Genomes of all known members of a *Plasmodium* subgenus reveal paths to virulent human malaria

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*Plasmodium falciparum*, the most virulent agent of human malaria, shares a recent common ancestor with the gorilla parasite *Plasmodium praefalciparum*. Little is known about the other gorilla- and chimpanzee-infecting species in the same (*Laverania*) subgenus as *P. falciparum*, but none of them are capable of establishing repeated infection and transmission in humans. To elucidate underlying mechanisms and the evolutionary history of this subgenus, we have generated multiple genomes from all known *Laverania* species. The completeness of our dataset allows us to conclude that interspecific gene transfers, as well as convergent evolution, were important in the evolution of these species. Striking copy number and structural variations were observed within gene families and one, stevor, shows a host-specific sequence pattern. The complete genome sequence of the closest ancestor of *P. falciparum* enables us to estimate the timing of the beginning of speciation to be 40,000–60,000 years ago followed by a population bottleneck around 4,000–6,000 years ago. Our data allow us also to search in detail for the features of *P. falciparum* that made it the only member of the *Laverania* able to infect and spread in humans.

The absence of in vitro culture or an animal model has precluded obtaining sufficient DNA for full genome sequencing and has hindered investigation of the *Laverania*. So far the full draft genome of *P. reichenowi* and a nearly complete draft sequence of *P. gaboni* are available. These data together with additional PCR-based approaches have provided important insights into the evolution of this subgenus, including the lateral gene transfer of the rh5 locus, the early expansion of the FIKK family gene, and the observation that the common ancestor also had var genes. However, the lack of whole-genome information for the whole subgenus (*particularly* *P. praefalciparum*) has severely constrained the scope of subsequent analyses. Our new data confirm and considerably extend these findings.

To investigate the evolutionary history of all known members of the *Laverania* subgenus and to address the question why *P. falciparum* is the only extant species to have adapted successfully to humans, we sequenced multiple genotypes of all known *Laverania* species.

**Genome sequencing from six *Laverania* species.** Fifteen blood samples that were positive for ape malaria parasites by PCR were taken during successive routine sanitary controls, from four gorillas and seven chimpanzees living in a sanctuary or quarantine facility before release (see Methods). Despite low parasitemia, a combination of host DNA depletion, parasite cell sorting and amplification methods enabled sufficient parasite DNA templates to be obtained for short- (Illumina) and long-read (Pacific Biosciences) sequencing.
From Bayesian whole-genome estimates, the ancestor of all parasite species: *P. praefalciparum*, *P. blacklocki*, *P. adleri*, *P. biloculinsi*, *P. gaboni* and *P. reichenowi*. The assemblies consisted of 44–97 scaffolds (Supplementary Table 1), with large contigs containing the subtelomeric regions and internal gene clusters that house multigene families known in *P. falciparum* and *P. reichenowi* to be involved in virulence and host–pathogen interactions. The high quality of the assemblies compared with those obtained previously is illustrated by the good representation of multigene families (Supplementary Table 2) and the large number of one-to-one orthologues obtained between the different reference genomes (4,350 among the seven species and 4,826 between *P. falciparum*, *P. praefalciparum* and *P. reichenowi*). Two to four additional genomes were obtained for each species except for *P. blacklocki* (Fig. 1a and Supplementary Table 1).

Speciation history in the *Laverania* subgenus. Conservation of synteny is striking between these complete genomes and enabled us to reconstruct the relationships between different *Laverania* species, to compare their relative genetic diversity (Fig. 2a; Supplementary Fig. 1) and to estimate the age of the different speciation events that led to the extant species. The latter has been problematic in the past because of the lack of both complete genome data and accurate estimates of mutation rate and generation time. Using the most divergent estimates of generation time and measured mutation rates from the *P. falciparum* literature, we found the data converge to 0.9–1.5 mutations per year per genome (Supplementary Note 1). We observed a similar substitution rate in vivo by examining existing sequence data for five geographically diverse isolates, covering a 200-kb region surrounding the *PjCRT* gene that is relatively conserved because of a selective sweep resulting from chloroquine use (Supplementary Note 1; Supplementary Fig. 2). The fact that these two figures are similar suggests that the in vitro mutation rate may have been underestimated because many mutations will be lost by genetic drift. Because no data are available for the other species, we have assumed hereon that these values generalize across the subgenus. From Bayesian whole-genome estimates, the ancestor of all current-day parasites of this subgenus existed 0.7–1.2 million years ago, a time at which the subgenus divided into two main clades, A (*P. adleri* and *P. gaboni*) and B, which includes the remaining species (Fig. 1a). Our range of values is far more recent than previous estimates10. Following the clade A/B subdivision, several speciation events occurred leading either to new chimpanzee or gorilla parasites. Interestingly, the divergence between *P. adleri* and *P. gaboni* in one lineage and *P. reichenowi* and the ancestor of *P. praefalciparum/P. falciparum* in the other lineage occurred at approximately the same time (140–230 thousand years ago; Fig. 1a; Table 2). On the basis of coalescence estimates, *P. falciparum* began to emerge in humans from *P. praefalciparum* around 40,000–60,000 years ago (Fig. 1a), later than the evolution of the first modern humans and their spread throughout Africa12. Our analysis also indicates significant gene flow between these two parasite species after their initial divergence (Supplementary Table 3).

