Origin of *Plasmodium falciparum* malaria is traced by mitochondrial DNA

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Abstract

The origin and geographical spread of *Plasmodium falciparum* is here determined by analysis of mitochondrial DNA sequence polymorphism and divergence from its most closely related species *P. reichenowi* (a rare parasite of chimpanzees). The complete 6 kb mitochondrial genome was sequenced from the single known isolate of *P. reichenowi* and from four different cultured isolates of *P. falciparum*, and aligned with the two previously derived *P. falciparum* sequences. The extremely low synonymous nucleotide polymorphism in *P. falciparum* (p = 0.0004) contrasts with the divergence at such sites between the two species (K = 0.1201), and supports a hypothesis that *P. falciparum* has recently emerged from a single ancestral population. To survey the geographical distribution of mitochondrial haplotypes in *P. falciparum*, 104 isolates from several endemic areas were typed for each of the identified single nucleotide polymorphisms. The haplotypes show a radiation out of Africa, with unique types in Southeast Asia and South America being related to African types by single nucleotide changes. This indicates that *P. falciparum* originated in Africa and colonised Southeast Asia and South America separately. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Plasmodium falciparum*; *Plasmodium reichenowi*; Mitochondrial genome; Genetics; Species; Evolution

Abbreviations: mt, mitochondrial; nt, nucleotide.

*Note: Nucleotide sequences reported in this paper are available in the EMBL, GenBank™ and DDBJ databases under the accession numbers AJ251941 and AJ276844-AJ276847.

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1. Introduction

The origins of major infectious diseases of humans are of relevance to understanding evolution of pathogen virulence and natural selection on the human genome [1]. The malaria parasite *Plasmodium falciparum* has had an unparalleled impact on human society and health, and remains a major agent of human mortality [3]. The malaria parasite has a small mitochondrial DNA (mtDNA) genome and is uniparentally inherited [13]. Replication involves recombination within but probably not between mt lineages [14]. A broad phylogeny of Plasmodium species based on sequences of the mitochondrial cytochrome b gene [15] was consistent with that derived from rRNA gene sequences in chromosomal DNA [6]. Here, the complete mt genome sequence was derived from *P. reichenowi* and four cultured isolates of *P. falciparum*, and analysed together with two previously derived *P. falciparum* sequences. This identifies, and provides a quantitative survey of, intra- and inter-specific nucleotide differences. The single nucleotide polymorphisms (SNPs) and their composite haplotypes within *P. falciparum* were then determined from 104 field isolates, revealing a stark geographical radiation of mtDNA haplotypes.

