Global sequence diversity of the lactate dehydrogenase gene in *Plasmodium falciparum*

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Abstract

Background: Antigen-detecting rapid diagnostic tests (RDTs) have been recommended by the World Health Organization for use in remote areas to improve malaria case management. Lactate dehydrogenase (LDH) of *Plasmodium falciparum* is one of the main parasite antigens employed by various commercial RDTs. It has been hypothesized that the poor detection of LDH-based RDTs is attributed in part to the sequence diversity of the gene. To test this, the present study aimed to investigate the genetic diversity of the *P. falciparum* ldh gene in Thailand and to construct the map of LDH sequence diversity in *P. falciparum* populations worldwide.

Methods: The ldh gene was sequenced for 50 *P. falciparum* isolates in Thailand and compared with hundreds of sequences from *P. falciparum* populations worldwide. Several indices of molecular variation were calculated, including the proportion of polymorphic sites, the average nucleotide diversity index (\(\pi\)), and the haplotype diversity index (\(H\)). Tests of positive selection and neutrality tests were performed to determine signatures of natural selection on the gene. Mean genetic distance within and between species of *Plasmodium ldh* was analysed to infer evolutionary relationships.

Results: Nucleotide sequences of *P. falciparum* ldh could be classified into 9 alleles, encoding 5 isoforms of LDH. *L1a* was the most common allelic type and was distributed in *P. falciparum* populations worldwide. *Plasmodium falciparum* ldh sequences were highly conserved, with haplotype and nucleotide diversity values of 0.203 and 0.0004, respectively. The extremely low genetic diversity was maintained by purifying selection, likely due to functional constraints. Phylogenetic analysis inferred the close genetic relationship of *P. falciparum* to malaria parasites of great apes, rather than to other human malaria parasites.

Conclusions: This study revealed the global genetic variation of the ldh gene in *P. falciparum*, providing knowledge for improving detection of LDH-based RDTs and supporting the candidacy of LDH as a therapeutic drug target.

Keywords: Genetic diversity, DNA sequencing, Lactate dehydrogenase, Rapid diagnostic tests, Malaria, Purifying selection

Background

Accurate diagnosis of malaria is critical for case management and prevents unnecessary anti-malarial drug treatment. Microscopic diagnosis is considered a gold standard method for clinical diagnosis. Microscopic examinations of blood films have many limitations, including the requirement of high parasitaemia levels, long sample preparation time, and, most importantly, the required skills for diagnostic interpretation. To overcome this problem, a number of diagnostic tools that make use of conserved malaria antigens have been developed. The antigen-detecting rapid diagnostic test (RDT) is regarded as an alternative malaria diagnostic method that has been proven to be accurate, sensitive, quick and easy to interpret. RDTs detect malaria parasite specific antigens in blood through immunochromatography and can give positive or negative results for a sample at thresholds pre-set by manufacturers.

Different types of RDTs use different types of monoclonal antibodies or combinations of antibodies to detect...
and/or differentiate *Plasmodium* antigens. Some antibodies aim to detect a particular species. For instance, anti-histidine-rich protein 2 (HRP2) enables the detection of *Plasmodium falciparum*. On the other hand, anti-lactate dehydrogenase (LDH) is an example of a pan-malarial antibody, enabling detection of all types of human *Plasmodium* species. There are multiple formats of LDH-based detecting RDTs that are routinely employed for clinical malaria diagnostics. This includes OptiMAL-IT (Cressier, Switzerland), which identifies and differentiates *P. falciparum* from non-*P. falciparum* species (*Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae*) on the basis of *P. falciparum* LDH in a patient's whole blood [1]. Several investigations have reported the performance of OptiMAL-IT assays in various endemic areas. While a general consensus has emerged that they performed well collectively, some studies have indicated that the efficacy of OptiMAL might vary from one geographical location to another [2–7]. Variation of RDT sensitivity in most studies has showed a correlation with low blood parasitaemia level, but some studies have showed variable sensitivity even in the presence of high blood parasitaemia [4, 6]. For example, the efficacy of the OptiMAL assay was evaluated in a cross-sectional survey in Thailand in 2001 [7]. The data showed that the sensitivity of the OptiMAL assay for *P. falciparum* was 22.5% with > 500 parasites/μl and 10.5% with > 100 parasites/μl. It is not clear from the studies whether the poor performance of RDTs was due simply to how they were engineered or whether it was due to the genetic variability of LDH itself. The analysis of polymorphism of the gene encoding HRP2 in *P. falciparum* natural isolates from different regions showed that the observed polymorphism could contribute significantly to the low sensitivities of *P. falciparum* HRP2-based RDTs [8, 9]. However, whether the genetic diversity of the gene encoding LDH of *P. falciparum* led to the poor performance of LDH-detecting RDTs has not been evaluated.

There are few publications that have investigated the genetic polymorphism of the *ldh* gene in natural *P. falciparum* populations. A study by Talman showed that there was a single haplotype of *ldh* sequences in 49 *P. falciparum* natural isolates from various geographical locations, which were identical to *P. falciparum* reference strain 3D7 [10]. Later, sequence analysis of *P. falciparum* *ldh* derived from *P. falciparum* populations in India, Iran and Madagascar [11–13] identified 4 novel *P. falciparum* *ldh* allelic types in parasite populations. Thailand is considered one of the key endemic areas for malaria and the hot spot for multi-drug resistance. To date, there is no published data of *P. falciparum* *ldh* genetic diversity in Thailand. To address this deficiency, the present study aimed to investigate the genetic diversity of *P. falciparum* populations in Thailand. The goals were then extended to perform systematic analysis of *P. falciparum* *ldh* sequences deposited in public databases. The outcome of this study enhances the understanding of the genetic diversity, genetic structure and distribution patterns of *P. falciparum* *ldh* in Thailand and worldwide parasite populations. This will aid in the selection of appropriate epitopes for improving RDTs. Moreover, as LDH is a promising drug target, the basis of genetic structure in this study will help to identify the proper inhibitors for the design of LDH-based anti-malarial drugs.

