Limited Polymorphism of the Kelch Propeller Domain in Plasmodium malariae and P. ovale Isolates from Thailand

Supatchara Nakeesathit,a,b Naowarat Saralamba,a,c Sasithon Puksrittayakamee,b Arjen Dondorp,a,d Francois Nosten,a,e Nicholas J. White,a,d Mallika Imwonga,e

Mahidol Oxford Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; Centre for Tropical Medicine, Nuffield Department of Medicine, Churchill Hospital, Oxford, United Kingdom; Shoklo Malaria Research Unit, Tak, Thailand

Artemisinin resistance in Plasmodium falciparum, the agent of severe malaria, is currently a major obstacle to malaria control in Southeast Asia. A gene named “kelch13” has been associated with artemisinin resistance in P. falciparum. The orthologue of the kelch gene in P. vivax was identified and a small number of mutations were found in previous studies. The kelch orthologues in the other two human malaria parasites, P. malariae and P. ovale, have not yet been studied. Therefore, in this study, the orthologous kelch genes of P. malariae, P. ovale wallikeri, and P. ovale curtisi were isolated and analyzed for the first time. The homologies of the kelch genes of P. malariae and P. ovale were 84.8% and 82.7%, respectively, compared to the gene in P. falciparum. kelch polymorphisms were studied in 13 P. malariae and 5 P. ovale isolates from Thailand. There were 2 nonsynonymous mutations found in these samples. One mutation was P533L, which was found in 1 of 13 P. malariae isolates, and the other was K137R, found in 1 isolate of P. ovale wallikeri (n = 4). This result needs to be considered in the context of widespread artemisinin used within the region; their functional consequences for artemisinin sensitivity in P. malariae and P. ovale will need to be elucidated.

Malaria remains one of the world’s most important infectious diseases, with an estimated 198 million cases and 584,000 deaths in 2013 (1). Malaria is caused by protozoa of the genus Plasmodium, with the following five species causing disease in humans: Plasmodium falciparum, P. vivax, P. malariae, P. ovale, and P. knowlesi. P. malariae is the third most common infecting species with incidences in areas of endemicity reported to be <4% to 20% of the total number of malaria infections (2). This parasite has a 72-h erythrocytic developmental cycle and is usually detected at low parasitemias in mixed infections with either P. falciparum or P. vivax. Although P. malariae does not form liver hypnozoites, it can persist in the circulation for many years. Infections with P. ovale are found in sub-Saharan Africa, the Middle East, Papua New Guinea, and Southeast Asia. P. ovale is a less common parasite, but it still has an estimated global incidence of >15 million cases annually (3). Like P. malariae, P. ovale causes infections with low parasitemias and is usually found with P. falciparum or P. vivax. P. ovale can cause relapse infections from dormant exoerythrocytic-stage parasites in the liver, called hypnozoites (4).

Artemisinin-based combination therapies (ACTs) have been adopted as the first line of treatment for uncomplicated falciparum malaria in most countries where malaria is endemic, including Thailand (1). However, over the last few years, artemisinin-resistant P. falciparum has emerged in western Cambodia and is now firmly established in the surrounding countries, including Thailand, Laos, Vietnam, and Myanmar. Recently, a molecular marker for artemisinin resistance was identified, namely, mutations in the P. falciparum kelch (Pfkelch) gene, which is located on chromosome 13 and encodes a 727-amino-acid protein. Mutations in the so-called propeller region of the Kelch protein (K13 propeller) closely correlate with delayed parasite clearance, which defines the artemisinin resistance phenotype. The kelch gene consists of three domains: a Plasmodium-specific domain, a BTB/POZ domain, and the kelch propeller domain (Fig. 1). The original studies reported the K13 propeller mutations C580Y, R539T, and Y493H in Cambodian P. falciparum strains, and interestingly, only one mutation per kelch gene seemed to be allowed (5). Since these initial studies, more than 60 single nucleotide polymorphisms (SNPs) in the K13 propeller have been identified, of which most correlate with the slow-clearance phenotype (6). Among Cambodian P. vivax strains, a V552I polymorphism in the orthologous kelch gene has been described for two isolates (7). It is not known whether this mutation in P. vivax causes resistance to artemisinins. Because P. malariae and P. ovale are frequently found as mixed infections with P. falciparum, these species are also exposed to ACTs when P. falciparum infections are being treated. It is therefore plausible that kelch mutations in P. malariae and P. ovale might have emerged and may confer artemisinin resistance. The kelch genes from these two parasites have not been isolated or studied previously. The present study aimed to isolate the “propeller region” of the Pmkelch and Pokelch genes, from P. malariae and P. ovale, respectively, and to determine the presence of polymorphisms in these genes.
MATERIALS AND METHODS