2. Materials and methods

2.1. Sequence analysis of *P. reichenowi* and *P. falciparum* mitochondrial genomes

Complete mt genome sequences were derived from *P. reichenowi* and *P. falciparum* from diverse sources (7G8 from Brazil, NF54 imported to the Netherlands from Africa, T9/96 and K1 from Thailand). This was performed by PCR amplification and sequencing of eight overlapping regions covering a complete linear copy of the mt genome. The nucleotide (nt) positions based on the EMBL sequence of the C10 *P. falciparum* clone (accession no. M76611), and pairs of oligonucleotide PCR primers were: (1) nt 85–741, fwd 5'CAACACCATCAATTGATTGGG-3', rev 5'-CAGAATAATTGACATGTGATGTC-3'; (2) nt 650–1626, fwd 5'-CCTTACTGACTCTATGAAC-3', rev 5'-TTGCAAGGCCTGAGACG-3'; (3) nt 1493–2289, fwd 5'-AGAACGTTAGATAATGTCCG-3', rev 5'-AAATCTCCGAATAATCCTGGCA-3'; (4) nt 2142–3135, fwd 5'-ATTATCGATAATACGTACTGAT-3', rev 5'-ACCTAATATAAC-TCCAGTAGTACC-3'; (5) nt 3049–3954, fwd 5'-TGTGTATGATACAGCTCTTCA-3', rev 5'-TCCAAATTACTGCCTAGGAAT-3'; (6) nt 3841–4748, fwd 5'-ATTATTTGATCTAGG-3', rev 5'-AGCATCCATCTACAGATGTTG-3'; (7) nt 4644–5534, fwd 5'-GCATATCTAATGGATGACGGG-3', rev 5'-CTTCTGATGACTCGTGAGG-3'; (8) nt 5459–(next tandem copy)185, 5'-CATCGTATGTTTAGCTTTG-3'. Amplification of fragment (5) using the above primers was inefficient for *P. reichenowi*, and sequences of the flankning overlapping fragments showed that nucleotide differences between the species existed within the template sequences, so another pair of primers was designed for this region of the *P. reichenowi*
sequence: fwd 5'-TTTGGTATGATACATAGCT-CTTCC-3', rev 5'-TCCAAATAACTGCTACTG-GAATAG-3'. Amplification was performed in a total volume of 100 μl with the following components: 2 mM MgCl₂, 1 × Bioline amplification reaction buffer, 200 μM dNTPs, 0.1 μM each oligonucleotide primer, 0.5 U BioTaq™ (Bioline) polymerase, and 2 μl solution of DNA template. A hot start step was performed at 95°C for 10 min, after which samples were placed on ice and BioTaq™ polymerase added. Temperature cycling was as follows; 95°C for 1 min, 60°C for 1 min, 72°C for 1 min for 3 cycles; 95°C for 1 min, 55°C for 1 min, 72°C for 1 min for 42 cycles, and 10 min at 72°C final extension. The amplified products (5 μl) were run on 1.5% agarose gels in TAE buffer to detect the correct band, using EcoR1+HindIII-digested Lambda DNA (Promega) as molecular mass markers.

Amplified products were cloned in pGEM®-T Easy Vector (Promega), with transformation and growth performed in JM 109 Escherichia coli High Efficiency Competent Cells. Inserts in purified recombinant plasmids were sequenced using Thermo Sequenase™ dye terminator cycle sequencing pre-mix kit (Amersham™) using SP6 and T7 universal primers. Samples were run on an ABI -377 DNA sequencer (Perkin–Elmer), and sequences were checked and assembled using Sequence Navigator software. To exclude any error, the P. reichenowi mt genome was sequenced and edited twice, starting from independent PCR amplifications. Any ambiguity, and putative polymorphism among P. falciparum isolates, was checked by additional amplification and sequencing (very important as the actual level of polymorphism within P. falciparum was considerably lower than the PCR misincorporation error rate). Inter-specific nucleotide divergence (K) and intra-specific polymorphism (π) among aligned sequences was calculated with the DnaSP 3.0 software [16].

2.2. P. falciparum populations sampled for mtDNA haplotypes

In order that haplotypes could be resolved, 104 P. falciparum isolates were chosen, each of which had appeared not to contain mixed genotypes at nuclear polymorphic loci. There were 73 from Africa (25 isolates from Ibadan, Nigeria; 18 from Yaounde, Cameroon, 18 from Kwazulu-Natal, South Africa), 11 from Southeast Asia (Malinsau in Sabah, Malaysian Borneo) and 20 from South America (12 from Porto Velho and 8 from Tailandia, Brazil). Genomic DNA was prepared from the original isolates without in vitro culture.