**Methods**

**Parasite sample**

A total of 50 isolates of the human malaria parasite *P. falciparum* used in the present study were maintained from the Malaria Research Laboratory, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. These parasites were isolated from Thai patients who were admitted to malaria clinical centres at the Thailand–Myanmar border in Tak (n = 1), Mae Hong Son (n = 10), Kanchanaburi (n = 14), and Ranong (n = 11), the ‘Thailand–Laos border in Ubon Ratchathani (n = 4) and the Thailand–Cambodia border in Trat (n = 10)’ [14] (see Additional file 1). After microscopic examination, the parasites were culture-adapted using a standard candle jar method [15]. The parasite lines were genotyped with microsatellites and merozoite surface protein-3 gene for species identification [16, 17].

**Amplification and DNA sequencing of the *Plasmodium falciparum* *ldh* gene**

Blood-stage malaria parasites were expanded in laboratories and harvested for genomic DNA preparation. The phenol/chloroform extraction method was employed, as previously described [14]. Genomic DNA was dissolved in TE buffer (pH 8.0) and stored at −20°C until use. PCR amplification of the *ldh* gene utilized two PCR primers with the sequences LDH-F1 5′-ATG GCA CCA AAA GCA AAA ATC GTT T-3′ and LDH-R1 5′-TTG CAT TTG TTT CTC TCT TTG TTG CA-3′, corresponding to nucleotide positions 1041824–1041850 and 1041358–1041382 of chromosome 13 from *P. falciparum* strain 3D7 (Accession Number: NC_004331), respectively. Total PCR volumes of 50 μl contained 200 μM of dNTPs, 2 mM of MgCl₂, 0.2 μM of primers, 200–300 ng of parasite DNA and 1 unit of Taq polymerase enzyme in 1× Taq PCR buffer (Biotechnrib, Germany). PCR amplification started with 95°C for 1 min, followed by 35 cycles of 95°C for 40 s, 60°C for 40 s and 72°C for 1.20 min, with a final extension at 72°C for 5 min. PCR products were analysed by standard agarose gel electrophoresis. All reactions produced a single band with an estimated...
size of ~1000 bp. Subsequently, DNA sequencing was performed using BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems) on an ABI3730XL DNA analyser. Sequencing primers were the PCR primers as well as two additional sequencing primers (LDH-F2 5′-CAT CAA GAT TGA AGT ATT ACA TAT CCT-3′ and LDH-R2 5′-TCT TGT AAA GGG ATA CCA CCT ACA GTA-3′) corresponding to nucleotide positions 1041936–1041962 and 1042384–1042409 of chromosome 13 from *P. falciparum* strain 3D7. Sequences of the *ldh* gene were visualized using BioEdit (version 7.2.6).

**Retrieval of *ldh* sequences from human, gorilla, chimpanzee, and simian malaria parasites**

Nucleotide sequences of *P. falciparum* *ldh* genes from India, Iran and Madagascar were available from the published literature [11–13] (see Additional file 2). *ldh* sequences of *P. vivax* in South Korea, China, India and Iran were previously described [11, 12, 18], while the *ldh* sequence of *P. ovale* strain Harding was retrieved from a previous report by Brown et al. [19] (see Additional file 3). *ldh* sequences of malaria parasites in gorillas and chimpanzees from Cameroon and Democratic Republic of Congo were retrieved from two reports by Liu [20, 21].

Furthermore, to retrieve deposited sequences of *Plasmodium* *ldh* gene from the NCBI database, BLAST searches were performed using the nucleotide sequence 5′ ATG GCA CCA AAA GCA AAA ATC GTT TTA GTT 3′ as a query sequence against the nucleotide collection (nr/nt) database of all plasmodia (Taxid, 5820). Then, complete or partial sequences with an annotation "lactate dehydrogenase" from *Plasmodium* species of humans (*P. falciparum*), gorillas (*Plasmodium gaboni*), chimpanzees (*Plasmodium reichenowi*) and macaques (*Plasmodium knowlesi*) were manually selected (see Additional file 3). Homology of the putative *ldh* sequences was determined by sequence alignment.

Additional unpublished *ldh* nucleotide sequences from *Plasmodium* parasites deposited in the PlasmoDB database [22] were obtained using a key word search for "lactate dehydrogenase". A total of 202 *P. falciparum* *ldh* sequences were deposited by Christopher V Plowe (Howard Hughes Medical Institute/Center for Vaccine Development, University of Maryland), Dan Neafsey (The Broad Institute), Elizabeth Winzeler (The Scripps Research Institute), Alfred Amambua-Ngwa (Medical Research Council, Fajara Banjul, The Gambia), and Dominic Kwiatkowski (Oxford/Wellcome Trust Sanger Institute) (unpublished data). The *P. knowlesi* *ldh* sequence was deposited by Arnab Pain [23]. The *Plasmodium cynomolgi* *ldh* sequence was deposited by Tachibana et al. [24]. The *P. gaboni* and *P. reichenowi* *ldh* sequences were deposited by Sundaraman et al. [25] and Otto et al. [26], respectively.