*P. malariae* and *P. ovale* strains were obtained from blood samples taken from previous studies performed in Thailand between 1995 and 2012 (*n* = 18), under ethical approval MUTM2011-049-05. This study was reviewed and approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand, with ethical approval MUTM2015-001-01. All 18 *P. malariae* and *P. ovale* isolates included in this study were from monoinfections with parasites varying from 26/500 white blood cells (WBC) to 1/1,000 red blood cells (RBC). Patients were treated with chloroquine according to standard guidelines. DNA extraction was performed with a DNA minikit (Qiagen, Germany) following the manufacturer’s instructions. Genomic DNAs were kept at −20°C until further use.

For isolation of the *P. malariae* and *P. ovale kelch* genes, degenerate primers (Table 1 and Fig. 2) were designed to target the conserved region of the *kelch* genes from the other 3 human malaria parasites, i.e., *P. falciparum* (accession number PF3D7_1343700), *P. vivax* (accession number PVX_083080), and *P. knowlesi* (accession number PKH_121080).

After partial sequences of the *kelch* gene were obtained from *P. malariae* and *P. ovale*, *Pmkelch* - and *Pokelch*-specific primers (Table 2 and Fig. 3) were subsequently designed to investigate polymorphisms in the gene. In this study, seminested and nested PCR approaches were used to increase the sensitivity and specificity of amplification. All primary PCRs and secondary reactions for DNA cloning were carried out in a total volume of 20 μL. Secondary reactions for direct DNA sequencing were performed in 100-μL mixtures. Reaction solutions contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 125 μM deoxyribonucleoside triphosphates (dNTPs), and 250 nM (each) primers for both the primary and secondary reactions, with 0.4 U of Taq polymerase (Invitrogen) included in each reaction mixture. In the primary reaction mixture, 1.5 μL of genomic DNA was used as the template; 2 μL of the products of the primary reaction was then used as the template for the secondary PCR. Cycling parameters for the PCRs were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles for primary PCR and 35 cycles for the secondary reaction (each cycle consisted of denaturing at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min), with a final extension step at 72°C for 5 min. The temperature profile for the PCRs with *P. malariae*- and *P. ovale*-specific primers was as follows: initial denaturation at 95°C for 1 min, followed by 25 cycles for primary PCR and 30 cycles for the secondary reaction (each cycle consisted of denaturing at 94°C for 1 min, annealing at 55°C for 2 min [primary reaction] and 1 min [secondary reaction], and extension at 72°C for 2 min [primary reaction] or 1 min 30 s [secondary reaction]), with a final extension step at 72°C for 5 min. The PCR product size was estimated by comparison with a 100-bp DNA ladder. PCR products were cloned into the pGEM-T Easy vector (Promega) according to the manufacturer’s instructions. The *Pmkelch* - and *Pokelch*-specific PCR products were purified using a FavorPrep gel/PCR purification kit (Farvogen, Taiwan) following the manufacturer’s instructions.

DNA sequencing was performed with both the forward and reverse strands to confirm the presence of polymorphisms. To ensure that the DNA sequences were from the *P. malariae* and *P. ovale kelch* genes, all DNA sequences were assessed with the Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/Blast.cgi. All sequences were then assembled, the *kelch* DNA and protein sequences were aligned with ClustalW software by use of Bioedit, and a phylogenetic analysis was constructed using the MEGA6 software program (Tokyo, Japan) (8). Phylogenetic relationships between the *kelch* genes of the different *Plasmodium* species were assessed by the neighbor-joining method. The *Toxoplasma gondii kelch* sequence (accession number XM_002365296) was used as the outgroup because small-subunit rRNA sequence data from other Apicomplexa have shown that *Toxoplasma gondii* is positioned prior to the stem that gives rise to *Plasmodium* spp. (9).