2.3. Genotyping mtDNA in natural populations of P. falciparum

The four polymorphic nucleotides which were identified by sequencing (positions 772, 1692, 4179, and 4952) were typed in the field isolates by PCR amplification followed by sequence specific oligonucleotide probing (PCR-SSOP). Four small fragments of the mt genome were amplified, each incorporating one of the polymorphic nucleotide sites. The nucleotide positions of the fragments, and the primer pairs were as follows: (1) nt 651–880, fwd 5'-CCTTACGTACTCTAGCTATGA-AC-3', rev 5'-ATATATGATACTTCTACCTACCGAA-TGG; (2) nt 1642–1730, fwd 5'-ATCCTAGACGTATCTACCTAGCTATGAC-3', rev 5'-CATCTCAACTCTACCTACCTACCTACCAAC-3'; (3) nt 4065–4289, fwd 5'-CATTCAATGTAGACCAAAATCC-3', rev 5'-TGGTAGAAAGTACCATTCAGGTA-3'; (4) nt 4849–5083, fwd 5'-GGAGTTGGCAAGTTAAGAGTT-3', rev 5'-GTCAATCAAA-TGCAATATAGCAG-3'. To increase the yield of fragment (2), nested PCR was performed on a template (nt 1635–1758) which had been prepared by a first round PCR with the following primers; fwd 5'-ATCTACGTGTATGTGGTCCGTCG-3', rev 5'-GGAGTTGGCAAAATCC-3'. Amplifications were performed in 10 μl volumes in 96-well plates, with the following components; 1.5 mM MgCl₂, 1 × Bioline amplification reaction buffer, 200 μM dNTPs, 0.1 μM each oligonucleotide primer, 0.5 U BioTaq™ (Bioline) polymerase and 1 μl solution of DNA template (with variable concentration of DNA in field isolates). Temperature cycling was as follows; 95°C for 1 min, 60°C for 1 min, 72°C for 1 min for 3 cycles; 95°C for 1 min, 55°C for 1 min, 72°C for 1 min for 42 cycles.
Products were denatured, and 1.5 μl dot-blotted onto replicate nylon membranes (MagnaGraph™) in 96-dot arrays. Membranes were blocked (in 4 × SSPE, 0.1% Lauroylsarcosine, 1.0% milk powder) at 37°C for 30 min. Oligonucleotide probes specific for the alternative alleles of each of the 4 single nucleotide polymorphisms were, 772T 5'-TAACCAGATTATTTCAAC-3', 772C 5'-TAACGACTATTTCACAAC-3', 1692G 5'-TATACTGTGATAGTTAATACA-3', 1692C 5'-TTATATTCTAATACTTACAAAG-3', 4192T 5'-CTATTTATATTTATCGAT-3', 4192C 5'-CTATTTACTATTATC-3'. Probes were 3'-labelled with digoxigenin (DIG) (Boehringer Mannheim) and incubated with membranes at a final concentration of 1 nM in separate tubes containing 5 ml TMAC hybridisation solution (3M Tetramethylammonium chloride, 50 mM Tris–HCl pH 8.0, 0.1% SDS, 2mM EDTA pH 8.0), rotating at 53°C for 90 min. Membranes were then washed while agitating for 2 × 10 min in 2 × SSPE per 0.1% SDS at room temperature (low-stringency washes) and 2 × 10 min (high-stringency washes) in TMAC solution at 56°C. Probes were detected using anti-DIG-AP Fab fragment conjugated with alkaline phosphatase and CSPD reagent as a substrate for alkaline phosphatase (Boehringer Mannheim) following the manufacturer’s guidelines. Membranes were exposed to Hyperfilm-ECL for 1–3 h and films were developed and scored independently by two investigators to determine the allelic pattern of hybridisation. Any rare discordance or uncertainty in allelic scoring led to a sample being repeated. This procedure robustly discriminates alleles differing by a single nucleotide, as described previously for studies on chromosomal gene loci in *P. falciparum* [17,18].

Haplotypes were resolved (the isolates had been intentionally chosen not to contain mixed clones), and their frequencies determined in each geographical population sample. The inter-population component of variance in haplotype frequencies, $F_{ST}$, was calculated as the $\theta$ value [19], and the statistical significance of its deviation from zero was derived by running 10 000 permutations on randomised data, using the FSTAT 1.2 program.

