Two Nonrecombining Sympatric Forms of the Human Malaria Parasite

Plasmodium ovale Occur Globally

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(See the editorial commentary by Su, on pages 1453–1454.)

Background. Malaria in humans is caused by apicomplexan parasites belonging to 5 species of the genus Plasmodium. Infections with Plasmodium ovale are widely distributed but rarely investigated, and the resulting burden of disease is not known. Dimorphism in defined genes has led to P. ovale parasites being divided into classic and variant types. We hypothesized that these dimorphs represent distinct parasite species.

Methods. Multilocus sequence analysis of 6 genetic characters was carried out among 55 isolates from 12 African and 3 Asia-Pacific countries.

Results. Each genetic character displayed complete dimorphism and segregated perfectly between the 2 types. Both types were identified in samples from Ghana, Nigeria, São Tomé, Sierra Leone, and Uganda and have been described previously in Myanmar. Splitting of the 2 lineages is estimated to have occurred between 1.0 and 3.5 million years ago in hominid hosts.

Conclusions. We propose that P. ovale comprises 2 nonrecombining species that are sympatric in Africa and Asia. We speculate on possible scenarios that could have led to this speciation. Furthermore, the relatively high frequency of imported cases of symptomatic P. ovale infection in the United Kingdom suggests that the morbidity caused by ovale malaria has been underestimated.

One of 5 parasite species that causes human malaria [1, 2], Plasmodium ovale (Stevens 1922) is widely distributed across tropical regions [3, 4], although there have been no confirmed cases in the Americas [5]. The global burden of ovale malaria is considered to be slight,
principally because the prevalence of *P. ovale* as detected by microscopy is low. Difficulty of diagnosis is partly the result of the low parasite density that is characteristic of clinical malaria caused by ovale malaria parasites [3, 6–8]. *P. ovale* can be differentiated from other parasite species by amplification of species-specific sequences within genes encoding the parasite’s small subunit ribosomal RNA (ssrRNA) [9]. However, a number of isolates microscopically identified as *P. ovale* could not be detected by this method [10] because of sequence variations in the ssrRNA gene target. It has since been demonstrated that the dimorphism into classic and variant ssrRNA types extended to other genes—specifically, the mitochondrial locus cytochrome b and the nuclear genes encoding both lactate dehydrogenase and the ookinete surface antigens of *P. ovale* [11–13]. Such dimorphism may simply reflect a regional discontinuity in the distribution of ovale malaria, and indeed most reports of variant parasites to date have been from regions of endemicity in Asia [11, 13].

We hypothesized that the dimorphic forms of *P. ovale* actually comprise distinct species and that these might be geographically restricted. Two independent studies were initiated at Mahidol University, Bangkok, Thailand (MUB), and at the Health Protection Agency Malaria Reference Laboratory, London School of Hygiene and Tropical Medicine, London, United Kingdom (UKMRL), to address this hypothesis. Multilocus genetic analysis was used to explore the genetic diversity exhibited among 55 *P. ovale* isolates from 15 countries. The combined results of these 2 studies led us to conclude that *P. ovale* consists of 2 nonrecombining species but that these are sympatric in both Africa and Asia.

**METHODS**

**Parasite isolates.** The UKMRL sample set was collected by the Health Protection Agency Malaria Reference Laboratory at the London School of Hygiene and Tropical Medicine, the reference center for all imported cases of malaria in the United Kingdom. DNA from *Plasmodium* isolates is extracted and stored for use in validating new molecular diagnostic techniques and for surveillance purposes. No patient identifiers or data were linked to this sample set apart from the presumed country of origin of the infection. The sequence data shown were obtained from *P. ovale* isolates imported in 2007.

The MUB samples were provided by study authors and collaborators in the country of origin and were sent to Mahidol University’s Department of Clinical Tropical Medicine. *P. ovale* DNA was isolated, and DNA sequences at loci of interest were determined by direct sequencing of polymerase chain reaction (PCR) products.