*P. falciparum* has strikingly low diversity (*π* = 0.0004), compared with the other *Laverania* species (0.002–0.0049) (Supplementary Fig. 1). It has been proposed that *P. falciparum* arose from a single transfer of *P. praefalciparum* into humans and based in part on the paucity of neutral SNPs within the genome, that *P. falciparum* emerged from a bottleneck of a single parasite around 10,000 years ago, after agriculture was established6,8. In light of our results, we estimate that the *P. falciparum* population declined around 11,000 years ago and reached a minimum around 5,000 years ago (Fig. 1b) with an effective population size (*Ne*) of around 3,000 (generally the census number of parasites is higher than *Ne*3,11; Supplementary Note 1). The hypothesis of a single progenitor is also inconsistent with the observation of several ancient gene dimorphisms that have been observed in *P. falciparum*. A previous analysis using *P. reichenowi* and *P. gaboni* sequence data provided some evidence that different dimorphic loci diverged at different points in the tree11. Looking at each of these *P. falciparum* loci across the *Laverania*, we found different patterns of evolution at the *msp1*, *var1csa*, and *msp3* loci (Supplementary Fig. 3a). Most strikingly, a mutually exclusive dimorphism (described as MAD20/K116) in the central 70% of the *msp1* sequence clearly pre-dates the *P. falciparum*–*P. praefalciparum* common ancestor, and dimorphism in *var1csa* (an unusual var gene of unknown function that is transcribed late in the asexual cycle) occurred before the split with *P. reichenowi*.

Fig. 1 | Overview of the dating of the evolution of the *Laverania*. a, Maximum likelihood tree of the *Laverania* on the basis of “Lav12sp” set of orthologues. All bootstrap values are 100. Coalescence-based estimates of the timing of speciation events are displayed on nodes, on the basis of intergenic and genic alignments. The number of genomes obtained per ape (Supplementary Note 1; Supplementary Fig. 2). The fact that these two figures are similar suggests that the in vitro mutation rate may have been underestimated because many mutations will be lost by genetic drift. Because no data are available for the other species, we have assumed hereon that these values generalize across the subgenus. From Bayesian whole-genome estimates, the ancestor of all current-day parasites of this subgenus existed 0.7–1.2 million years ago, a time at which the subgenus divided into two main clades, A (*P. adleri* and *P. gaboni*) and B, which includes the remaining species (Fig. 1a). Our range of values is far more recent than previous estimates10. Following the clade A/B subdivision, several speciation events occurred leading either to new chimpanzee or gorilla parasites. Interestingly, the divergence between *P. adleri* and *P. gaboni* in one lineage and *P. reichenowi* and the ancestor of *P. praefalciparum/P. falciparum* in the other lineage occurred at approximately the same time (140–230 thousand years ago; Fig. 1a; Table 2). On the basis of coalescence estimates, *P. falciparum* began to emerge in humans from *P. praefalciparum* around 40,000–60,000 years ago (Fig. 1a), later than the evolution of the first modern humans and their spread throughout Africa12. Our analysis also indicates significant gene flow between these two parasite species after their initial divergence (Supplementary Table 3).

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In contrast, the gene eba-175 that encodes a parasite surface ligand involved in red blood cell invasion contains a dimorphism that arose after the emergence of \textit{P. falciparum} (Supplementary Fig. 3b). The time to the most recent common ancestor of eba-175 has been estimated as 130–140 thousand years in an analysis\textsuperscript{16} that assumed \textit{P. falciparum} and \textit{P. reichenowi} diverged 6 million years ago. However, on the basis of our new estimate for \textit{P. falciparum–P. reichenowi} divergence, we recalibrated their estimate of the most recent common ancestor of the eba-175 alleles to be around 4,000 years ago, which is in good agreement with our divergence time for \textit{P. falciparum} (Supplementary Note 1). The recent dimorphism cannot, however, explain the observation of an ancient dimorphism.

**Fig. 2 | Overview of the analyses of core genes over all Laverania genomes.** a. Summary of evolution of core genes. From outer to inner track: scatterplot of branch-site test for each genome (see Supplementary Table 4 for \textit{P. falciparum} data); per-species \(d_\text{S}/d_\text{A}\) values (0.5 < \(d_\text{S}/d_\text{A}\) < 2); orthologues are represented by vertical black lines under the chromosome track, with dots representing \textit{P. falciparum} 3D7 var genes on the forward (blue) or reverse strands (red), or var pseudogenes (black); average of the relative polymorphism (\(\pi\)) across species, with the underlying \(\pi\) for each species calculated from multiple strains ("Lav15st" dataset) and normalized by the average for that species; signatures of convergent evolution on the basis of host-specific fixed differences analysis with the chromosome 4 region that includes the Rh5 locus highlighted (black box). b. Magnified view of the Rh5 region that is enriched with host-specific fixed differences. Convergent evolution analysis was performed using orthologues conserved across the \textit{Laverania}. Filled circles represent the subset of differences that were fixed within all the isolates available ("Lav15st" set) and for which we could reject neutral evolution (for the gene list see Supplementary Table 5).
identified 172 genes (out of 4,826) with signatures of positive selection in the human parasite species only (Supplementary Table 4).

Among the 172 genes, almost half (>250 aa) showed signals of gene flow between parasites infecting the same host species (P. falciparum life cycle) and 25 with significant copy number variation among lineages. Data from P. praefalciparum include the subtelomeric gene families from the two infecting genotypes. Assembly of P. billcollinsi is incomplete in the subtelomeres.