3. Results

3.1. mtDNA sequence polymorphism in *P. falciparum* and divergence from *P. reichenowi*

Full mt genome sequences were derived from the single known isolate of *P. reichenowi* and four isolates of *P. falciparum* (7G8, NF54, T9/96 and K1), and aligned together with sequences from an additional two *P. falciparum* isolates which had previously been reported (C10 of uncertain origin, CAMP from peninsular Malaysia) [12,20]. In the complete alignment of 5965 base pairs, there were 139 nucleotides which differed between *P. reichenowi* and each of the six *P. falciparum* isolates, and four nucleotides which differed among any of the *P. falciparum* isolates (Fig. 1).

The divergence and polymorphism at different types of nucleotide sites in the mt genome is summarised in Table 1. The nucleotide differences are most abundant at synonymous sites within protein coding genes, which supports the idea that there are probably strong constraints on changes at other sites in the mt genome [20]. At synonymous nucleotide sites, the divergence between the species (12%) is 300 times greater than the polymorphism (0.04%) within *P. falciparum*. Thus, the time since the common ancestor of both species is probably at least 2 orders of magnitude longer than the time since the origin of modern *P. falciparum* populations. If the mitochondrial lineages separated with the species approximately 5–10 million years ago [6], *P. falciparum* mt sequences probably derive from an origin within the last 50 000 years.

3.2. Geographical distribution and radiation of mtDNA haplotypes in *P. falciparum*

To investigate the geographical origin of *P. falciparum*, haplotypes of mtDNA were studied by typing the four single nucleotide polymorphisms described above (nucleotides 772, 1692, 4179, and 4952), in 104 isolates from different
locations. Fig. 2 shows an example of the typing of alleles by PCR-SSOP (illustrated for eight isolates typed at position 772) and all 104 isolates were typed in a similar manner at each of the 4 nucleotide positions. Five haplotypes were found, with frequencies in each of the populations shown in Table 2. Three haplotypes are seen in Africa (the most frequent being CGCC) and there is significant heterogeneity in frequencies among the three African populations \( F_{ST} = 0.21, P < 0.001 \). The populations sampled from other continents have different haplotypes (TGCC in Malaysia,

Fig. 1. Scheme of the \textit{P. falciparum}/\textit{P. reichenowi} mitochondrial (mt) genome. Nucleotide positions are numbered as by Feagin et al. [12] (accession no. M76611), putative positions of genes are shown as shaded boxes (grey, protein coding genes; black, LSU rRNA fragments; white, SSU rRNA fragments), with transcription in forward orientation shown above the line and transcription from the opposite strand shown below (the mt genome is poly-cistrionically transcribed from each strand [32,33]). Nucleotide positions of these genes were derived from accession no. M76611 (version 12, updated 04-MAR-2000), and it is possible that additional rRNA gene fragments may be described in the future [34]. Positions of the single nucleotide differences between the species and polymorphisms among the six isolates of \textit{P. falciparum} are shown underneath. The mt genome sequences of \textit{P. reichenowi} and \textit{P. falciparum} NF54, 7G8, K1 and T9/96 have the accession numbers AJ251941 and AJ276844 — AJ276847 (\textit{P. falciparum} C10 and CAMP sequences previously reported have accession numbers M76611 and M99416). The four single nucleotide polymorphisms are at positions 772 (T\textsuperscript{C}), 1692 (G\textsuperscript{A}), 4179 (T\textsuperscript{C}), and 4952 (T\textsuperscript{C}). The haplotypes are CGCC (T9/96 and CAMP), TGCC (K1 and C10), CGCT (NF54), and CATC (7G8). The \textit{P. reichenowi} sequence also has a single nucleotide deletion at position 5451, and the reported sequence of CAMP has a single nucleotide deletion at position 5883.