DNA sequences of all *P. malariae* and *P. ovale* isolates were analyzed for GC content and codon usage by use of the MEGA6 program and the Sequence Manipulation Suite package (SMS) (http://www.bioinformatics.org/sms2/index.html).

Nucleotide sequence accession numbers. The DNA sequences of *Pmkelch* and *Pokelch* have been submitted to GenBank under accession numbers KT792967 to KT792971.

RESULTS

Isolation of *Pmkelch* and *Pokelch*. Several pairs of degenerate primers were designed based on the conserved region of the *kelch* genes from *P. falciparum*, *P. vivax*, and *P. knowlesi*, with the aim of amplifying overlapping *kelch* fragments. Five overlapping fragments of the *P. malariae kelch* gene and 4 fragments of the *P. ovale wallikeri kelch* gene (*PoWkelch*) were obtained, cloned, and sequenced (Fig. 2). The sizes of the assembled sequences of *Pmkelch* and *PoWkelch* were 2,087 and 2,063 bp, respectively. The *Pmkelch* gene encoded 695 amino acids, while *PoWkelch* encoded 687 amino acids. Four fragments of the *P. ovale curtisi kelch* gene (*PoCkelch*) were obtained by use of the *Pokelch*-specific primers, showing a 1,977-bp gene that encoded the amino acids of the partial *P. ovale curtisi Kelch* protein.

The coding sequences of the *P. malariae*, *P. ovale wallikeri*, and *P. ovale curtisi kelch* genes were aligned with other *Plasmodium* spp. *kelch* sequences (see Fig. S1 and S2 in the supplemental material).
and compared between species. The proportions of amino acid homology between species are shown in Table 3, showing overall homologies of 82.7% to 98.5%, whereas compared to the human kelch gene, there was only 10.5% to 15% homology. It appeared that the BTB/POZ domain of the protein was the most highly conserved region (97.9 to 100%), whereas most polymorphisms were found in the Plasmodium-specific domain. Overall, kelch gene-encoded amino acid homologies were high between Plasmodium species, ranging from 93.3 to 99.6%. Homologies of both the P. malariae and P. ovale kelch genes to those of other Plasmodium species were similar to the homology in amino acids reported for a comparison between the P. falciparum and P. knowlesi kelch genes, which was reported as 88% for the overall kelch gene and 97% for the kelch propeller domain (5, 10).

Guanine-cytosine (GC) compositions of the different codons in the Plasmodium sp. kelch genes are shown in Table 4. P. malariae, P. ovale wallikeri, and P. ovale curtisi contained low GC contents, i.e., 28.9%, 27.9%, and 28.4%, respectively, in contrast to the high GC content found in the kelch genes of P. vivax and P. knowlesi. The GC contents of the kelch genes of P. malariae and P. ovale are similar to those of other P. malariae and P. ovale genes, e.g., dhfr-ts (accession numbers AY846634 and EU266606), ppk28-dhps (accession number KJ400027), 18s rRNA (accession numbers M54897 and L48987), MSP1 (accession numbers FJ824669 and FJ824670), and Plasmepsin (accession number AF001210), within the range of 23.5 to 36.63% GC content. The kelch codon usage of each Plasmodium species is shown in Table 5. Codons containing only G and C were found in 1.15% (8/695 codons), 0.87% (6/687 codons), and 1.05% (7/658 codons) of codons in the P. malariae, P. ovale wallikeri, and P. ovale curtisi genes, respectively, which is similar to the case in P. falciparum (1.51% [11/727 codons]). The most prevalent amino acid codon in the kelch genes of P. malariae, P. ovale wallikeri, and P. ovale curtisi was an Asn codon (AAT), resulting in 63, 57, and 55 residues, respectively. Amino acid usages were similar between the different Plasmodium species.

To exclude the possibility of interspecies cross-reactivity in the isolation of Pmkelch and Pokelch in our experiments, we first tested the specificity of amplification by applying the specific primers targeting the kelch genes of these parasites. Dashed lines indicate the PCR products from the primary amplification reactions, and the thick lines indicate the PCR products obtained after the secondary amplification reactions.