Evolution through introgression, gene transfer and convergence. Frequent mixed-species infections in apes and mosquitoes provide clear opportunities for interspecific gene flow between these parasites. A recent study reported a gene transfer event between P. adleri and the ancestor of P. falciparum and P. praefalciparum of a region on chromosome 4 including key genes involved in erythrocyte invasion (rh5 and cyrP). Because such events preserve the phylogenetic history of the genes involved, we systematically examined the evidence for introgression or gene transfer events across the complete subgenus by testing the congruence of each gene tree to the species tree for genes with one-to-one orthologues. Beyond the region that includes rh5 (Fig. 2b; Supplementary Fig. 4a), few signals of gene flow between parasites infecting the same host species were obtained (n = 11), suggesting that these events were rare or usually strongly deleterious (Supplementary Fig. 5).

The Laverania subgenus evolved to infect chimpanzees and gorillas, but on a genome-wide scale, the convergent evolution of host-specific traits has not left a signature (Supplementary Note 2). We therefore examined each CDS independently and were able to identify genes with differences fixed within specific hosts, falling into three categories: 53 in chimpanzee-infective parasites, 49 in gorilla-infective and 12 with fixed traits in both host species (Fig. 2; Supplementary Table 5a). For at least 67 genes, these differences were unlikely to have arisen by chance (P < 0.05), and Gene Ontology (GO) term enrichment analysis revealed that several of these genes are involved in host invasion and pathogenesis (Supplementary Table 5b) including rh5 (which has a signal for convergent evolution even when the introgressed tree topology is taken into consideration; Supplementary Fig. 4b). Rh5 is the only gene identified in P. falciparum that is essential for erythrocyte recognition during invasion, via binding to Basigin. P. falciparum rh5 cannot bind to gorilla Basigin and binds poorly to the chimpanzee protein16. We notice that one of the convergent sites is known to be a binding site for the host receptor Basigin in the mosquito stages) also showed a signal of adaptive evolution (Supplementary Table 4).

near the human and ape loci for glycoporphin17, an EBA-175 binding protein. The formation and maintenance of all of these dimorphic loci has therefore been shaped by different balancing selection pressures over time.

P. falciparum-specific evolution. During its move away from gorillas, P. falciparum had to adapt to a new vertebrate host (human) and new vector species (for example, Anopheles gambiae)18. To infer P. falciparum–specific adaptive changes, we considered the P. falciparum/P. praefalciparum/P. reichenowi genome trio and then applied two lineage-based tests to find positive selection that occurred in the P. falciparum branch (see Methods). The two tests identified 172 genes (out of 4,826) with signatures of positive selection in the human parasite species only (Supplementary Table 4). Among the 172 genes, almost half (n = 82) encoded proteins of unknown function. Analysis of those with functional annotation indicated that genes involved in pathogenesis, entry into host, actin movement and organization and in drug response were overrepresented. Other genes, expressed in different stages of the P. falciparum life cycle (for example, sera4 and emp3, involved during the erythrocytic stages; trsp and lisp1, involved in the hepatic stages; and plc4, CelTOS and Cap380, involved in the mosquito stages) also showed a signal of adaptive evolution (Supplementary Table 4).
The gene eba-165 encodes a member of the erythrocyte binding-like superfamily of proteins that are involved in erythrocyte invasion. Although eba-165 is a pseudogene in *P. falciparum*\(^2\), it is not a pseudogene in the other *Laverania* species and may therefore be involved in erythrocyte invasion, like other erythrocyte binding-like members. The protein has three convergent sites in gorillas. One falls inside the F2 region, a domain involved in the interactions with erythrocyte receptors. The role of this protein and of these convergent sites in the invasion of gorilla red cells remains to be determined. Finally, genes involved in gamete fertility (the 6-cysteine protein P230) or implicated in *Plasmodium* invasion of erythrocytes (doc2\(^2\)) also displayed signals of convergent evolution. Twelve parasite coding sequences had fixed differences at the same amino acid position in chimpanzees and gorillas. Of these, P230 was the only one found with a position that was different and fixed across all three host species. P230 is involved in gamete development and trans-specific reproductive barriers\(^3\), possibly through enabling male gametes to bind to erythrocytes before exflagellation\(^4\). Host-specific residues observed in P230 might affect the efficiency of the binding to the erythrocyte receptor and result from co-evolution between the parasite molecule and the host receptor.

**Subtelomeric gene families.** So far, the only in-depth data on the subtelomeric gene families of the *Laverania* have come from *P. reichenowi* and *P. falciparum*. These important families are well represented in our assemblies (Supplementary Tables 2 and 6a), and we provide a comprehensive picture of their evolution.

Most gene families were probably present in the ancestor of all *Laverania*. The same general pattern of one-to-one orthology throughout the subgenus indicates that many underwent gene duplication early (for example, FKKK) or before (for example, ETRAMP, PHIST and SURFIN) the development of a distinct *Laverania* lineage. Only a subset displayed contractions or expansions between specific *Laverania* species (Fig. 3; Supplementary Table 6a; Supplementary Fig. 7). For these latter families, clade A and most species of clade B clearly differ in their composition. *P. blacklocki* (clade B) is intermediate in its composition. Some gene families, such as the group of exported proteins hyp4, hyp5, mc-2tm and EPE1, have expanded only in *P. praefalciparum* and *P. falciparum* (and even more in *P. falciparum* for hyp4 and hyp5). Because all four are components of Maurer's clefts, an organelle involved in protein export\(^5\), some evolution of function in this organelle may have been an important precursor to human infection. The family of acyl-CoA synthetase genes, reported to be expanded and diversified in *P. falciparum*\(^6\), is in fact expanded across the *Laverania* and has four fewer copies in *P. falciparum* (Supplementary Fig. 6). Other genes that show clade- or group-specific expansion include DBLmsp, glycophrin-binding protein and CLAG (Supplementary Fig. 7).