**Genomic data.** Data were accessed via the PlasmoDB Blast server (http://plasmodb.org/plasmo/), using *Plasmodium falciparum* sequences as bait. Where frame shifts were evident in *Plasmodium gallinaceum* or *Plasmodium reichenowi* contigs, these were altered to match the coding sequence of the *P. falciparum* homologue. Prerelease unannotated draft *P. ovale* genome sequence data from the CDC/Nigeria isolate of *P. ovale* [14] were searched by simple text queries for genes encoding putative erythrocyte-binding proteins and for regions coding amino acid repeats, which might be suitable for analysis of intraspecific polymorphism. The CDC/Nigeria isolate is a classic type.

**Locus amplification and sequencing.** Conserved primers based on published sequences from the homologous loci in *Plasmodium vivax* and *Plasmodium malariae* were used to first identify *pog3p* sequences; *podhfr-ts* sequences were amplified using degenerate primers designed specifically for this study. Primers for *potra* and *porbp2* were also designed specifically for this study, on the basis of the unpublished draft genome sequence of the CDC/Nigeria isolate (classic type). All primer sequences used for amplification of each locus are given in Table 1. PCR products were purified and DNA sequences determined using BigDye chemistry (Applied Biosystems) followed by fractionation on capillary sequencers, as described elsewhere [15]. All novel DNA sequences were submitted to GenBank; accession numbers are shown in Table 2.

**Phylogenetic analysis.** Split decomposition graphs were constructed from *pocytb* and *pog3p* data, using the computer program SplitsTree (version 4.0) [16]. Data from *pocytb* and *pog3p* sequences were analyzed independently by 2 investigators using different methods to estimate convergence times of *P. ovale* classic and variant types. First, unrooted trees were generated from maximum likelihood and parsimony methods, using the DNAML and FITCH programs in the PHYLIP program package (version 3.69) [17]. Different tree structures, together with alternative root positions and the imposition of a strict molecular clock, were tested for goodness of fit using the likelihood ratio and Kishino and Hasegawa tests within the CODEML program (version 4.3) [18, 19]. The same program was used to date the divergence between the 2 *P. ovale* sequences, using a calibration date between *P. falciparum* and *P. reichenowi* of 5 million years ago (MYA); 95% confidence intervals were

Table 1. Primer Sequences Used for Amplification of Each *Plasmodium ovale* Locus

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer Sequences Used for Amplification of Each <em>Plasmodium ovale</em> Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pocytb</em></td>
<td>forward primer: 5′-TCTGACATCTGATGTATCAT-3′; reverse primer: 5′-TTCCAAGGAGATGCATGTA-3′</td>
</tr>
<tr>
<td><em>pog3p</em></td>
<td>forward primer: 5′-CTCACTCGTACGCTGCTGCT-3′; reverse primer: 5′-TTCCACCATCGTACGCTGCT-3′</td>
</tr>
</tbody>
</table>

This table is available in its entirety in the online version of the Journal of Infectious Diseases.

Table 2. GenBank Accession Numbers for All DNA Sequences Determined in the Present Study

<table>
<thead>
<tr>
<th>Locus</th>
<th>GenBank Accession Numbers for All DNA Sequences Determined in the Present Study</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pocytb</em></td>
<td>EF488686, EF488687, EF488688, EF488689, EF488690, EF488691, EF488692, EF488693, EF488694, EF488695</td>
</tr>
<tr>
<td><em>pog3p</em></td>
<td>EF488696, EF488697, EF488698, EF488699, EF488700, EF488701, EF488702, EF488703, EF488704, EF488705</td>
</tr>
</tbody>
</table>

This table is available in its entirety in the online version of the Journal of Infectious Diseases.
Table 3. Segregation of 5 Dimorphic Characters among 31 UKMRL *Plasmodium ovale* Isolates and of 3 Dimorphic Characters among 20 MUB *P. ovale* Isolates