Table 1
Mitochondrial DNA divergence between \textit{P. falciparum} and \textit{P. reichenowi} and polymorphism within \textit{P. falciparum}

<table>
<thead>
<tr>
<th>Type of nucleotide site (( n = ) number analysed)</th>
<th>Divergence between \textit{P. falciparum} and \textit{P. reichenowi}</th>
<th>Polymorphism within \textit{P. falciparum}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of fixed differences</td>
<td>Divergence (K)</td>
</tr>
<tr>
<td>Synonymous (( n = 817 ))</td>
<td>93</td>
<td>0.1201</td>
</tr>
<tr>
<td>Non-synonymous (( n = 2693 ))</td>
<td>19</td>
<td>0.0082</td>
</tr>
<tr>
<td>rRNA genes (( n = 1101 ))</td>
<td>9</td>
<td>0.0082</td>
</tr>
<tr>
<td>Intergenic (( n = 1354 ))</td>
<td>18</td>
<td>0.0147</td>
</tr>
<tr>
<td>Total (( n = 5965 ))</td>
<td>139</td>
<td>0.0241</td>
</tr>
</tbody>
</table>

* The six complete mtDNA genome sequences of \textit{P. falciparum} and one of \textit{P. reichenowi} are analysed (5965 aligned nucleotide positions, after omission of 2 positions containing deletions). Divergence (K) and diversity (\( \pi \)) indices show the proportion of nucleotides which differ in pairwise comparisons of sequences, for each given type of nucleotide position. The Jukes and Cantor correction is applied, which has a marginal effect for these low levels of sequence differences.

b These intergenic positions are not known to code for protein or rRNA (EMBL accession no. M76611, updated 04-MAR-2000) but it is possible that rRNA transcripts will be mapped to some of them [34]. A small number of other positions encoded both rRNA and protein sequences on opposite strands and are categorised here as the latter.
Fig. 2. Example of typing one of the single nucleotide polymorphisms (nt position 772), using polymerase chain reaction amplification followed by allele sequence-specific oligonucleotide probing (PCR-SSOP). Results are shown from a corresponding portion of replicate membranes probed with oligonucleotides recognizing alleles 772T and 772C (the oligonucleotides differ at the ninth position). The membrane portions each contain PCR products from eight isolates (four isolates from Malaysia on the top row, and 4 from South Africa on the bottom row). It can be clearly seen that the isolates on the top row hybridise to probe 772T and not to 772C, so these parasites have nucleotide T at position 772; conversely the isolates on the bottom row hybridise to probe 772C and not 772T, so these parasites have nucleotide C at this position. Such unequivocal discrimination can be routinely obtained for all single nucleotide polymorphisms (protocol outlined in Section 2). Observed variation in the signal intensity for different isolates is a normal feature of varying parasite DNA abundance between field isolates, which presents no difficulty for scoring relative hybridisation of allelic probes for each individual isolate.

Table 2
Mitochondrial DNA haplotype frequencies in geographical samples of *P. falciparum* field isolates

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>CGCC</td>
<td>0.60</td>
<td>0.50</td>
<td>1.00</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CACC</td>
<td>0.36</td>
<td>0.50</td>
<td>–</td>
<td>–</td>
<td>0.10</td>
</tr>
<tr>
<td>CGCT</td>
<td>0.04</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TGCC</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.00</td>
<td>–</td>
</tr>
<tr>
<td>CATC</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.90</td>
</tr>
</tbody>
</table>

and CATC in Brazil) and only two Brazilian isolates have an African haplotype (CACC). Thus, the inter-continental variance in haplotype frequencies is extremely high (*F*_{ST} = 0.65, *P* < 0.001).

The mtDNA haplotypes are inter-related by single nucleotide differences, and a simple network of haplotype relationships may be constructed. This shows a radiation out of Africa (Fig. 3). Most African isolates have the haplotypes CACC or CGCC which are at the centre of the network, and there is a related African haplotype CGCT which is rare (seen only in NF54 and one Nigerian isolate). Outside of Africa, most isolates have continent-specific types which are at the termini of the network (CATC in 7G8 and most Brazilian field isolates; TGCC in the Malaysian field isolates and cultured Thai isolate K1), and the remainder have the expected putative ancestral types (CACC in two Brazilian field isolates, and CGCC which was seen in Southeast Asian cultured isolates CAMP and T9/96).