One striking interclade difference concerns the largest gene family that is probably common to all other malaria species: the *Plasmodium* interspersed repeat family (pir, which includes the rif and stevor families in *P. falciparum*) (Figs. 3 and 4). This family has been proposed to be involved in important functions such as antigenic variation, immune evasion, signalling, trafficking, red cell rigidity and adhesion\(^7\), and yet has expanded only in clade B, after the *P. blacklocki* split (Fig. 3). The rif genes comprise a small conserved group and a much larger group of more diverse members that contains just 13 genes from clade A species and at least 180 members per clade B species (Fig. 4). There is, however, no evidence for host-specific adaptation in these sequences.

In contrast, a subset of stevor genes showed strong host-specific sequence diversification (Fig. 4; Supplementary Fig. 8). On the basis of full-length alignments, there is a deep phylogenetic split between stevor genes, but when only short conserved protein motifs are considered a group of Stevor proteins (stevor II, Fig. 4a) forms a cluster composed almost entirely of members from gorilla-infecting species. Because stevor genes are known to be involved in host–parasite interactions (such as binding to host glycophrin C in *P. falciparum*\(^8\)), this host-specific sequence may reflect sequence differences in host-specific factors in gorillas.

**Evolution of var genes.** The var genes, crucial mediators of pathogenesis and the establishment of chronic infection through cytoadherence and immune evasion, are the best studied *P. falciparum* multigene family and are unique to the *Laverania*\(^9\). They are two-exon genes, and their products have three types of major domain; exon 1 encodes Duffy binding-like (DBL) and cysteine-rich interdomain regions (CIDR), and exon 2 encodes acidic terminal sequence (ATS)\(^10\). Similar to *P. falciparum*, our data are consistent with all *Laverania* species having var genes (Fig. 3) that retain a two-exon structure and are organized into subtelomeric or internal var gene clusters. There are, however, three notable features of var evolution within the subgenus.

First, there is a deep division in how the repertoire is organized between the major clades. The var genes of clade B parasites, with the exception of *P. blacklocki*, resemble those of *P. falciparum* in terms of genomic organisation, domain types and numbers (Fig. 5; Supplementary Table 7). In contrast, the repertoires of clade A parasites and *P. blacklocki* (treated as one group hereafter in this section) differ in their domain composition, contain a novel CIDR-like domain (CIDRn; Fig. 5a; Supplementary Fig. 9) and have lower sequence diversity per domain but cluster into more subgroups than clade B domains (Fig. 5b; Supplementary Fig. 10). The paucity of domains similar to those in *P. falciparum* (such as CIDRxs) that are involved in cytoadherence to some specific and common host receptors means that if endothelial cytoadherence was important in clade A, some alternative receptors must have been utilized.

Second, in total there are ten internal var gene clusters (confirmed by contiguous sequence data), but eight are oppositely oriented between the two clades (Supplementary Fig. 11; Supplementary Table 8). Clade B parasites also show a much greater number of associated GC-rich RNAs of unknown function elements than clade A (Supplementary Table 8).

Third, the ATS domains cluster tightly within clade A. Within clade B there is clear evidence of species-specific diversification, except in *P. praefalciparum* and *P. falciparum*, reflecting their recent speciation. There is one intact ATS from *P. falciparum*, as well as several pseudogenes that cluster with clade A (Fig. 5c). Moreover, of seven internal var arrays (Supplementary Fig. 11) in *P. falciparum*, containing a functional var gene, five terminate with one of these pseudogenes (on the opposite DNA strand), suggesting that they may be remnants from ancient rearrangements. The intact *P. falciparum* gene is var2csa, a var-like gene that is highly conserved between *P. falciparum* isolates\(^11\), involved both in cytoadherence in the placenta in primigravidae and proposed to be a central intermediate in var gene switching during antigenic variation\(^12\). We therefore propose var2csa is a remnant of an ancient multigene family that has been maintained as a single complete gene in *P. falciparum*, for the dual purposes of var switching and placental cytoadherence.

There is other evidence of retention of ancient var gene sequence across the subgenus. First, in clade B we find a nearly full-length var pseudogene that has highest similarity to *P. adleri* and *P. gaboni* var genes, within an internal var cluster on chromosome 4 in *P. falciparum* and *P. praefalciparum*, but on the opposite strand to the other var genes. It is found in all *P. falciparum* isolates, but not in *P. reichenowi*. Second, in *P. gaboni* and *P. adleri*, three genes have the N-terminal Duffy binding-like α/CIDRα architecture typical of clade B genes, and their domains cluster within clade B on the basis of similarity (Fig. 5b, larger nodes). Directly adjacent to two of these var genes are two rif pseudogenes that also show greatest similarity to those from clade B. Last, we find a further nine rif pseudogenes of...
clade A parasites that cluster with clade B rif genes (Fig. 4). If these observations reflect retention of ancient copies, their high sequence conservation suggests that they are under extremely unusual selection pressure. Alternatively, they may represent relics of gene transfer between species that occurred after the clade A/B split.