**TABLE 2** Specific oligonucleotide primers used for amplification of P. malariae and P. ovale kelch gene sequences

| Primer  | Sequence (5’ to 3’) | Product size (bp) |
|---------|---------------------|------------------|
| PmKelchF1* | AAAAAATTTTACGACATTTTCGA | 800 |
| PmKelchR1 | TGTCCAACTTTCTCTTCTTCATA | |
| PmKelchR2 | AACTGCAAGTTTTCGTTTTGGGA | 750 |
| PmKelchR4* | TAAACCGGAGTGACAAATCTT | |
| PmKelch_F | TGATGAAAGAAATGGACTGCC | 1,241 |
| PmKelch_OR | TTGGGACAGCAAGAGGACAGAGG | |
| PmKelch_NR | AAGCAGAAGGGCCGAAATTT | |
| PmKelchR1 | GCGATGAAAGAAATGGACTGCC | 680 |
| PmKelchR2 | CACGTGCGTCCGATGAGAAATA | 730 |
| PmKelchR4* | AAAACCGGAGTGACAAATCTT | |
| PmKelchF2 | TTTTGAAACTTCAAGACATACAC | 1,066 |
| PmKelchR2 | ATGTCCTATTCGCCAATC | |
| PmKelch_NR | GGGGCCAGATAATGAGG | |

* Primers used in the primary reaction mixtures.
All of the differences between these two species were tested and confirmed by using the same primer set and approach. The species is based on each domain. P. ovale and P. malariae were isolated by using the same primer set and approach.

**DISCUSSION**

This study isolates and explores, for the first time, the kelch orthologue genes from P. malariae, P. ovale curtisi, and P. ovale wallikeri. Analysis of the kelch genes from 13 P. malariae isolates from Thailand showed limited polymorphism, with only one nonsynonymous mutation, P533L, found in a single isolate. This mutation is equivalent to the previously reported P553L mutation in all Plasmodium species, with the newly isolated strains (13 P. malariae, 4 P. ovale wallikeri, and 1 P. ovale curtisi strain) located according to their relationships.

FIG 3 Diagrams of the kelch propeller genes from P. malariae (A) and P. ovale (B). The positions of specific oligonucleotide primers used for Pmkelch and Pokelch gene polymorphism investigation are indicated. Dashed lines indicate the PCR products from the primary amplification reactions, and the thick lines indicate the PCR products obtained after the secondary amplification reactions.

(n = 20) and P. vivax (n = 20), as well as to samples from healthy volunteers (n = 20). Parasitemias varied between 4,000 parasites/µl and 35,000 parasites/µl. None of the P. falciparum, P. vivax, or control samples showed any DNA amplification, confirming the P. malariae and P. ovale species-specific amplification obtained using these primers. Second, the specificity of amplification was demonstrated by showing that the 4 amplified fragments of Pmkelch were from the same gene. Specific Pmkelch primers (PmkelchF1 and PmkelchR4) (Table 2) which can bind to the 5' and 3' ends of the gene were used to amplify the 2-kb sequence of this gene. The 2-kb Pmkelch fragment was then cloned into a pGEM vector and sequenced with all 4 forward Pmkelch primers. The same approach was used to determine the specificity of amplification of Pokelch. The assembled DNA sequences obtained by primer walking, with plasmid replication in Escherichia coli, showed that all 4 fragments were from the Pmkelch gene or the Pokelch gene. From the plasmid amplification, 3 SNPs (F195S, K649E, and S659T) were observed in the Pmkelch gene, and 6 SNPs (N93D, K104I, N154S, E235G, D531Y, and A599T) were observed in the Pokelch gene. However, by direct sequencing of the PCR products, these 9 SNPs appeared to all be false-positive findings, which is a known potential artifact of this DNA cloning process. Third, all SNPs appeared to all be false-positive findings, which is a known potential artifact of this DNA cloning process. Third, all SNPs appeared to all be false-positive findings, which is a known potential artifact of this DNA cloning process. Third, all SNPs appeared to all be false-positive findings, which is a known potential artifact of this DNA cloning process. Third, all SNPs appeared to all be false-positive findings, which is a known potential artifact of this DNA cloning process. Third, all SNPs appeared to all be false-positive findings, which is a known potential artifact of this DNA cloning process.
### TABLE 3 Comparison of homologies of malarial kelch genes