4. Discussion

This study gives strong molecular evidence for a recent African origin of *P. falciparum*, and subsequent colonisations of Southeast Asia and South America. The nucleotide diversity throughout the mt genome of *P. falciparum* (0.03% overall, 0.04%
Fig. 3. Geographical distribution of *P. falciparum* mtDNA haplotypes, shown by their nucleotides at each of the 4 polymorphic sites (positions 772, 1692, 4179, 4952). Solid lines indicate single nucleotide differences between haplotypes. Dashed arrows indicate putative colonisation events of haplotypes from Africa into Southeast Asia and South America. The continental distribution of haplotypes was derived from field isolates (Table 2), and from putative origins of cultured isolates which have been fully sequenced (see text of Section 3).
at synonymous positions) is approximately two orders of magnitude lower than the divergence with *P. reichenowi* (2.41% overall, 12.01% at synonymous positions). The best estimate of the split between *P. falciparum* and *P. reichenowi* is 5–10 million years ago [6], so it is likely that existing *P. falciparum* mtDNA sequences derive from a common origin in the order of 50,000 years ago. The precision of this estimation is limited by the very low polymorphism, but it is concordant with an estimate based on low synonymous nucleotide polymorphism in some nuclear genes [5] which allowed for the possibility of an even more recent date. It should be noted that the result obtained here, which compares intra- and inter-species divergence, is robust to any effect of high AT content and biased codon usage in the mitochondrial genome, as this is similar in all *Plasmodium* species studied [20].

The relationship among mtDNA haplotypes in *P. falciparum*, and their geographical distribution, shows a clear radiation out of Africa. The two common African types are central in the simple haplotype tree, which strongly suggests that they are ancestral to the other types. The branch termini consist of one type common in Southeast Asia, an unrelated type common in South America, and a rare type in Africa. This indicates that Southeast Asia and South America were colonised separately by *P. falciparum*, a conclusion which is further supported by the detection of the respective putative ancestral types in these regions (Fig. 3). The data are not incompatible with a hypothesis that *P. falciparum* first emerged in early agricultural communities in Africa [21], aided by recent evolution in larval ecology of the African mosquito vector species *Anopheles gambiae* sensu stricto [22].

Several issues present themselves for future research. It remains possible that an intensive sequencing survey in Africa might reveal more mtDNA diversity than that seen among the complete sequences to date, which were mainly from non-African parasites. Accurate dating of colonisation events, or tracing the spread of *P. falciparum* within each continent, will probably require more sequence diversity than that afforded by the study of the mt genome. It is complex to study ancestral relationships among single nucleotide polymorphism haplotypes in the nuclear genome due to frequent recombination [17,23], so the 35-kb circular plastid genome [24] or rapidly mutating microsatellite loci [23,25] could be best markers for such studies.

It is surprising that the terminal mtDNA haplotypes should have become so common in South America and Southeast Asia, in comparison to their putative ancestors, and it is unknown if positive selection has operated on these haplotypes. The almost complete partitioning of mtDNA haplotypes between continents is similar to the distribution of alleles of a gamete surface protein gene *Pfs*48/45 [26], but contrasts with the more broadly distributed alleles of asexual blood-stage antigen genes surveyed so far [27,28]. This might indicate continent-specific divergent selection on both the mtDNA and *Pfs*48/45, or balancing selection on most asexual blood-stage antigen genes surveyed. In strong contrast with the low overall diversity in *P. falciparum* mtDNA, and in some nuclear genes [5], there are very divergent and apparently ancient alleles in several antigen genes [7–10]. Thus, it is likely that some of the antigen polymorphisms have been maintained for a long time by balancing selection [29,30], which should encourage investigation of their alleles as potential targets of acquired immune responses [27,31].

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References


