>
<td>23.4</td>
<td></td>
</tr>
</tbody>
</table>

of resistance in the border counties of China remains unknown. Therefore, there is an urgent need to monitor drug resistance of vivax malaria in this area.

Several genetic polymorphisms can provide reliable data about the prevalence of drug resistance related P. falciparum and P. vivax. The P. falciparum chloroquine resistance transporter gene (pfcr) T76 mutation and multidrug resistance 1 gene (pfmdr1) Y86 mutation have been linked to chloroquine and amodiaquine resistance (13,14). Similarly, the P. vivax multidrug resistance 1 gene (pvmdr1) F976 mutation, which was shown to be associated with reduced susceptibility to chloroquine, was selected to evaluate the resistance of P. vivax (15). As for artemisinin resistance, the slowly clearing infections were strongly associated with single point mutations in the “propeller” region of the P. falciparum kelch protein gene on chromosome 13 (kelch13) (16). Recently, the WHO stated that artemisinin resistance should be suspected when ≥5% of patients carry K13 resistance-associated mutations (17).

Here we report an assessment of antimalarial resistance marker polymorphisms including pfmdr1, pfcr, pvmdr1, and K13 propeller in samples collected from the China-Myanmar border region. The results might provide basic evidence for further molecular surveillance of drug-resistant P. falciparum and P. vivax strains in this region.

MATERIALS AND METHODS
Study site. The study was conducted in Yingjiang county, one of the 18 counties at the China-Myanmar border, in western Yunnan province. It has a long borderline of 214.6 km with the Kachin state of Myanmar, which is a tier II area according to the Global Plan for Artemisinin Resistance Containment (GPARC) by the WHO (17). The population of Yingjiang county is 307,960, and cross-border trade, logging, quarry, and of the total indigenous cases in China. Approximately 200 μl of finger-prick blood was spotted on a piece

FIG 1 Screening, enrollment, and follow-up of subject patients.

TABLE 1 Selection of P. falciparum and P. vivax polymorphisms

The results might provide basic evidence for further molecular surveillance of drug-resistant P. falciparum and P. vivax strains in this region.

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pfmdr1. The pfmdr1 gene was sequenced successfully in 3 isolates of all P. falciparum samples (11.5%, 3/26) that covered codons 86 and 184. Among all of the mutational types, no N1042D, S1034C, and D1246Y mutations were found. Mutations at codon N86Y (11.5%, 3/26) were common; Y86Y184 was the most prevalent (66.7%, 2/3) of all haplotypes (Tables 1 and 2). Further, one patient harboring the N86Y mutation was found in Nabang, China; the other two patients with this mutation were from Myanmar (P = 1.0000) (Table 1).

pfcr. Sequencing of the pfcr gene was successful in 9 isolates (34.6%, 9/26) that covered codons 74, 75, and 76. Of all three mutated codons, K76T was the most prevalent (23.1%, 6/26) (Table 1). Two patients with the mutated genotype K76T were ob-
served in Nabang, China; the other four patients with K76T were from Myanmar (P = 1.0000). Four different pfcr alleles were found, among which M74N75T76 was the most common (55.6%, 511/9) (Table 2). One patient harboring I74N75K76 was detected in Myanmar. Patients with other mutated codons were all returning from Myanmar (Table 2). The other three patients with mutated F446I were returning from Yingjiang county and 15.8% (3/19) in samples from Myanmar.