| Species comparison | % amino acid homology |
|--------------------|-----------------------|
| Overall            | Plasmodium-specific domain | BTB/POZ domain | Kelch domain |
| KEAP1 variant 1-PP | 12.6 5.7               | 7.2 22.1       |              |
| KEAP1 variant 1-PV | 13.6 8.1               | 7.2 21.8       |              |
| KEAP1 variant 1-PK | 13.6 8.1               | 7.2 21.8       |              |
| KEAP1 variant 1-PM | 13.4 7.4               | 7.2 21.8       |              |
| KEAP1 variant 1-POC| 13.8 7.9               | 7.2 22.1       |              |
| KEAP1 variant 1-POW| 14.0 8.1               | 7.2 22.1       |              |
| KEAP1 variant 2-PP | 12.6 5.7               | 7.2 22.1       |              |
| KEAP1 variant 2-PV | 13.6 8.1               | 7.2 21.8       |              |
| KEAP1 variant 2-PK | 13.6 8.1               | 7.2 21.8       |              |
| KEAP1 variant 2-PM | 13.4 7.4               | 7.2 21.8       |              |
| KEAP1 variant 2-POC| 13.8 7.9               | 7.2 22.1       |              |

*KEAP1, human Kelch-like ECH-associated protein 1; KLHL, human Kelch-like family member; PP, P. falciparum; PV, P. vivax; PK, P. knowlesi; PM, P. malariae; POW, P. ovale wallikeri; POC, P. ovale curtisi.*

### TABLE 4 GC contents of malarial kelch genes

| GC content (%) | kelch gene domain and malaria species |
|----------------|------------------------------------------|
| Overall        | Codon position 1 | Codon position 2 | Codon position 3 |

| Plasmodium-specific domain |
|----------------------------|
| P. falciparum | 21.8 30.4 | 22.0 13.0 |
| P. vivax     | 34.1 35.3 | 23.0 45.9 |
| P. knowlesi  | 30.5 30.8 | 22.7 38.1 |
| P. malariae  | 28.9 34.5 | 30.1 19.1 |
| P. ovale wallikeri | 27.9 35.9 | 29.7 21.2 |
| P. ovale curtisi | 28.4 36.3 | 29.3 19.7 |

| BTB/POZ domain |
|----------------|
| P. falciparum | 28.9 37.1 | 34.0 15.5 |
| P. vivax     | 39.9 41.2 | 34.0 44.3 |
| P. knowlesi  | 37.1 42.3 | 34.0 35.1 |
| P. malariae  | 28.5 38.1 | 34.0 13.4 |
| P. ovale wallikeri | 26.5 39.2 | 34.0 6.2 |
| P. ovale curtisi | 27.3 38.1 | 34.0 11.3 |

| Kelch domain    |
|-----------------|
| P. falciparum   | 32.0 42.5 | 37.2 16.5 |
| P. vivax        | 44.0 43.5 | 37.9 50.5 |
| P. knowlesi     | 40.1 43.2 | 37.9 39.3 |
| P. malariae     | 31.2 40.5 | 37.7 15.4 |
| P. ovale wallikeri | 34.9 42.7 | 37.7 24.2 |
| P. ovale curtisi | 33.6 42.1 | 37.7 21.0 |

There were 5 samples containing P. ovale included in this study: 4 P. ovale wallikeri and 1 P. ovale curtisi isolate. In the P. ovale wallikeri strains, only one nonsynonymous mutation in kelch, K137R, was found. The K137R mutation is located in the kelch BTB/POZ domain. Multiple-sequence alignment of Plasmodium Kelch amino acids showed that there are a number of polymorphisms within the Plasmodium-specific domain. Collection of more Pokelch genes from more samples would provide insight into the specific sequence for each Plasmodium species.