**Conclusion**

We have produced high-quality genomes and used mutation rates and generation times, covering the full range of most recent estimates, to calculate the date of speciation for all known members of the *Laverania*, with only a small margin of error. In our analysis, we have shown that the successful infection of humans by *P. falciparum* occurred recently and involved numerous parasites rather than a single one as previously proposed. After the establishment in its new host, the parasite population went through a bottleneck around 5,000 years ago during the period of rapid human population expansion because of farming (Fig. 1b).

We summarize the major genomic events during the evolution of the *Laverania* in Fig. 6.

As a result of our analyses, we propose the following series of events for the emergence of *P. falciparum* as a major human pathogen. First, the crucial lateral transfer event of the rh5 locus between clade A and B parasites may also have involved var and rifin genes in other parts of the genome that, because of their orientation on the opposite strand, were not lost during later recombination. Next, facilitatory mutations are likely to have occurred in both parasites rather than a single one as previously proposed. After the establishment in its new host, the parasite population went through a bottleneck around 5,000 years ago during the period of rapid human population expansion because of farming (Fig. 1b).

Fig. 5 | Evolution of var gene domains in the *Laverania*. a Heatmap of numbers of var gene domains in each *Laverania* species. Duffy represents regions closest to the Pfam Duffy binding domain. CIDRn is a new domain discovered in this study in clade A. Only domains from var genes longer than 2.5 kb were considered. Heatmap colours blue-yellow-white indicate decreasing copy numbers. b Graphical representation of similarity between domains, using domains from var genes longer than 2.5 kb. Domains are coloured by species and clustered by a minimum BLAST cutoff of 45% global identity. Larger circles denote var genes in the opposite orientation. c Maximum likelihood trees of the ATS. Apparent ATS sequences from clade A that cluster with clade B are indicated (**).
We find evidence for gene flow between lineages throughout this period. The expansion of the human population with the advent of farming probably led to strong evolutionary pressure for mosquito species (specifically *An. gambiae*) to feed primarily on humans. Therefore, the existing human infective (*P. falciparum*) genotypes would be selected for human and appropriate vector success, and the fittest would rapidly expand. Subsequent rapid accumulation of mutations that favoured growth in humans, and in the anthropophilic vectors such as *An. gambiae*, are likely to have occurred to increase human-specific reproductive success. The resulting specific parasite genotypes that expanded (and appeared as an emergence from a bottleneck) would have had a much lower probability of a direct transfer back to apes. With experiments on gorillas and chimpanzees not possible, it will be difficult to directly prove the precise combination of different alleles that allowed the emergence of *P. falciparum*. However, for the genes that we have implicated in this process, existing data (www.genedb.org) suggest they are expressed throughout the life cycle, but that only half have been characterized. This opens up new opportunities for future studies on host specificity and host adaptation in *Plasmodium*.

Methods

Sample collection. All but two infected blood samples from chimpanzees (*Pan troglodytes troglodytes*) and gorillas (*Gorilla gorilla gorilla*) were obtained from the sanctuary "Parc de La Lékédi", Bakoumba (Haut-Ogooué, Gabon), during routine sanitary controls of the animals. This park holds various primate species, including gorillas, chimpanzees and monkeys (*Cercopithecinae*), that have been orphaned because of bushmeat-poaching activities and have been confiscated by the Gabonese Government, quarantined at the Centre International de Recherches Médicales de Franceville (CIRMF; Gabon) and finally released into semifree ranging enclosures in the sanctuary. Every 6 months, chimpanzees (12 individuals) and gorillas (2 individuals) are anesthetized for medical checkup. Blood samples were collected from the animals during sanitary controls (July 2011, September 2012, May 2013 and December 2013). Two additional infected blood samples were obtained from gorilla orphans (GG05, GG06) seized by the Gabonese Government, quarantined at the Centre International de Recherches Médicales de Franceville (CIRMF; Gabon) and finally released into semifree ranging enclosures in the sanctuary. Every 6 months, chimpanzees (12 individuals) and gorillas (2 individuals) are anesthetized for medical checkup. Blood samples were collected from the animals during sanitary controls (July 2011, September 2012, May 2013 and December 2013). Two additional infected blood samples were obtained from gorilla orphans (GG05, GG06) seized by the Gabonese Government in 2011 and 2013, and sent to the CIRMF for quarantine before being released in a sanctuary. All animal work was conducted according to relevant national and international guidelines. From each animal, 15 ml of whole blood was collected in EDTA tubes. For all samples but three, white blood cell depletion was performed on 10 ml of the freshly collected samples using cellulose columns as described by Auburn et al. Remaining blood was subsequently used for DNA extraction and detection of *Plasmodium* infections as described in Ollomo et al. Overall, 15 blood samples from 7 chimpanzees and 4 gorillas were found to contain the *Laverania* samples used in the present study (Supplementary Table 1).

Ethical consideration. The animals’ well-being was guaranteed by the veterinarians of the “Parc de la Lékédi” and the CIRMF who proceeded to the sanitary controls and the blood sampling. Because these blood samples were collected as part of the standard protocol for the sanitary controls (and not specifically for our experiment), our study did not need the approval of an Institutional Animal Care or Use Committee. Note also that our study did not involve randomization nor blinding.

Sample preparation. Three methods were used for DNA amplification before sequencing (Supplementary Table 1). For all but one sample, whole-genome amplification (WGA) was performed with a REPLI-g Mini Kit (Qiagen)
following a modified protocol to enrich genomic DNA. The genome of *P. blacklocki* was generated using selective WGA (oWGA) as indicated by Oyola et al. using 20 primers, followed by a WGA. Finally, for the PfrPrG03 (a *P. praefalciparum* isolate) and PdgB02 (a *P. adleri* isolate) samples, we used a cell sorting approach.