<table>
<thead>
<tr>
<th>Locus</th>
<th>Nucleotide polymorphismsa</th>
<th>P ovar cule stateb</th>
<th>Classicc</th>
<th>Variantd</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssrRNA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pocytb</td>
<td>162, 201, 375, 402, 492, 534, 744, 756, 774, 885, 903, 948</td>
<td>A. UKMRL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>porbp2</td>
<td>85, 121, 145, 193, 249, 294, 310, 323, 375, 431, 459, 516, 538, 561, 563, 581, 602, 628, 741, 745</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** All 5 loci were successfully analyzed for the each of the 31 isolates shown in Table 3A: the remaining 4 isolates in the UKMRL data set yielded data for 4 or fewer loci. DNA was not available for pocytb sequence analysis for the majority of MUB isolates. Only polymorphisms occurring in at least 3 isolates are shown in Table 3A and 3B. bp, base pair; MUB, Mahidol University, Bangkok; NA, not applicable; PCR, polymerase chain reaction; ssrRNA, small subunit ribosomal RNA; UKMRL, Health Protection Agency Malaria Reference Laboratory, London School of Hygiene and Tropical Medicine, United Kingdom.

a Nonsynonymous polymorphisms are shown in boldface type. Numbering is from the first nucleotide of rRNA, from the putative initiation ATG (identified in *Plasmodium* species) to nucleotide 774 of the variant.
encodes a tryptophan-rich antigen containing a variable-length 3-amino acid repeat) were analyzed. A similar study was independently initiated for 20 isolates collected at MUB and a number of collaborating centers, but for these sequence analysis was confined to the ssrRNA gene, podhfr-ts (which encodes dihydrofolate reductase–thymidylate synthase), and, for some isolates, the mitochondrial gene pocytb.

There was segregation of all dimorphic characters, such that each of the 55 parasite isolates was either of variant type or of classic type for all determined characters (Tables 3 and 4). Thus, there was no evidence of inter- or intragenic recombination between the classic and variant haplotypes sampled from 2 distinct biogeographic regions of the world. This confirmed that the multigenic haplotypes are stable and is consistent with the existence of a bona fide species barrier, illustrated by split decomposition graphs for pocytb (from Africa and Asia) and pog3p (from Africa only), which indicate a strong bifurcation separating the 2 P. ovale forms (Figure 1). Contrary to a priori predictions, classic and variant P. ovale parasites were found to be sympatric across Africa, occurring together in 5 of the countries from which our isolates were obtained (Table 4). The MUB isolates were found to harbor variant and classic pocytb alleles identical to those in the UKMRL sample set, and these segregated perfectly with ssrRNA genes of the variant and classic types. The only exception was a single isolate from Papua New Guinea—this isolate harbored the variant allele of podhfr-ts, but its ssrRNA gene included 8 residues characteristic of the classic allele from a total of 25 polymorphic sites, with the remaining 17 sites being identical to the variant allele (Table 4). Further analyses of this sample were not possible because of a lack of material. The pocytb sequences from 3 classic and 3 variant P. ovale isolates from Myanmar and Thailand described by Win et al [11] showed perfect concordance with the 22 classic and 18 variant pocytb sequences described here. We conclude that these 2 parasite types are sympatric in Africa and Asia and that allopatry cannot explain the lack of recombination between the multilocus haplotypes we have described.

We reconstructed gene trees for Plasmodium cyt b sequences, using both maximum likelihood–based and distance-based methodologies to ensure congruency of topologies [17, 23]. Alternative roots and the goodness of fit of molecular clocks on both local and global scales were tested [18, 19]. The single optimum bifurcating tree with P. gallinaceum as the outgroup supported a global molecular clock (Figure 2). After calibrating this clock on the assumption of a divergence time of P. falciparum and P. reichenowi of 5 MYA [15, 25], the 2 P. ovale forms converged on a mean estimate of 2.1–2.2 MYA (95% highest posterior density interval, 0.9–3.6 MYA). This finding was supported by an alternative strict-clock analysis [20] that used divergence among the rodent malaria parasites Plasmodium yoelii, Plasmodium chabaudi, Plasmodium berghei, and Plasmodium vinckei to calibrate the clock. Calibration of the original analysis with a more recent P. falciparum/P. reichenowi divergence estimate of 3 MYA [26] suggested a slightly more recent divergence of the P. ovale forms, at 1.3 MYA (95% highest posterior density interval, 0.67–2.1 MYA).