Genetic analysis of nucleotide composition within the kelch genes of all human Plasmodium species showed similar trends in GC content and codon usage. Overall, GC contents were similar, ranging from 26.8 to 35.2% (Table 4), which is slightly different from those of other genes. For comparison, the GC content of the pppk-dhps gene of P. malariae is similar to that for P. falciparum (23.5 to 27.8%), whereas the content in P. vivax is slightly higher (43.2%) (16). The homologies of the kelch genes of all human malarial species showed that P. falciparum separates from the other species. Multiple-sequence alignment of amino acids within the Kelch Plasmodium-specific domain (see Fig. S1 and S2 in the supplemental material) clearly showed the conservation of amino acid sequences among P. vivax, P. malariae, P. ovale, and P. knowlesi, whereas P. falciparum showed an alternative codon. The same pattern was found for non-Plasmodium-specific domains.
TABLE 5 Codon usage in the malarial kelch gene

| Codon | Amino acid | No. of occurrences* |
|-------|------------|---------------------|
| AAG   | Lys        | PF 27            |
| AAA   | Lys        | PV 27            |
| AAT   | Asn        | PK 13            |
| AAC   | Asn        | PM 13            |
| AGG   | Arg        | POW 7            |
| ACA   | Thr        | POC 7            |
| ACT   | Thr        | PV 7             |
| ACC   | Thr        | PK 7             |
| AGG   | Arg        | PM 7             |
| AGA   | Arg        | POW 7            |
| ATA   | Ile        | PK 21            |
| ATT   | Ile        | PM 21            |
| ATG   | Met        | PK 21            |
| CAG   | Gln        | PK 14            |
| CAC   | His        | PM 14            |
| CCG   | Pro        | PV 14            |
| CCA   | Pro        | PK 14            |
| CCT   | Pro        | PM 14            |
| CGC   | Arg        | POW 14           |
| CGA   | Arg        | POC 14           |
| CTG   | Leu        | PK 14            |
| CTA   | Leu        | PM 14            |
| CTT   | Leu        | POW 14           |
| CTC   | Leu        | POC 14           |
| GAG   | Glu        | PV 14            |
| GAA   | Glu        | PK 14            |
| GAT   | Asp        | PM 14            |
| GAC   | Asp        | POW 14           |
| GGC   | Ala        | PV 14            |
| GCA   | Ala        | PK 14            |
| GCT   | Ala        | PM 14            |
| GCC   | Ala        | POW 14           |
| GGG   | Gly        | POC 14           |
| GGA   | Gly        | PK 14            |
| GGT   | Gly        | PM 14            |
| GTG   | Val        | POW 14           |
| GTA   | Val        | POC 14           |
| GTT   | Val        | PV 14            |
| GTC   | Val        | PK 14            |
| TAG   | Stop       | PM 14            |
| TAA   | Stop       | POW 14           |
| ATT   | Tyr        | PK 14            |
| TAC   | Tyr        | PM 14            |
| TCG   | Ser        | POW 14           |
| TCA   | Ser        | POC 14           |
| TCT   | Ser        | PV 14            |
| TCC   | Ser        | PK 14            |
| TGA   | Stop       | PM 14            |
| TGG   | Trp        | POW 14           |
| TGT   | Cys        | POC 14           |
| TCG   | Cys        | PK 14            |
| TTG   | Leu        | PM 14            |
| TTA   | Leu        | POW 14           |
| TTT   | Phe        | POC 14           |

*PF, P. falciparum; PV, P. vivax; PK, P. knowlesi; PM, P. malariae; POW, P. ovale wallikeri; POC, P. ovale curtisi.

TABLE 6 Nonsynonymous mutations observed for P. malariae, P. ovale, and other human malaria parasites

| Isolate no. | Isolate or accession no.* | Organism |
|-------------|---------------------------|----------|
| 1           | K137R                     | P. falciparum |
| 2           | K152                      | P. vivax   |
| 3           | K152                      | P. malariae |
| 4           | K137R                     | P. ovale   |
| 5           | K137R                     | P. ovale wallikeri |
| 6           | K137R                     | P. ovale curtisi |

*Accession numbers for PM2, PM048, PoW1, PoC13, and PoW20 are KT929676 to KT792971, respectively.

This characteristic facilitated the generation of the phylogenetic tree topology (Fig. 4), which showed that P. falciparum was first separated as a single branch distant from the other Plasmodium species. kelch gene homology confirmed the close genetic relationship between P. ovale wallikeri and P. ovale curtisi (94.9%), in line with previous reports comparing other genes between these species (3, 11). This is in accordance with the presence of sympatric P. ovale wallikeri and P. ovale curtisi in Thailand.