**Population genetic analysis of the *Plasmodium falciparum* *ldh* gene**

*ldh* sequences from *Plasmodium* parasites were aligned and edited using the MUSCLE alignment algorithm [27] and the Alignment Editor tool in MEGA 7.0 [28]. The numbers of *ldh* alleles, non-synonymous and synonymous SNP sites, and *ldh* isoforms (variants of polypeptides) in each *Plasmodium* species were analysed using the BioEdit sequence editor (version 7.2.6) and DnaSP (version 5.10.1) [29]. Nucleotide diversity (π) was extrapolated from the number of nucleotide differences between two sequences per nucleotide site as previously described [30]. The haplotype diversity index (H) was calculated from a relative frequency of each haplotype and the numbers of sequences in the dataset [31]. The mean numbers of non-synonymous substitutions per non-synonymous sites (*dN*) and synonymous substitutions per synonymous sites (*dS*) were calculated using the method of Nei and Gojobori [32] with the Hasegawa–Kishino–Yano nucleotide substitution model. *dN*-*dS* values were calculated to investigate evidence of positive selection. Significant positive values at *P* < 0.05 indicated an overabundance of non-synonymous mutation, suggesting directional selection [33, 34].

Departures from the predictions of the neutral mode of molecular evolution were determined using Tajima’s *D*, Fu and Li’s *D* *s* and *F* *s* indices, implemented in DnaSP 5.10.1 software [35, 36]. The results of the neutrality tests were deemed to be statistically significant if the *P* value was less than 0.05 (*P* < 0.05). In addition, sliding window plots, with a window length of 90 bases and a step size of 3 bp, were generated for *dN,* *dS*, neutrality test analyses to identify regions of *ldh* where a significant departure from neutrality was observed (*P* < 0.05). Divergence in the distributions of *ldh* alleles of *P. falciparum* populations in Africa, Asia and South America was tested for using Wright’s fixation index (*F* *s*) with Arlequin version 3.5 [37]. Significant differences of genetic variance for any parasite population pairs at *P* < 0.05 indicated population differentiation.

**Phylogenetic analysis**

Unique *ldh* haplotypes of the malaria parasites from humans (*P. falciparum, P. malariae, P. ovale, P. vivax*), gorillas (*Plasmodium praefalciparum, Plasmodium alderi, Plasmodium bilcollini*), chimpanzees (*P. reichenowi, Plasmodium blacklockii, P. gaboni*), and macaques (*P. knowlesi, P. cynomolgi*) were selected from the putative *ldh* sequences in the NCBI and PlasmoDB databases. Sixty-one *ldh* sequences were included in the phylogenetic tree reconstruction (Additional files 2, 3). The best-fit substitution model for the multiple sequence alignment generated by MUSCLE was determined using the Bayesian
Information Criteria (BIC) strategy in jModelTest [38]. Evolutionary relationships of the aligned sequences were determined using neighbour-joining (NJ) and maximum likelihood (ML) approaches in MEGA 7.0 [28] based on the p-distance method and general time reversible substitution model with a gamma distributed shape parameter (GTR+I+G), respectively. The robustness of the tree topology was tested with 1000 bootstrap replicates.

Results

Nucleotide sequences of the \(ldh\) gene in Thai \(P. falciparum\) isolates

The \(ldh\) gene of \(P. falciparum\) was amplified from genomic DNA of 50 \(P. falciparum\) isolates and sequenced using a Sanger sequencing method as described in “Methods”. An analysis of the 918-bp partial sequences of the \(ldh\) gene, corresponding to nucleotide positions 31–948 in \(P. falciparum\) strain 3D7, revealed a single allelic type of \(ldh\), named \(L1a\) (Table 1 and Additional file 1). The \(L1a\) allele was also identical to the sequence of \(P. falciparum\) strain 3D7.

Nucleotide sequences of the \(ldh\) gene in worldwide \(P. falciparum\) isolates

Having demonstrated the fixation of the \(ldh\) allele in Thai \(P. falciparum\) populations, the global diversity of the \(ldh\) gene in \(P. falciparum\) was further investigated. Nucleotide sequences of the \(ldh\) gene in \(P. falciparum\) from 23 countries worldwide were retrieved from the PlasmoDB and NCBI databases and the literature (Table 2 and Additional file 2), thereby generating a database of \(ldh\) sequences containing 268 worldwide \(P. falciparum\) isolates. A comparison of the 918-bp nucleotide sequences revealed 15 single nucleotide polymorphism (SNP) sites, revealing 9 \(ldh\) gene alleles with a haplotype diversity index \((H)\) of 0.203, an average pairwise nucleotide difference between sequences \((k)\) of 0.355 and an average nucleotide diversity at each locus \((\pi)\) of 0.0004.

In Table 1, 7 SNPs detected at positions 36 (TC A/TCG), 399 (GT A/GTC), 450 (TT A/TTG), 513 (CC A/CCC), 783 (TCA/TCG), 858 (GTT/GTC) and 891 (GAG/GAA) resulted in synonymous mutations at amino acid residues 12 (S), 133 (V), 150 (L), 171 (P), 261 (S), 286 (V) and 297 (E), respectively. The nucleotide substitution frequencies at positions 12, 133, 150, 171, 261, 286, and 297 were A/G (99.8%/0.2%), A/C (99.8%/0.2%), A/G (99.8%/0.2%), A/C (99.8%/0.2%), A/G (99.8%/0.2%), T/C (99.8%/0.2%) and G/A (99.8%/0.2%). The other 8 SNPs were detected at nucleotide positions 73 (CAG/AAG), 85 (GGA/CGA), 259 (GGA/CGA), 407 (TTA/TCA), 451 (GGT/CGT), 560 (GTT/GGT), 563 (CTT/CCT) and 814 (GAT/AAT), resulting in non-synonymous amino acid

| Allele | Isoform | Nucleotide position (corresponding amino acid position) |
|--------|---------|--------------------------------------------------------|
|        |         | 36 (12) | 73 (25) | 85 (29) | 259 (87) | 399 (133) | 407 (136) | 450 (150) | 451 (151) | 513 (171) | 560 (187) | 563 (188) | 783 (261) | 814 (272) | 858 (286) | 891 (297) |
| L1a    | LDH-1   | TCA     | CAG     | GGA     | GGA     | GTA     | TTA     | TTA     | GTT     | CCA     | GTT     | CIT     | TCA     | GAT     | GTT     | GAG     |
|        |         | S       | Q       | G       | G       | V       | L       | L       | V       | P       | L       | E       | S       | D       | V       | E       |
| L1b    |         | TCG     | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       |
| L1c    |         | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       |
| L1d    |         | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       |
| L1e    |         | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       |
| L2     | LDH-2   | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       |
| L3     | LDH-3   | –       | AAG     | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       | –       |
| L4     | LDH-4   | –       | CGA     | CGA     | GTC     | –       | –       | CGT     | –       | –       | –       | –       | –       | –       | –       | –       |
| L5     | LDH-5   | –       | –       | –       | –       | TCA     | –       | –       | –       | –       | –       | CCT     | –       | –       | –       | –       |