TABLE 3 Polymorphisms observed in the K13 propeller in P. falciparum isolates

<table>
<thead>
<tr>
<th>Codon position</th>
<th>Amino acid reference</th>
<th>Nucleotide reference</th>
<th>Amino acid mutation</th>
<th>Nucleotide mutation^a</th>
<th>Prevalence (% [no.])</th>
<th>Location(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>446 (n = 5)^b</td>
<td>F</td>
<td>Ttt</td>
<td>I</td>
<td>Att</td>
<td>19.2 (5/26)</td>
<td>2 in China, 3 from Myanmar</td>
</tr>
<tr>
<td>511 (n = 2)^b</td>
<td>I</td>
<td>Ata</td>
<td>M</td>
<td>atG</td>
<td>7.7 (2/26)</td>
<td>Myanmar</td>
</tr>
<tr>
<td>537 (n = 1)</td>
<td>N</td>
<td>Aat</td>
<td>I</td>
<td>aTt</td>
<td>3.8 (1/26)</td>
<td>Myanmar</td>
</tr>
<tr>
<td>574 (n = 1)</td>
<td>P</td>
<td>Cct</td>
<td>L</td>
<td>cTt</td>
<td>3.8 (1/26)</td>
<td>Myanmar</td>
</tr>
<tr>
<td>676 (n = 1)^b</td>
<td>A</td>
<td>Gcc</td>
<td>D</td>
<td>gAc</td>
<td>3.8 (1/26)</td>
<td>China</td>
</tr>
</tbody>
</table>

^a Mutations are in bold type.

^b A mutated site which was not reported before.

DISCUSSION

Yunnan province is located in southern China, and malaria is one of the most important public health problems (20). The incidence of malaria transmission is more severe in the China-Myanmar border counties. The total number of malaria cases in Yunnan province according to the annual reported data was 576 in 2013, including 460 P. vivax cases, 106 P. falciparum cases, and 10 cases of other species. Most of them (n = 463, 80.4%) were observed in the 18 China-Myanmar border counties. The situation in Yingjiang county was the most severe, with an incidence rate of 2.3 per 10,000 people, and 18 local transmission cases were reported, representing 21.2% of the total local cases in China, with 54 other cases imported from Myanmar. Based on these facts, it was selected as the study site.

Resistance to antimalarial drugs has been a long-standing problem in the GMS. MDR P. falciparum strains that have emerged in the Thai-Cambodian border region, as well as the emerging resistance to chloroquine (CQ), to sulfadoxine-pyrimethamine (SP), and then to mefloquine (MQ), are gradually spreading in the tropical world (21). Malaria in the China-Myanmar border region is a topic of regional and national public health concern. The development and spread of MDR P. falciparum have led to the adoption of artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated P. falciparum malaria in this region to improve treatment outcomes. However, widespread artemisinin resistance has been observed in the GMS (4, 22); this poses a great threat of resistant parasites being brought into China with the migrant population, immediately affecting therapy efficacy. In the China-Myanmar border area, artemisinins have been used for more than 30 years, mostly as monotherapies prior to 2005. Earlier in vitro assays had already detected a trend of declining sensitivity to artemisinins in the border area of Yunnan province (23, 24). Therefore, an understanding of whether artemisinin-resistant parasites have spread to the neighboring regions or emerged elsewhere in this area is essential for coordinating containment efforts.

In addition, P. vivax malaria persists in areas of Yunnan province along the China-Myanmar border (25), and CQ was adopted more than 50 years ago in China as the first-line drug used for treatment of the blood-stage P. vivax infection. Although high-level resistance of P. vivax to CQ and SP was reported more than a decade ago in the north of Myanmar (26, 27), the resistance in the border counties of China remains unknown.

The malaria parasite encodes many transporters, and some of them such as pfndr1 and pfcr have been strongly connected with antimalarial drug resistance (28). The pfndr1 genotype is correlated with resistance of P. falciparum to CQ, MQ, and artemisinins, whereas knockdown of pfndr1 expression leads to increased susceptibility (29). The N86Y pfndr1 mutation that confers CQ resistance is also associated with decreased sensitivity to artemisinins, and...
inins (18). In Thailand and Cambodia, the pfmdr1 gene has become increasingly prevalent in field parasite populations and was responsible for the declining efficacy of ACTs (30, 31). In our assay, two mutated pfmdr1 alleles, N86Y and Y184F, were observed, and one patient with a Y976F allele amplification was found in the port city of Nabang, China. These findings are contradictory to those of Wang et al., who reported that pfmdr1 N86Y had not been observed in this region (32). The possibility that patients take antimalarials with insufficient compliance cannot be excluded, although MQ was not frequently used in this region (33). Moreover, we have found one patient with the double mutation of pfmdr1 N86Y and K13 propeller F446I, which may further indicate significance of pfmdr1 mutations in artemisinin resistance. This uncommon pfmdr1 polymorphism from this region offers opportunities for investigations of the mechanisms of artemisinin resistance.