**Sample sequencing.** All samples were first sequenced with Illumina. Amplification-free Illumina libraries of 400–600 bp were prepared from the enriched genomic DNA using NEBNext and run on MiSeq and HiSeq 2000 (v3 chemistry) Illumina machines.

After the Illumina sequencing, six samples with a combination of the least number of multiple infections (see later) and the lowest level of host contamination were chosen for long-read sequencing using Pacific Biosciences (PacBio). The DNA of the samples (after WGA) was size selected to 8 kb and sequenced with the CL/P5 chemistry of SMRT cells (PacBio sequencing runs) used varied between samples (Supplementary Table 1).

**Genome assembly, genome QC, split of infection and annotation.** *Determination of multiple infections.* To initially quantify multiple infections and so allow samples to be selected for PacBio sequencing from those comprising a low number of species, Illumina reads from each sample were mapped against a concatenation of all available Cox 3 and CytB genes of the Laverania from National Center for Biotechnology Information, using SNP-o-Matic (parameter chop = 5) to position reads only where they aligned perfectly. SNP-o-Matic returns all the positions of repetitive mapping reads. This output allows us to count the read depth of these two genes across all species and, therefore, determine the number and relative amount of different malaria species per sample.

Whole-genome amplification bias. The uneven coverage that resulted from WGA bias, host contamination and multiple infections presented a challenge for sequence assembly. To overcome the bias and host contamination, we sequenced each DNA sample deeper than normally necessary. Lower coverage of the subtelomeres was obtained for the SWGA sample (*P. blacklocki*), meaning that the subtelomeres in that assembly were not as complete as those in the assemblies for other species.

**Long-reads (Pacific Bioscience) assemblies.** Six reference genomes were assembled using HGAP (Hierarchical Genome Assembly Process), with different settings for the genome size parameter, ranging from 23 (P. reichenowi) to 72 Mb (P. billcollinsi). This parameter encodes how many long reads are corrected for use in the assembly and depends on the host contamination and the amount of different isolates in the samples. The obtained contigs from HGAP were ordered with ABACAS (Algorithm-Based Automatic Contiguation of Assembled Sequences) against a *P. falciparum* 3D7 reference that has no subtelomeric regions. Assembly errors and WGA artefacts were manually corrected using ACT. After this step, three iterations of iCORN2 (Iterative Correction of Reference Nucleotides) were run, followed by another ABACAS step, allowing overlapping contigs to be merged (parameter: ABA_CHECK_OVERLAP = 1). For the PrG01, PbgB01 and PdgG01 assembly, we also ran Phelly to close some of the sequencing gaps.

**Host decontamination.** To detect and remove sequence data derived from host DNA, we compared contigs with the chimpanzee or gorilla genomes using BLAST (Basic Local Alignment Search Tool). Contigs were considered as host contamination if more than 50% of their BLAST hits had more than 95% identity to any of the great ape genomes. Unordered contigs with a GC content >32% were searched against the non-redundant nucleotide database, to detect and remove further contaminants.

**Resolving multiple infections.** The first assembled genome was a single *P. reichenowi* infection, PrG01. We detected low levels of *P. vivax*-like and virus contamination (TT virus, AB038624.1), which were excluded. For quality control, the assembly was compared against the existing PCDC reference genome. The number of PIRs was similar, and there were no breaks in synteny. There were, however, significantly fewer sequencing gaps, and 17 Rep20 regions could be found (a known repeat close to the telomeres in *P. falciparum*). Thus, the assembly of PacBio data (PrG01; Supplementary Table 2) appears to be of higher quality than the existing *P. reichenowi* PCDC reference.

The *P. adleri* sample consists of a single infection. Because a large number of cycles of amplification were used, a greater number of SMRT cells were sequenced (Supplementary Table 1) to overcome the problem of uneven coverage resulting in underrepresented regions. An estimated genome size of 60 Mb was chosen for the HGAP analysis to ensure that all regions were covered.

PbgG01 was a *P. galmoni* isolate with a *P. vivax*-like co-infection. To detect contigs of *P. vivax*, we searched unordered contigs (those that could not be placed against P5D7 using ABACAS) against the protein sequences of *P. falciparum* 3D7 and the *P. vivax* PvP01 reference genome using TBLASTx. For each contig, the relative number of genes hitting against the two genomes was used to assign it to *P. galmoni* or *P. vivax*. In most cases, all genes for a given contig consistently hit only one genome so that the attribution to either species was clear. Overall, 14 Mb of *P. vivax*-like sequences was obtained that will be described elsewhere.

The *P. billcollinsi* genome (PhbcG01) was obtained from co-infection with a *P. galmoni* genome (PgalG02). Rather than ordering the contigs against P5D7 with ABACAS, contigs were ordered against a combined reference comprising *P. galmoni* (PbgG01) and the P5D7 (parameters: overlap 500 bp, identity 90%). The species designation of contigs was confirmed with TBLASTx searches of annotated genes against a combination of the proteomes of PgalG01 and PrCDC. For subtelomeric gene families, contigs were attributed to species if the hit was significant for one species, not the other. Some of the contigs could not be attributed unambiguously and were discarded. Due to sequencing gaps, some of the core genes are missing from the final assembly.