Table 1 Single nucleotide polymorphisms (SNPs) in the gene encoding lactate dehydrogenase (LDH) of \(P. falciparum\)

Positions of polymorphic nucleotides (underlined letter) in the \(ldh\) gene are numbered according to the \(ldh\) sequence of \(P. falciparum\) reference strain 3D7. Amino acid positions of LDH are shown in parentheses (bold, non-synonymous amino acid substitution; italic, synonymous substitution). A nucleotide substitution at position 15 (GCA/GCC) reported in \(P. falciparum\) Mzr-1 isolate from India (NCBI sequence ID: JN547219, [11]) was excluded in the present study. Dashes (–) indicate the nucleotide sequence of the \(L1a\) allele.
substitutions at residues 25 (Q/K), 29 (G/R), 87 (G/R), 136 (L/S), 151 (G/R), 187 (V/G), 188 (L/P) and 272 (D/N), respectively. The nucleotide substitution frequencies at positions 73, 85, 259, 407, 451, 560, 563 and 814 were C/A (99.8%/0.2%), G/C (99.8%/0.2%), G/C (99.8%/0.2%), T/C (99.8%/0.2%), G/C (99.8%/0.2%), T/G (99.8%/0.2%), T/C (99.8%/0.2%), and G/A (91.1%/8.9%), respectively.

Of the 7 synonymous SNP sites, 2 SNPs at positions 36 and 891 were unique to L1b, whereas SNPs at positions 513 and 858 were unique to L1c (Table 1). The other 2 SNPs at positions 450 and 783 were unique to L1d and L1e, respectively. L2, L3, L4 and L5 alleles of the ldh gene were characterized by 8 non-synonymous SNPs. Two SNPs at positions 814 and 73 were unique to the L2 and L3 alleles, respectively. Four SNP sites at positions 85, 259, 451 and 560 were specific to the L4 allele, while 2 SNP sites at positions 407 and 563 were specific to the L5 allele.

Table 2 Geographical distribution of ldh alleles in natural isolates of Plasmodium falciparum

| Origin of P. falciparum | L1a | L1b | L1c | L1d | L1e | L2 | L3 | L4 | L5 |
|------------------------|-----|-----|-----|-----|-----|----|----|----|----|
|                       |     |     |     |     |     |    |    |    |    |
| Cambodia²              | 1   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| P. R. China³           | 0   | 0   | 0   | 1   | 0   | 1  | 0  | 1  | 1  |
| Indonesia⁴             | 0 (133)³ | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| India⁵                 | 2 (46)³ | 0   | 1   | 0   | 0   | 0  | 0  | 0  | 0  |
| Iran⁶                  | 6   | 1   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| Laos⁴                  | 2   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| Malaysia⁵              | 1   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| The Philippines⁶       | 1   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| Thailand⁷              | 53  | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| Vietnam⁸               | 3   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| The Gambia⁹            | 54  | 0   | 0   | 0   | 0   | 1  | 1  | 0  | 0  |
| Ghana⁹                 | 0   | 0   | 0   | 0   | 0   | 1  | 0  | 0  | 0  |
| Madagascar¹            | 0 (126)³ | 0   | 0   | 0   | 0   | 1  | 1  | 0  | 0  |
| Mali¹                  | 14  | 0   | 0   | 0   | 0   | 8  | 0  | 0  | 0  |
| Mozambique¹³           | 1   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| Senegal¹³              | 54  | 0   | 0   | 0   | 0   | 8  | 0  | 0  | 0  |
| Tanzania¹³             | 1   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| Uganda¹³               | 7   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| Brazil¹³               | 4   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| Colombia¹³             | 1   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| El Salvador¹³          | 1   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| French Guiana¹³        | 22  | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| Honduras¹⁵             | 2   | 0   | 0   | 0   | 0   | 0  | 0  | 0  | 0  |
| Total                  | 230 | 1   | 1   | 1   | 1   | 31 | 1  | 1  | 1  |

The numbers of nucleotide sequences from the PlasmoDB and NCBI databases are shown

* Published sequences of P. falciparum ldh in Indonesia, Madagascar, India and Iran were taken from the literature [11–13, 39]

* Sequence data of 50 P. falciparum isolates in the present study and 3 sequences of P. falciparum strain K1 submitted to the NCBI database

* Unpublished nucleotide sequences of the ldh gene were retrieved from the PlasmoDB and NCBI databases and used to deduce the amino acid sequences (see Additional file 2)

* Numbers in brackets indicate ldh sequences of P. falciparum that were identical to L1a of P. falciparum 3D7 which were reported in literature [11–13, 39], but without the NCBI sequence IDs