Phylogenetic analysis showed the close relatedness of kelch gene sequences between Plasmodium species infecting humans, but it also showed clear clustering patterns for the Pmkelch and Pokelch genes defining the different species. This shows that the isolated kelch genes from both P. malariae and P. ovale are species specific and are not variants of the gene from any other human Plasmodium species. This separation of clades according to Plasmodium species is consistent with patterns described previously for other genes, such as msp1 (17).

In conclusion, this study is the first report of the isolation and analysis of the kelch-orthologous genes of P. malariae, P. ovale wallikeri, and P. ovale curtisi. Only a single point mutation in kelch was observed among 13 P. malariae isolates. Its functional consequences for artemisinin sensitivity in P. malariae and P. ovale remain to be elucidated. To obtain a more complete picture of the genetic epidemiology of the artemisinin resistance-associated kelch gene in all human malaria species, kelch orthologue gene polymorphisms will need to be studied in a larger sample of P. malariae, P. ovale wallikeri, and P. ovale curtisi strains.
ACKNOWLEDGMENTS
This study was supported by Mahidol University and the Wellcome Trust of Great Britain.

We have no conflicts of interest to declare.

FUNDING INFORMATION
This work, including the efforts of Arjen Dondorp and Nicholas J. White, was funded by Wellcome Trust. This work, including the efforts of Malika Imwong, was funded by Mahidol University.

REFERENCES
1. WHO. 2014. WHO malaria report 2014. World Health Organization, Geneva, Switzerland.
2. Bruce MC, Macheso A, Galinski MR, Barnwell JW. 2007. Characterization and application of multiple genetic markers for Plasmodium malariae. Parasitology 134:637–650. http://dx.doi.org/10.1017/S0031182006001958.
3. Sutherland CJ, Tanomsing N, Nolder D, Oguile M, Jennison C, Pukrittayakamee S, Do Rosario VE, Arez AP, Pinto J, Michon P, Escalante AA, Nosten F, Burke M, Lee R, Blake M, Otto TD, Barnwell JW, Pain A, Williams J, White NJ, Day NP, Snounou G, Lockhart PJ, Chiiodini PL, Imwong M, Polley SD. 2010. Two nonrecombining sympatric forms of the human malaria parasite Plasmodium ovale occur globally. J Infect Dis 201:1544–1550. http://dx.doi.org/10.1086/652240.
4. Collins WE, Jeffery GM. 2005. Plasmodium ovale: parasite and disease. Clin Microbiol Rev 18:570–581. http://dx.doi.org/10.1128/CMR.18.3.570-581.2005.
5. Arisy F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V, Bouchier C, Ma L, Lim P, Leang R, Duong S, Sreng S, Suon S, Chouer CM, Bout DM, Menard S, Rogers WO, Genton B, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A, Barale JC, Fairhurst RM, Benoit-Vical F, Mercereau-Puijalon O, Menard D. 2014. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature 505:50–55. http://dx.doi.org/10.1038/nature12876.
6. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B, Sopha C, Chouer CM, Nguon C, Sovannaroth S, Pukrittayakamee S, Jittamala P, Chotivanich K, Chuksamut K, Suchatsoonthorn C, Runcharoen R, Hien TT, Thuy-Nhien NT, Thanh NV, Phu NH, Htut Y, Han KT, Aye KH, Mokoulu OA, Olaosebikan RR, Folaranmi OO, Mayxay M, Khamthavong M, Hongvanthong B, Newton PN, Onyamboko MA, Fanello CI, Tshefu AK, Mishra N, Valecha N, Phyo AP, Nosten F, Yi P, Tripura R, Borrmann S, Bshahreil M, Peshu J, Fai MA, Ghose A, Hossain MA, Samad R, Rahman MR, Hasan MM, Islam A, Miotto O, Amato R, MacInnis B, Stalker J, Kwiatkowski DP, Bozdech Z, Jeeapant A, Cheah PY, Sakulthawat T, Chalk J, Intharabut B, Silamut K, Rose J, Vihokhern B, Kunasel C, Imwong M, Tarning J, Taylor WJ, Yeung S, Woodrow CJ, Flegg JA, Das D, Smith J, Venkatesan M, Plewes CV, Stepnieska K, Guerin PJ, Dondorp AM, Day NP, White NJ. 2014. Spread of artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med 371:411–423. http://dx.doi.org/10.1056/NEJMoa1314981.
7. Popovici J, Rao S, Eal L, Bin S, Kim S, Menard D. 2015. Reduced polymorphism in the Kelch propeller domain in Plasmodium vivax isolates from Cambodia. Antimicrob Agents Chemother 59:730–733. http://dx.doi.org/10.1128/AAC.03908-14.
8. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Ecol 30:2725–2729. http://dx.doi.org/10.1093/molbev/ms3197.
9. Egea N, Lang-Unnasch N. 1996. Phylogeny of the large extrachromosomal DNA of organisms in the phylum Apicomplexa. J Eukaryot Microbiol 43:158. http://dx.doi.org/10.1111/j.1550-7408.1996.tb04497.x.
10. Talundzic E, Chenet SM, Goldman IF, Patel DS, Nelson JA, Plucinski