We conclude that the classic and variant P. ovale forms are in fact 2 distinct nonrecombining globally sympatric species, and we propose to name these species Plasmodium ovale curtisi (classic type) and Plasmodium ovale wallikeri (variant type), in honor of Christopher F. Curtis (1939–2008) and David Walliker (1940–2007), respectively. We have adopted the trinomial forms to maintain ovale in the nomenclature for the sake of continuity and to minimize any confusion that might arise from altering the name of a long-established taxon of clinical importance, particularly because it is unclear which of these should be considered the agent of “true” ovale malaria, originally described by Stevens in 1922.

It is difficult to conceive of an explanation for our results if all the parasites analyzed are of the same species. Although self-fertilization may occur between identical parasite genotypes (particularly under conditions of low transmission), no truly clonal natural population of any Plasmodium species parasite has been described. We conclude that sexual recombination is an obligate event for P. ovale parasites, as it is for all other Plasmodium species. The following points summarize our evidence that the 2 forms of P. ovale should be considered distinct species:

| Table 4. Genotype Summary for All Plasmodium ovale Isolates Evaluated in the Present Study |
| This table is available in its entirety in the online version of the Journal of Infectious Diseases |

**Table 4. Genotype Summary for All Plasmodium ovale Isolates Evaluated in the Present Study**

**Figure 1.** Split decomposition graphs of 41 pocytb (A) and 35 pog3p (B) sequences. Graphs were constructed using the computer program SplitsTree (version 4.0) [16]. Scale bars show relative sequence dissimilarity. Data on pocytb are from both the Health Protection Agency Malaria Reference Laboratory, London School of Hygiene and Tropical Medicine, United Kingdom (UKMRL), sample set (n = 34) and the Mahidol University, Bangkok, sample set (n = 6). Data on pog3p are from the UKMRL sample set only; DNA was not available for sequencing of pocytb in 2 UKMRL isolates. The numbers of sequences in each node are shown in italics.
Figure 2. Unrooted phylogenetic tree representing the relationship of Plasmodium ovale curtisi and Plasmodium ovale wailikeri to 14 other taxa, on the basis of cytb sequence data. Bootstrap values calculated from 1000 pseudorandom data sets are given where >85%; the position of the root derived for molecular clock fitting is shown (black arrow). Alternative trees derived from maximum likelihood and parsimony methods, together with alternative root positions, were tested for goodness of fit by maximum likelihood (CODEML program) [19]. With the topology shown, a strict molecular clock was not rejected \( (P = 0.48) \). Calibration of the molecular clock using a divergence time between Plasmodium reichenowi and Plasmodium falciparum of 5 million years ago (MYA) gave an estimated divergence between P. ovale curtisi and P. ovale wailikeri of 2.1 MYA (95% confidence interval, 1.0–3.5 MYA). A similar estimate (2.2 MYA [95% posterior density interval, 0.9–3.6 MYA]) for the divergence of the 2 P. ovale forms was obtained in independent strict-clock analyses, using BEAST software [20]. In these analyses, additional Plasmodium vivax and chimpanzee parasite sequences (CPZcam89 and CPZcam91) [21] were included. A general time-reversible model with invariant sites and \( \gamma \) correction was selected using Modeltest [22], and 3 tree nodes were calibrated assuming a normal distribution and that Asian primate parasites and African simian parasites (represented by Plasmodium gonderi) diverged 8.0 \( (\pm 1.0) \) MYA; that parasites of Pan and Homo species (represented by P. reichenowi and P. falciparum) diverged 4.1 \( (\pm 0.4) \) MYA; and that P. vivax strains diverged from each other 0.25 \( (\pm 0.05) \) MYA. To investigate potential site saturation in the third position, LogDet values [24] were calculated. None were found to be undefined. Those distances with larger values concerned the paths between P. falciparum, P. reichenowi, and the rodent Plasmodium sequences. These paths were not associated with the node calibrations used.