The sample used to produce the *P. praefalciparum* genome (PfrPrG01) had a high level of host contamination, a low level of co-infection with *P. adleri* and co-infection with two distinct *P. praefalciparum* genotypes. For the core genome, we used iCORN2 to select the dominant genotype at each position. Where it was not possible to phase the genotypes, due to a lack of variation, we assumed that they were identical. In the subtelomeres, however, it was possible to distinguish, but not phase, the two *P. praefalciparum* genotypes, resulting in approximately twice the number of var genes as seen in *P. falciparum*. Due to contamination of construction vectors (*E. coli*) and host, 29 SMRT cells were sequenced and the HAGP parameter for the assembly size was set to 60 Mb. Contigs were screened against *P. adleri* and *P. falciparum* to exclude a *P. adleri* co-infection. All of the contigs that had a *P. falciparum* BLAST hit or had no clear hit (such as those containing species-specific genetic material) were attributed to *P. praefalciparum*. For the polyplody sample (Supplementary Table 1) including five *P. galmoni* genomes were mapped against the P5D7, *P. falciparum* and *P. adleri* assemblies. Contigs were excluded where more normalized hits to the three *P. adleri* samples were found than to one of the two other *P. praefalciparum* samples. Similarly, this method was used to eliminate the remote possibility that any of the contigs in the *P. praefalciparum* assembly were in fact derived from *P. falciparum* co-infection.

The *P. blacklocki* sample was from a single infection. Due to SWGA, the PacBio sequence data covered regions not covered by Illumina, but because of the bias of the primers, the subtelomeres were not covered fully. However, the internal var gene clusters are all assembled. Some of the core genes from this species are also missing.

**Annotation.** The genomes were annotated as described by Otto et al. In short, the annotation of *P. falciparum* (version July 2015) was transferred with RATT, and new gene models were called with Augustus. Obvious structural errors in core genes were manually corrected in Artemis.

**Mapping: generation of further samples.** To generate the gene sequence for different samples, we mapped Illumina reads against a set of reference genomes using BWA and default parameters. For the gorilla samples, we mapped against the combined PacBio reference genomes of *P. adleri*, *P. blacklocki* and *P. praefalciparum*, and for the chimpanzee samples, the combined references of *P. galmoni* (PbgG01), *P. billcollinsi* and *P. reichenowi* (PrG01). SNPs with Phred score ≥100 were called using GATK UnifiedGenotyper V2.0.35 (parameters: -pnrn POOL -ploidy 2 -gln POOLBOTH). From these SNP calls we constructed the new gene set, masking regions in genes with less than 10 x coverage of 'properly' (correct distance and orientation) mapped paired reads. To generate the sequences of the other 13 isolates, we obtained homologous SNP calls (consensus program from bcftools 1.2). We quality-controlled the SNP calling by regenerating PrCDC and PbgG02 gene sets from PrG01 and PbgG01, respectively, and confirmed that they were placed with nearly no differences in a phylogenetic tree.

**Orthogonal group determination and alignment.** Orthogonal groups were identified using OrthoMCL v1.44 across: (i) the seven core Laverania genomes; (ii) the seven core genomes, the Laverania isolates PbgG02, PrCDC and *P. falciparum* IT, as well as two outgroup genomes *Plasmodium vivax* Sali and *Plasmodium knowlesi* IT (PPf; and (iii) the previously published *P. reichenowi* CDC and five *P. falciparum* isolates (3D7, IT, DD2, HB3 and 7G89). From these groups, different complete sets of 1:1 orthologues were extracted:

1. “Lav12sp” set of 3,369 orthologs across the seven core Laverania species; (ii) the seven core genomes, the *Laverania* isolates PbgG02, PrCDC and *P. falciparum* IT, as well as two outgroup genomes *Plasmodium vivax* Sali and *Plasmodium knowlesi* IT (PPf; and (iii) the previously published *P. reichenowi* CDC and five *P. falciparum* isolates (3D7, IT, DD2, HB3 and 7G89).
 evolution), the fourth one for the analyses of within-species polymorphism and the fifth one for the analysis of P. falciparum adaptive evolution.

To reduce the rate of false-positive results in the evolutionary analyses caused by misalignments (for example, Jordan & Goldman), we performed codon-based multiple alignments using PRANK and the -codon and +F options, because it was shown to outperform other programs in the context of the detection of positive selection. Before aligning codons, low-complexity regions were excluded in the nucleotide sequences using dustmasker and in amino acid sequences using segmasker from National Center for Biotechnology Information-BLAST. Poorly aligned regions were excluded using Gblocks, with default settings.

**Analysis of interspecific gene flow, introgression or gene transfer.** *Species tree inference.* Two ML trees were performed using RAxML (default parameters) to illustrate the phylogenetic relationships between the *Laverania* species and genotypes studied in this article using the “Lav12sp” and the “Lav25st” set of orthologues. For each tree, multiple nucleotide alignments of each orthologous group were constructed as described earlier. Trees were then constructed from the concatenated alignments of the “Lav12sp” set of orthologues for the species tree and the “Lav25st” set for the strain tree using RAxML and the following options: -m GTRGAMMA -a -t 100.

**Tree topology test.** Interspecific gene flow was investigated by testing congruence between each gene tree topology and the species tree topology. We performed the Shimodaira–Hasegawa test using RAxML and the following options: -m GTRGAMMA -f a -# 100. To determine what number of host-specific differences were fixed within each host species (gorilla or chimpanzee) presented an excess of convergence. Analyses were performed under different models of amino acid evolution: LG, WAG, JONES and DAYHOFF.