The pfcrt K76T mutation has been widely used as a reliable marker for CQ resistance, and it was found to have a high prevalence in China (34). Furthermore, it also influences P. falciparum susceptibility to MQ, halofantrine, and artemisinin (18). This is consistent with our study showing that pfcrt K76T played a predominant role in the pfcrt genotypes. Molecular assays have shown high prevalence of the M74N75K76 (55.6%) genotype, which was similar to the findings of Huang et al. (35). No significant difference was observed between the local samples from Yingjiang county and those from Myanmar, since migration occurs in both directions. Despite the fact that China has not used CQ to treat P. falciparum infections for more than 30 years, the stable and high prevalence of this mutation may be a result of the continued use of CQ as a first-line drug for P. vivax infection over several decades. Another factor that may contribute to the high prevalence of pfcrt K76T is the use of CQ as a first-line drug for P. vivax infection for several decades in Myanmar, especially in the Myanmar-Thailand border area, where a high prevalence of pfcrt K76T was found, thus suggesting that the natural selection against CQ pressure for the maintenance of the pfcrt mutation in P. falciparum is still retained in the region (36, 37). However, we also found three other haplotypes (I74N75K76, M74E75K76, and M74E75T76) in our study, suggesting that selection of other pfcrt haplotypes was still needed.

Resistance to common antimalarial drugs has been reported for P. vivax in the GMS, including Myanmar and Vietnam, and also in Indonesia (38–40). A trend for a gradual decline in the in vitro sensitivity of this parasite to CQ had also been reported during 2005 to 2008 around the China-Myanmar border and in central China (41, 42). Our findings revealed that the presence of the pvmdr1 Y976F mutation in Yingjiang county in China is consistent with previous reports of declining sensitivity to CQ; this was also observed in Myanmar and Xishuangbanna (Yunnan province), which exhibit high frequencies of the pvmdr1 Y976F allele (43, 44). The long history of CQ use and the frequent population movement among borders may contribute to the CQ-resistant P. vivax strains detected in Yingjiang county. Close surveillance at sentinel sites in this region should continue so that the emergence and spread of P. vivax resistance can be carefully monitored.

Artemisinin and its derivatives have been used for falciparum malaria treatment in China since the late 1970s (45). In vitro assays showed that the susceptibility of P. falciparum to artemisinins was declining in China, but no evidence of the artemisinin resistance has been detected (46). In our study, five nonsynonymous mutations were found, and three of them were not reported previously. Furthermore, our study showed that F446I was the predominant allele (19.2%, 5/26), and two cases with this mutation were found in the port city of Nabang, China. Another mutation, K13 propeller A676D, was also observed in China, China (Fig. 2). However, the C580Y allele, which was widely found in Cambodia (16), was not found in this study. Nevertheless, our results indicate that the mutated K13 propeller gene alleles exist in the China-Myanmar border area, and their presence should raise concerns regarding the risks of emerging artemisinin resistance in the GMS. We recommend further clinical trials associated with K13 propeller mutations, which might be useful for identifying additional genetic loci involved in monitoring the threat of artemisinin resistance.

The prevalence of the K13 propeller polymorphism detected in Yingjiang county indicates that ACTs should be used in the China-Myanmar border area, and rational use of antimalarials against P. falciparum strains imported from Southeast Asia should be adopted. In addition, routine monitoring and surveillance, as recommended by the WHO Global Plan for Artemisinin Resistance Containment, should continuously be strengthened. Additional clinical investigations to complement sentinel surveillance, in-
clustering either analysis of the drug markers or risk factors or new approaches to monitor resistance, are required.

In conclusion, the present data might be helpful for enrichment of molecular surveillance of antimarialar resistance and for developing and updating guidance for the use of antimalarials in the region.

ACKNOWLEDGMENTS
We thank the staffs of the provincial and county centers for disease control and prevention in China for assistance. We declare no conflicts of interest.

REFERENCES