- Perfect segregation of 5 loci into 2 distinct types was found among 55 isolates.
- Both types are widespread in both Africa and Asia and were documented here as being sympatric in many African countries and in at least 1 Southeast Asian country (Myanmar).
- Indirect evidence from imported ovale malaria cases in the United Kingdom suggests that, as Trape and colleagues have previously argued [7, 8], transmission intensity of ovale malaria is not uniformly low, as is usually assumed. This strongly supports the notion that the 2 P. ovale types have the opportunity to mate and recombine but do not do so.
- The extent of the diversity in the cytochrome b gene (12 single-nucleotide polymorphisms), which presents as complete dimorphism between classic and variant P. ovale, is difficult to explain away as within-species polymorphism for a gene that is highly conserved among members of the genus Plasmodium.
- Only minor differences are observed among diffr-ts sequences of P. falciparum, P. vivax, and P. malariae isolates collected from different continents. However, our P. ovale curtisi and P. ovale wailikeri isolates carry very distinct DHFR-TS enzymes (29 nonsynonymous mutations and a different amino acid repeat length in the coding region), suggesting real biochemical differences between them.
- Definitive proof of a biological species barrier ideally comes from cross-mating experiments; these are in fact virtually impossible, because P. ovale parasites cannot currently be cultured. Although P. ovale curtisi is established in experimental chimpanzee models, P. ovale wailikeri is not; consequently, primate-based crosses could not be performed ethically at this time either. Thus, molecular analyses offer the only avenue to analyze the relationship between the dimorphic forms of P. ovale.

Therefore, we consider that the weight of evidence favors the proposition that the 2 P. ovale types are actually 2 distinct species. Allopatry may have contributed to the initial putative speciation event between P. ovale curtisi and P. ovale wailikeri, with the 2 geographically separated populations moving back into sympatry subsequent to divergence, perhaps as a result of migration of their simian or hominid hosts. Alternatively, assuming that the common ancestral parasite lineage of both species was in early hominids, 2 independent host transitions separated in time may have occurred between ape and human lineages. The intervening period of evolution in different hosts could have led to speciation, which then prevented recombination when both lineages later occupied the same primate hosts.

The nature of the mechanism(s) keeping P. ovale curtisi and P. ovale wailikeri apart in the present is of great interest but remains speculative. There are 3 possible scenarios. First, subtle regional, ecological, or seasonal differences in their distribution could be maintaining a physical barrier between the 2 species. Second, the 2 species may have mutually exclusive mosquito or human host specificities. The occurrence of P. ovale curtisi and P. ovale wailikeri across Asia and Africa, where many different Anopheles species transmit malaria to humans, makes it less likely that nonoverlapping vector specificities alone prevent the 2 parasite species from recombining. A possible restriction at the level of the human host is suggested from the differences noted for the porbp2 genes of the 2 species. This locus encodes a putative erythrocyte/reticulocyte-binding molecule. The majority of differences were nonsynonymous (a pattern in stark contrast to the other loci analyzed [Table 3]), which implies...
that *P. ovale curtisi* and *P. ovale wallikeri* may differ in host red cell invasion phenotype. Thus, if each species were an obligate invader of erythrocytes with distinct but mutually exclusive characteristics, the 2 parasite types would never occupy the same human host. However, an erythrocyte-based restriction would not necessarily prevent hybridization between the 2 species because of incomplete feeding. This occurs when a mosquito feeds in >1 human host in the same night, allowing ingestion of both species and thus the chance for recombination. This scenario requires that people carrying infective gametocytes of *P. ovale curtisi* and of *P. ovale wallikeri* are in very close proximity to each other on the same night.

Laboratory-based investigations of erythrocyte invasion are limited for *P. ovale*, because this species cannot at present be maintained in vitro or manipulated genetically. It is interesting to note that host restriction does not prevent infection in the chimpanzee; the *P. ovale curtisi* isolate currently undergoing genome sequencing was propagated in a chimpanzee [14], and recent data confirm that *P. ovale wallikeri* retains the ability to naturally infect chimpanzees [21]. It of course remains possible that host partitioning due to erythrocyte-invasion requirements also occurs between the 2 species in *Pan*.