**Gene-based detection of convergent evolution throughout the Laverania.** For each orthologue of the “Lav7sp” set, the number and percentage of fixed amino acid differences between parasites infecting the same host were calculated, that is, the number of positions showing the same amino acid within a host species but different amino acid between host species. Alignments of all of the available sequences (“Lav12sp”) from all of the sequenced isolates were then used to determine what number of host-specific differences were fixed within each host and each species. To evaluate whether the observed number of host-specific fixed differences in an alignment can be attributed to neutral evolution/purifying selection alone (with no positive selection), we used a simulation-based approach. For each coding sequence, 1,000 sequences of the same size were simulated to evolving along the same tree with the same specified branch lengths, substitution model, codon frequencies and omega (dN/dS). Using the program Evolver from PAML v4.8a, the program Codeml from PAML v4.8a was first used to estimate the tree, the codon frequencies and the average omega values for each of the coding sequences and amino acid differences. For each simulated dataset, the number of fixed amino acid differences between the parasites infecting the same host was estimated. The probability of observing n fixed differences was then computed as the proportion of the simulated dataset of 1,000 sequences that showed at least the same number of fixed differences as observed in the real data.

A branch-site test was also applied, for each gene, on each terminal branch of the entire species tree using the “Lav7sp” dataset. dN/dS ratio estimates per branch and genes were obtained using Codeml (PAML v4.4c) with a free-ratio model of evolution (Fig. 2).

**Gene Ontology enrichment analyses.** Analysis of GO term enrichment was performed using GOseq with default parameters. GO annotations from GeneDB were used, but with unreviewed automated annotations excluded.

**Gene family analyses.** To estimate the differential abundance of gene families across species, we counted the Gene products and the Pfam domains, and analysed them by the variance of the expression. Unless otherwise stated, trees were constructed using PhyML (default parameters) or RAxML (model estimated) from alignments generated with Muscle, with default settings. Many of the findings were confirmed manually through ACT and bamview. The analysis of the var genes was performed on var genes larger than 2.5 kb. Domains were called with the HMMer models from varDom. To estimate the statistical significance of the Observed AIC scores, with filtering low-complexity regions. Representation was done in R through the heatmap.2 program from gplot (see also Supplementary Note 3).

**Allelic dimorphisms.** For the analysis of dimorphism in msp, all sequences available for the *Laverania* were downloaded from Uniprot. Data were subsampled to obtain a similar number of sequences for each group. Phylogenetic trees were constructed with PhyML, using default parameters and drawn in FigTree. The eba-175 alignment was visualized with Jalview.

**Divergence dating.** Alignments of the *Laverania* included intergenic regions where Aragon 402–681 mitotic events per year (Supplementary Note 1) and a mutation rate of 3.78E–10 for four mitotic events per year (Supplementary Note 2) was taken from PDD7 line without drugs, equivalent to around 0.9–1.5 mutations per genome per year. Although we observed similar mutation rates in clinical samples (Supplementary Note 1), these estimates have potential errors; therefore, we report ratios of divergence times in the figures that are robust to errors in these parameters. For coalescence-based estimates of speciation times, G-Phocs was used and multiple sequentially Markovian coalescent (MSMC) on segregating sites was used to estimate the *P. falciparum* bottleneck.

**Data availability.** All sequences have been submitted to the European Nucleotide Archive. The accession numbers of the raw reads and the assembly data can be found in Supplementary Table 9. The genomes are being submitted to EBI, project ID PRJEB13584. The genomes are available from plasmodb.org and ftp://ftp.sanger.ac.uk/pub/project/pathogens/Plasmodium/Laverania/.

**Computer code.** Custom computer code is available on request.

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**Author contributions**

T.D.O., B.O., F.R., C.N., M.B. and F.P. designed the study. C.A., A.P.O., L.B., E.W., B.N., N.D.M., C.P., P.D., V.R. and E.P. collected and assessed samples. C.A. performed the WGA and cell sorting on one sample. S.O.O. performed the WGA on the samples. M.S. organized the sequencing. T.D.O. did assembly and annotation. U.B. did manual curation. A.G. and F.P. performed the evolutionary analyses on core genomes. T.D.O., C.N. and M.B. performed the analyses of gene families and dimorphisms. T.C. performed the dating analyses. T.D.O., A.G., C.N., M.B. and F.P. wrote the manuscript. All authors read and approved the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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## Experimental design

1. **Sample size**  
   Describe how sample size was determined.  
   Not relevant, as we sequenced all the blood samples we obtained and then looked for malaria infections.

2. **Data exclusions**  
   Describe any data exclusions.  
   No data were excluded.

3. **Replication**  
   Describe whether the experimental findings were reliably reproduced.  
   n/a

4. **Randomization**  
   Describe how samples/organisms/participants were allocated into experimental groups.  
   n/a

5. **Blinding**  
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.  
   n/a

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**  
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a  
   Confirmed
   - The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - The test results (e.g. \(P\) values) given as exact values whenever possible and with confidence intervals noted
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.
Software

Describe the software used to analyze the data in this study.

The software used are publicly available, like the HGAP assembler and the PAGIT tool kit. All tests were performed using R.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

One of the achievements of the work was to obtain malaria DNA from primate blood samples. No more DNA is available, but all the sequencing reads are in the public databases.

Animals and human research participants

Provide details on animals and/or animal-derived materials used in the study.

The blood samples were obtained during routine sanitary controls of chimpanzees and gorillas living in sanctuaries. The ethics and the age of the primates are reported in the supplemental material.

Policy information about studies involving human research participants

Describe the covariate-relevant population characteristics of the human research participants.

n/a