Signatures of negative purifying selection on ldh
Because of the observed low levels of polymorphism, the next goal was to determine signatures of purifying selection on this gene. Three neutrality tests revealed significantly negative Tajima's D, Fu and Li's D* and Fu and Li's F* statistics of −1.967 (P < 0.05), −7.065 (P < 0.02) and −6.196 (P < 0.02), respectively. These results indicated that the ldh gene of P. falciparum was under negative purifying selection. To determine whether specific region(s) of the ldh region were under selection, a sliding window plot analysis was performed, with a window of 90 bp and a step size of 3 bp. Significantly negative D* and F* statistics were detected at nucleotide
positions 333–558 (Fig. 1). A sliding window plot of \( D \) statistics also showed negative values (0 to \(-1.802\)), although the values did not show a significant departure from zero (Fig. 1c). Examination of the patterns of synonymous and non-synonymous substitutions was also indicative of purifying selection, which is not surprising given the low levels of observed polymorphism and the results from the Fu and Li’s \( D^* \) and \( F^* \) tests. The average values of the numbers of non-synonymous substitution per non-synonymous site (\( d_N \)) and number of synonymous substitution per synonymous site (\( d_S \)) were \( 0.02763 \pm 0.2040 \) and \( 0.0138 \pm 0.0854 \), respectively. The \( d_N - d_S \) values of the \( ldh \) gene did not show a significant departure from zero (Fig. 2). Taken together, these results supported the view that the genetic conservation in the \( ldh \) gene of \( P. falciparum \) was mainly attributed to purifying selection.

**Distribution of \( ldh \) alleles in \( Plasmodium falciparum \) populations**

The 918-nucleotide fragments of the \( ldh \) gene were translated to 306 amino acid residues, corresponding to amino acid positions 11–316 in \( P. falciparum \) strain 3D7. An analysis of amino acid sequences revealed 5 distinct isoforms of LDH (Table 1). Five alleles, named \( L1a \), \( L1b \), \( L1c \), \( L1d \), and \( L1e \), produced the same amino acid sequence, corresponding to the LDH-1 isoform. The other 4 allelic types, \( L2 \), \( L3 \), \( L4 \) and \( L5 \), encoded different LDH amino acid sequences and were designated the LDH-2, LDH-3, LDH-4 and LDH-5 isoforms, respectively. Different alleles of \( ldh \) showed different geographical distributions. Table 2 shows that \( L1a \) was the most abundant allele, and was distributed across almost all \( P. falciparum \) populations. \( L1b \), \( L1c \), and \( L1d \) were detected in \( P. falciparum \) populations in Iran, India and China, while \( L1e \) was present in Mali. The second most abundant allele was \( L2 \), which was reported in Iran, The Gambia, Madagascar, and Uganda. In contrast, 3 alleles, \( L3 \), \( L4 \) and \( L5 \), were rare \( ldh \) alleles that were detected in single parasite isolates. \( L3 \) was reported only in Madagascar, while the \( L4 \) and \( L5 \) isoforms were found in \( P. falciparum \) populations in China.

The distribution of different LDH isoforms also reflected different levels of genetic diversity of \( ldh \) in \( P. falciparum \) populations. The results showed that genetic diversity of the \( ldh \) gene was lowest in South America, where one isoform of LDH was detected in all \( P. falciparum \) populations. In contrast, \( P. falciparum \) populations in Iran and many African countries, including the Gambia, Ghana, Senegal, and Uganda carried the 2 most abundant isoforms of LDH (LDH-1 and LDH-2). Madagascar was the only endemic area in which 3 isoforms (LDH-1, LDH-2, LDH-3) were detected.

To further determine whether parasite populations in Asia, Africa and South America are genetically isolated, pairwise inter-population comparisons were performed for each parasite population using Wright’s fixation index (\( F_{st} \)). Table 3 shows that the \( F_{st} \) values from the pairs of \( P. falciparum \) populations between South America and Asia and between South America and Africa were low and non-significant. The analysis indicated that the \( P. falciparum \) population in South America was genetically similar to \( P. falciparum \) populations in Africa and Asia. In contrast, a significant \( F_{st} \) value was detected between

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**Fig. 1** Sliding window plots of Tajima’s \( D \) values, Fu and Li’s \( D^* \) values and Fu and Li’s \( F^* \) value of \( Plasmodium falciparum \) lactate dehydrogenase. The plots of Tajima’s \( D \) values (a), Fu and Li’s \( D^* \) values (b) and Fu and Li’s \( F^* \) values (c) were analysed using default parameters, with a window length of 100 bp and step size of 25 bp. Significantly negative \( D^* \) and \( F^* \) statistics were detected at nucleotide positions 333–558 (corresponding to amino acid residues 111–186), suggesting a signature of negative (purifying) selection. The red line indicates \( p \) values < 0.05.
Evolutionary relationships of *Plasmodium* spp. *ldh* genes in mammals

A final goal of the present study was to compare levels of genetic diversity of *ldh* sequences in different *Plasmodium* species infecting humans and non-human primates. Nucleotide sequences of *ldh* genes of the human malaria parasites *P. vivax*, *P. ovale* and *P. malariae* were retrieved from the NCBI database, as described in “Methods”. The 49 *ldh* sequences of *P. vivax* contained 21 non-synonymous SNPs and 10 synonymous SNPs, which could be classified into 11 alleles, while the 3 *ldh* sequences of *P. ovale* contained 5 non-synonymous SNPs and 27 synonymous SNPs classified into 3 alleles (Table 4 and Additional file 3). There was only one *ldh* sequence from *P. malariae*. The level of *ldh* nucleotide diversity (\( \pi \)) in *P. ovale* (\( \pi = 0.0208 \)) was greater than those of *P. falciparum* (\( \pi = 0.0004 \)) and *P. vivax* (\( \pi = 0.0021 \)), although the number of available sequences was much lower. The non-synonymous mutations were outnumbered by synonymous mutations in *P. ovale* *ldh* sequences, which again suggest that the *P. ovale* *ldh* gene is under strong purifying selection. In addition to the *ldh* sequences of the human malaria parasites, *ldh* sequences from parasites of non-human primates were obtained from the NCBI database, including 9 *ldh* sequences from 3 species of malaria parasites in the gorilla: *P. praefalciparum* \((n = 4)\), *P. alderi* \((n = 4)\) and *P. billcollinsi* \((n = 1)\), 29 *ldh* sequences from 3 species of malaria parasites in chimpanzees: *P. reichenowi* \((n = 11)\), *P. gaboni* \((n = 15)\) and *P. blacklocki* \((n = 3)\), and 2 *ldh* sequences from 2 species of malaria parasites in macaques: *P. cynomolgi* and *P. knowlesi*.