FIG 4 Phylogenetic relationships among Plasmodium sp. kelch genes. Toxoplasma gondii was used as an outgroup to root the tree.
11. Tanomsing N, Imwong M, Sutherland CJ, Dolecek C, Hien TT, Nosten F, Day NP, White NJ, Snounou G. 2013. Genetic marker suitable for identification and genotyping of Plasmodium ovale curtisi and Plasmodium ovale wallikeri. J Clin Microbiol 51:4213–4216. http://dx.doi.org/10.1128/JCM.01527-13.

12. Tun KM, Imwong M, Lwin KM, Win AA, Hlaing TM, Hlaing T, Lin K, Kyaw MP, Plewes K, Faiz MA, Dhorda M, Cheah PY, Pukrittayakamee S, Ashley EA, Anderson TJ, Nair S, McDew-White M, Flegg JA, Grist EP, Guerin P, Maude RJ, Smithuis F, Dondorp AM, Day NP, Nosten F, White NJ, Woodrow CJ. 2015. Spread of artemisinin-resistant Plasmodium falciparum in Myanmar: a cross-sectional survey of the K13 molecular marker. Lancet Infect Dis 15:415–421. http://dx.doi.org/10.1016/S1473-3099(15)70032-0.

13. Miotto O, Amato R, Ashley EA, MacInnis B, Almagro-Garcia J, Almargtunga C, Lim P, Mead D, Oyola SO, Dhorda M, Imwong M, Woodrow C, Manske M, Stalker J, Drury E, Campino S, Amenga-Etego L, Thanh TN, Tran HT, Ringwald P, Bethell D, Nosten F, Phylo AP, Pukrittayakamee S, Chotivanich K, Chuoor CM, Nguon C, Suon S, Newton PN, Mayxay M, Khanthavong M, Hongvanthong B, Htu Y, Han KT, Kyaw MP, Faiz MA, Fanello CI, Onyamboko M, Mokuolu OA, Jacob CG, Takala-Harrison S, Plowe CV, Day NP, Dondorp AM, Spencer CC, McVeain G, Fairhurst RM, White NJ, Kwiatkowski DP. 2015. Genetic architecture of artemisinin-resistant Plasmodium falciparum. Nat Genet 47:226–234. http://dx.doi.org/10.1038/ng.3189.

14. Cheeseman IH, McDew-White M, Phylo AP, Sriprawat K, Nosten F, Anderson TJ. 2015. Pooled sequencing and rare variant association tests for identifying the determinants of emerging drug resistance in malaria parasites. Mol Biol Evol 32:1080–1090. http://dx.doi.org/10.1093/molbev/msu397.

15. MalariaGEN Plasmodium falciparum Community Project. 2016. Genomic epidemiology of artemisinin resistant malaria. eLife 5:e08714. http://dx.doi.org/10.7554/eLife.08714.

16. Tanomsing N, Mayxay M, Newton PN, Nosten F, Dolecek C, Hien TT, White NJ, Day NP, Dondorp AM, Imwong M. 2014. Genetic variability of Plasmodium malariae dihydropteroate synthase (dhps) in four Asian countries. PLoS One 9:e93942. http://dx.doi.org/10.1371/journal.pone.0093942.

17. Birkenmeyer L, Muerhoff AS, Dawson GJ, Desai SM. 2010. Isolation and characterization of the MSP1 genes from Plasmodium malariae and Plasmodium ovale. Am J Trop Med Hyg 82:996–1003. http://dx.doi.org/10.4269/ajtmh.2010.09-0022.