A third mechanism for maintenance of the 2 distinct species could be mating incompatibility between the gametocytes of *P. ovale curtisi* and *P. ovale wallikeri* or the production of noninfective sporozoites from cross-fertilization events. However, the occurrence of such phenomena among *Plasmodium* parasites has not been documented, to our knowledge. Our observations suggest that propagation of these 2 parasites may involve frequent self-fertilization, given that relatively low within-species diversity is observed. This may assist in the maintenance of 2 distinct *P. ovale* species, because the multigenic haplotypes we have described are remarkably stable across large geographic distances. However, this cannot explain the original divergence of these 2 types, and we assume that *P. ovale* undergoes obligate sexual recombination (as in all *Plasmodium* species studied to date), a view supported by the presence of homologues of sexual-stage antigens from *P. vivax* and *P. malariae* [13]. Some deviation from strict segregation of genetic markers was found for the ssrRNA gene, particularly for a single *P. ovale wallikeri* parasite collected in Papua New Guinea (Tables 3A and 4), but in our view this represents intraspecific polymorphism in a nonprotein coding gene, because the 8 substitutions observed that were not of the *P. ovale wallikeri* type were not contiguous. More detailed studies of *P. ovale* species in Melanesia are planned and may be crucial to understanding these newly recognized sibling species.

The existence of 2 distinct species, *P. ovale curtisi* and *P. ovale wallikeri*, cocirculating across global malaria transmission zones raises a number of important questions. Do these species differ in clinical presentation? Are both species adequately identified by pangenous rapid diagnostic tests [12]? Do they have different drug susceptibility profiles, and do they differ in their relapse periodicity? The eventual publication of the genome sequences of *P. ovale curtisi* should help obtain meaningful data on the genetic diversity of different isolates from the 2 species and assist in answering these questions. The ability of *P. ovale* to relapse from persisting hepatic forms makes this species important in the context of malaria control and its eventual eradication. *P. ovale* and *P. falciparum* often exist in the same host simultaneously [27, 28]. The widespread use of highly effective artemisinin combination therapies to treat *P. falciparum* infections may provide an opportunity for *P. ovale* to emerge from the liver after drug levels subside in treated people, as does *P. vivax* [29–31]. *P. ovale* has been described as having relapse intervals from 17 days to >2 years after exposure [4, 32, 33], but it is possible that the 2 species differ in their relapse periodicity and frequency. Prospective studies of treated cases of *P. ovale curtisi* and *P. ovale wallikeri* infection with adequate follow-up are required to address this question.

Our present investigation also led us to agree with Trape and colleagues that the contribution of *P. ovale* infections to malaria morbidity is significantly underestimated, probably as a result of misdiagnosis [7, 8]. Recent improvements in the quality of malaria microscopy in countries of nonendemicity, together with the use of PCR to confirm species identification, indicates that *P. ovale* infections are more prevalent among imported cases than would be expected on the basis of surveys conducted in malaria-endemic countries [5, 32–34]. For example, among 39,300 malaria cases imported into the United Kingdom from 1987 through 2006, Smith et al [5] reported 2101 confirmed symptomatic cases of *P. ovale* infection, acquired mainly in Africa. More recent data from the UKMRL showed that 45 of the 488 clinical cases of single-species malarial infection imported into the United Kingdom from Nigeria in 2007 were caused by *P. ovale*. If this prevalence of 9.2% held true for all clinical malaria recorded in Nigeria, where the annual malaria burden in 2006 was reported to be 57.5 million cases [35], this would correspond to 5 million symptomatic cases of *P. ovale* malaria per annum. Extending this estimate to the other malaria-endemic countries would predict an Africa-wide burden that exceeds 15 million cases annually. Thus, *P. ovale curtisi* and *P. ovale wallikeri* together constitute a substantial public health problem, and there is ample justification to allocate scientific and financial resources to further understanding to aid control of these hitherto-neglected malaria parasites.

**Acknowledgments**

We thank a number of colleagues for useful discussions, particularly Douglas Futuyma, Chris Newbold, and Richard Culleton. We are grateful to Linda Duval and Frederic Arvey for providing the chimpanzee isolate *cytb* sequences CPZcam89 and CPZcam91.
References