### Table 3 Pairwise *F*\(_{st}\) values of *ldh* haplotypes between *Plasmodium falciparum* populations in Asia, Africa and South America

|                      | South America | Africa |
|----------------------|---------------|--------|
| Africa               | 0.00167 (0.12613 ± 0.0309) | –      |
| Asia                 | – 0.01592 (0.99999 ± 0.0030) | 0.01569* (0.00000 ± 0.0000) |

Asterisk (*) indicates significant *F*\(_{st}\) value

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**Table 4 Genetic diversity of the gene encoding lactate dehydrogenase in malaria parasites of humans and non-human primates**

| Species                  | Host                      | n   | Size (bp) | nsSNP | sSNP | Allele | Isoform | \( \pi \) |
|--------------------------|---------------------------|-----|-----------|-------|------|--------|---------|---------|
| *P. falciparum*          | Homo sapiens              | 268 | 918       | 8     | 7    | 9      | 5       | 0.0004  |
| *P. vivax*\(^a\)         | Homo sapiens              | 49  | 931       | 21    | 10   | 11     | 9       | 0.0021  |
| *P. ovale*\(^b\)         | Homo sapiens              | 3   | 951       | 5     | 27   | 3      | 3       | 0.0208  |
| *P. malariae*\(^c\)      | Homo sapiens              | 1   | 898       | ND    | ND   | 1      | 1       | ND      |
| *P. praefalciparum*\(^d\) | Gorilla gorilla gorilla  | 4   | 821       | 1     | 1    | 3      | 2       | 0.0013  |
| *P. alderi*\(^e\)        | Gorilla gorilla gorilla  | 4   | 770       | 0     | 3    | 4      | 1       | 0.0022  |
| *P. billcollinsi*\(^f\)   | Gorilla gorilla gorilla  | 1   | 770       | ND    | ND   | 1      | 1       | ND      |
| *P. reichenowi*\(^g\)    | Pan troglodytes           | 11  | 822       | 0     | 9    | 10     | 1       | 0.0025  |
| *P. gaboni*\(^h\)        | Pan troglodytes           | 15  | 770       | 1     | 9    | 14     | 3       | 0.0030  |
| *P. blacklocki*\(^i\)    | Pan troglodytes           | 3   | 770       | 1     | 1    | 3      | 2       | 0.0017  |
| *P. cynomolgi*\(^j\)     | Macaca fascicularis       | 1   | 770       | ND    | ND   | 1      | 1       | ND      |
| *P. knowlesi*\(^k\)      | Macaca fascicularis       | 1   | 770       | ND    | ND   | 1      | 1       | ND      |

\(n\) number of nucleotide sequences, \(nsSNP\) non-synonymous SNPs, \(sSNP\) synonymous SNPs, ND not determined

\(^a\) *ldh* sequences of *P. vivax* in South Korea, China, India and Iran were taken from the literature [11, 12, 18]

\(^b\) *ldh* sequence of *P. ovale* strain Harding derived from Brown et al. [19]

\(^c\) *ldh* sequences from gorilla and chimpanzee malaria parasites in Cameroon and Democratic Republic of Congo were taken from Liu et al. [20, 21]

\(^d\) Unpublished sequences deposited in the PlasmoDB database (see Additional file 3 for sequence ID)
P. knowlesi (Additional file 3). An analysis of non-human primate malaria ldh sequences showed that the highest numbers of ldh alleles were detected in malaria parasites isolated from chimpanzees, P. gaboni and P. reichenowi. There were, however, no differences in the levels of the nucleotide diversity of ldh in P. gaboni and P. reichenowi compared to other non-human primate malaria parasites, including P. praefalciparum, P. alderi and P. blacklocki. It should also be noted that the levels of nucleotide diversity of ldh genes in the non-human malaria parasites were much higher than that of P. falciparum but were similar to that of P. vivax. These data demonstrate, therefore, the different levels of genetic diversity in ldh genes across malaria parasite species of humans and non-human primates.

Phylogenetic trees of 61 ldh gene alleles from the 12 species of Plasmodium species in human and non-human primates were constructed using the neighbour-joining and maximum likelihood methods. As shown in Fig. 3, the phylogenetic tree from the maximum likelihood method yielded 2 branches of ldh genes. The same tree topology was generated using neighbour-joining method (Additional file 4). The major branch is represented by the sequences of the 3 human malaria parasites, P. falciparum, P. malariae and P. ovale, as well as the gorilla and chimpanzee Plasmodium sequences. The tree also inferred that the P. falciparum ldh gene clustered closely together with the gorilla and chimpanzee malaria parasites. It should be noted that P. ovale and P. malariae formed a sister branch to the P. falciparum lineage, but the sequences were more diverse and distantly related to P. falciparum. Interestingly, the minor branch of the ldh gene tree was represented by the sequences of P. vivax ldh and the malaria parasites in macaques, P. knowlesi and P. cynomolgi. These results imply that the malaria parasites in humans may originate from different sister species of non-human primate malaria.

**Discussion**

LDH is considered a major target for therapeutic drugs and for the development of RDTs. The sequence analysis of ldh genes for human and non-human malaria parasites has been a subject of intensive research. However, there is limited sequence information available for the ldh gene, especially for P. falciparum populations in Thailand, the hotspot of multidrug resistance. Thus, the primary goal of the present study was to investigate the genetic diversity of the LDH gene in natural isolates of P. falciparum collected from 5 localities near the Thailand–Myanmar, Thailand–Laos and Thailand–Cambodia borders. The main finding was that Thai P. falciparum isolates all possessed identical sequences for the ldh gene, named L1a. Similar genotyping results of the P. falciparum ldh gene were found in Indonesia, where all P. falciparum isolates carried the L1a allele [39]. This suggested the fixation of the ldh gene in P. falciparum populations in Thailand and
Indonesia. The findings were contrary to previous analyses of *ldh* sequences in *P. falciparum* in India, Iran and Madagascar [11–13], in which multiple *ldh* alleles (L1b, L1c, L2 and L3) were reported.

To further investigate the global diversity of *ldh*, the deposited *ldh* sequences of *P. falciparum* from various geographical origins in the NCBI and PlasmoDB databases were analysed. Interestingly, the analysis identified 4 unreported alleles of *P. falciparum ldh*. The L1d and L1e alleles differed from L1a by 1 synonymous mutation and were found in *P. falciparum* isolates in China and Mali, respectively. L4 and L5 were detected in a *P. falciparum* isolate, FCC1/HN, maintained at the Institute of Tropical Medicine, First Military Medical University, Guangdong, China. Because L4 and L5 were found in laboratory isolates, further investigation will be required to determine whether the mutations occur in natural parasite populations or arose through technical errors. Overall, there were at least 9 alleles of the *ldh* gene in *P. falciparum* worldwide, and the L1a allele was the most common, circulating in all endemic regions in Asia, Africa and South America.

The present study also applied neutrality tests to determine the signatures of natural selection on the *ldh* gene of *P. falciparum*. The significant negative values of Tajima’s *D*, Fu and Li’s *D* and Fu and Li’s *F* tests implied that the genetic conservation in LDH was due to negative purifying selection. This result was also in agreement with a *dS*–*dF* analysis. These results supported the view that LDH is highly conserved likely due to functional constraints. During the erythrocytic cycle, malaria parasites depend exclusively on anaerobic metabolism for adenosine triphosphate (ATP) production and consequently exhibit high levels of glucose consumption [40–43]. LDH is an enzyme responsible for the conversion of pyruvate to 1-lactate while regenerating NAD⁺ from NADH+H⁺ for continued use in glycolysis. The analysis showed that all 7 non-synonymous SNPs that defined 5 isoforms of LDH were located in the regions that are unlikely to cause significant conformation changes, as shown in molecular 3D structure modelling studies of LDH variants in *Plasmodium* species [19, 44, 45]. The analysis also showed that the 9 alleles of *P. falciparum ldh* had identical amino acids at catalytic residues (R95, D155, R158, H182, see Fig. 4), the active site (K84), cofactor binding sites (P235 and P239) and the substrate specific loop (DKEWN, amino acid positions 90–94) [46]. This demonstrates the significance of functional constraints that limited genetic variability of this gene, supporting the view that the *Plasmodium* LDH enzyme should be an attractive target for development of selective inhibitors.

In addition to its role as an anti-malaria drug target, *Plasmodium* LDH is mainly used in screening of clinical malarias in endemic countries. *ldh* genes of *P. falciparum* were cloned and expressed in *Escherichia coli* for productions of polypeptides [46], which were subsequently used in the production of monoclonal antibodies. Different monoclonal antibodies against LDH have been incorporated into RDTs, such as OptiMAL®, enabling detection and differentiation of *Plasmodium* species infecting humans. The sensitivity and specificity of each of these tests have been assessed over a range of clinical conditions. In the case of OptiMAL for *P. falciparum* detection, the results of field studies appear more variable with an overall sensitivity between 31.4 and 100%. It has been suggested that the genetic diversity of *P. falciparum ldh* might influence the sensitivity of RDT kits [1–5, 7]. However, global analysis of *P. falciparum* LDH sequences revealed that the allele L1a appeared to be the major allele of LDH, representing between 80 and 100% of isolates in *P. falciparum* populations of various geographical origins. Thus, it is highly unlikely that the poor
sensitivity of the LDH-based detection would be influenced by genetic polymorphism in the ldh locus. Additionally, some of this variability may be explained by the relatively poor performance of RDTs at low parasitaemia (> 500 parasites/μl) [7]. Alternatively, it was shown that P. falciparum trophozoites had a relatively higher mRNA expression as well as LDH enzyme activity than rings and schizonts, coinciding with the time of maximal metabolic activity by the parasites, and trophozoites were the most susceptible stage of the anti-LDH drugs [47]. Thus, it is suggested that the sensitivity of RDT-tests could be influenced by the stages of erythrocytic development. Whether different erythrocytic stages of P. falciparum may account for variability in the sensitivity of LDH-based RDTs will require further investigation.

Currently, LDH peptides representing different regions of P. falciparum LDH-1 were chosen for productions of antibodies, which were subsequently incorporated in immunodiagnostic assays [3, 48–51]. The comparative sequence analysis of LDH epitopes and different isoforms of P. falciparum LDH indicated that some epitopes, incorporating amino acid residues 3–28, 82–105 and 85–99, contain polymorphic amino acid residues, which differentiate LDH-1 from LDH-3 and LDH-4 (see Additional file 5). Since LDH-3 and -4 isoforms represent < 1% in P. falciparum populations (see Table 2), this implies that most commercial immunodiagnostic assays and/or RDTs based on monoclonal antibodies against LDH-1 for P. falciparum detection should remain effective in all P. falciparum populations. Furthermore, when the LDH epitope sequences were compared with LDH sequences of other human Plasmodium species, it was found that LDH epitopes of P. falciparum contains a number of polymorphic sites that were different from P. vivax LDH. This was consistent with a previous finding that antibodies generated using these epitopes could detect P. falciparum with high specificity, but not P. vivax [48]. However, the comparative sequence analysis also revealed that some regions of LDH were conserved in all Plasmodium species, including KEWNRDDLPLNNK (amino acid residues 74–87), LKRYITVGGIPQEEF (amino acid residues 172–186), ASPYVAPAAAIEMAE (amino acid residues 216–231), CSTLLEQYGH (amino acid residues 243–253). Thus, these sequences may be selected for productions of pan-malarial antibodies for detection of all Plasmodium species.

Wright's fixation index (Fst) indicated that allelic distribution patterns of P. falciparum ldh in South America were similar to those in Africa and Asia. This result was mainly because all 3 parasite populations shared the same allele of ldh, L1a. This finding may suggest the evidence of gene flow between the parasite populations on different continents. This finding was supported by a microsatellite and SNP study that addressed the multiple introductions of African P. falciparum to South America [52]. The Fst analysis also indicated that ldh sequences in Africa and Asia were genetically distinct. This finding suggested that novel ldh alleles of P. falciparum in Africa and Asia may have arisen independently. The data showed that LDH-2 was more common in Africa, while it was extremely rare in P. falciparum in Asia. Further investigation will be required to determine whether LDH-2 has selective advantages over other isoforms.

Finally, sequence analysis of ldh from different species of human-derived Plasmodium revealed different levels of genetic diversity. The data indicated that the nucleotide sequence diversity of P. falciparum was lower than that of P. ovale and P. vivax. The present study was in general agreement with the recent reports of comparative genomic analysis showing more polymorphism in the P. vivax genome than in P. falciparum [53]. The results also revealed that the genetic diversity P. falciparum ldh was relatively lower than that of simian parasites. The paucity of genetic polymorphism in P. falciparum ldh may be indicative of a recent bottleneck and is analogous to the hypothesis by Rich stating that the origin of extant global populations of P. falciparum that have recently evolved from a single ancestral population [54].

The genealogy of Plasmodium species inferred from the phylogenetic tree constructed using ldh sequence revealed ldh sequences clustered according to the species of the malaria parasites. An exception was that ldh sequences of P. falciparum and P. praefalciparum were clustered in the same clade, thereby confirming the close evolutionary proximity between the two species. This finding was consistent with mitochondrial and genomic DNA studies, which have demonstrated the shared common origin of these parasites and supported the view that P. praefalciparum was the most recent common ancestor of P. falciparum [20, 21]. This result was because all P. praefalciparum ldh sequences (excluding HM235127 and HM235119) had sequences identical to the L1a allele of P. falciparum. The tree also indicated that the human malaria parasite P. falciparum was more evolutionary related to the malaria parasite in gorillas and chimpanzees than any other species of human malarias. It should be noted that P. ovale and P. malariae, which were clustered with P. vivax according to the phylogenetic tree based on the mitochondrial DNA sequences [55], were not monophyletic with P. vivax in ldh-based trees. Instead, P. vivax ldh formed a monophyletic clade with the malaria parasites of macaques, supporting the evolutionary closeness of P. vivax, P. cynomolgi and P. knowlesi.
Conclusion
This study extended the understanding of genetic variation in ldh and the prevalence of ldh alleles in natural populations of P. falciparum in Thailand and other endemic areas worldwide. ldh sequences of P. falciparum in Thailand are mono-allelic for the L1a allele. The L1a allele was also the major allele of ldh in P. falciparum in Asia, Africa and South America, implying that the contribution of genetic diversity of P. falciparum ldh to the poor sensitivity of RDT is highly unlikely. The genetic conservation of ldh in P. falciparum makes this gene an excellent target for anti-malaria drug development as well as a key target for RDT detection. Sequence information of ldh also recovered a close evolutionary relationship of the human malaria parasite P. falciparum and parasites of the African great apes.

Additional files

**Additional file 1.** Nucleotide sequence IDs of the ldh gene of Plasmodium falciparum in Thailand.

**Additional file 2.** Nucleotide sequence IDs of the ldh gene of Plasmodium falciparum from different geographical locations.

**Additional file 3.** Nucleotide sequence IDs of the ldh gene of the malaria parasites of humans and non-human primates.

**Additional file 4.** Neighbour joining tree of 61 allelic sequences of the gene encoding lactate dehydrogenase (ldh) from 12 Plasmodium parasite species. The sequences are named according to parasite species and allelic type. The first two letters indicate parasite species: PF (Plasmodium falciparum), Pm (Plasmodium malariae), Po (Plasmodium ovale), Pv (Plasmodium vivax), Pp (Plasmodium praefalciparum), Pr (Plasmodium reichenowi), Pb (Plasmodium billieti), Pf (Plasmodium blacki), Pa (Plasmodium aldibi), Pg (Plasmodium gambiae), PK (Plasmodium knowlesi) and Pc (Plasmodium cynomolgi). Species showed on the right hand side are labelled with color representing parasite host: Homo sapiens (blue), Gorilla gorilla (black), Pan troglodytes (green) and Macaca fascicularis (red). The tree was constructed using the aligned sequences of 768 nucleotides, corresponding to nucleotide position 52–819 after P. falciparum strain 3D7. Bootstrap values are shown next to the nodes. Scale bar shows nucleotide substitution per site.

**Additional file 5.** Sequence alignment of Plasmodium falciparum LDH epitopes in immunodiagnostic assays and LDH sequences of human Plasmodium species. Only LDH sequences that were different from LDH epitopes were shown. Letters in grey indicate polymorphic amino acid residues. Dot (.) indicates an amino acid residue identical to that of LDH epitopes.

Authors’ contributions
PS and SP performed data analysis and participated in manuscript preparation. SP and PH provided a conceptual framework for the project, guidance for interpretation of the data, performed data analysis, and participated in manuscript preparation, revision and coordination. All authors read and approved the final manuscript.

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