Out of Africa

International Journal for Parasitology 47 (2017) 87–97

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Invited Review

Out of Africa: origins and evolution of the human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*

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ARTICLE INFO

Article history:
Received 21 April 2016
Received in revised form 25 May 2016
Accepted 28 May 2016
Available online 2 July 2016

Keywords:
Malaria
African apes
Plasmodium falciparum
Plasmodium vivax
Laverania
Zoonotic transmission
Evolution

ABSTRACT

Plasmodium falciparum and *Plasmodium vivax* account for more than 95% of all human malaria infections, and thus pose a serious public health challenge. To control and potentially eliminate these pathogens, it is important to understand their origins and evolutionary history. Until recently, it was widely believed that *P. falciparum* had co-evolved with humans (and our ancestors) over millions of years, whilst *P. vivax* was assumed to have emerged in southeastern Asia following the cross-species transmission of a parasite from a macaque. However, the discovery of a multitude of *Plasmodium* spp. in chimpanzees and gorillas has refuted these theories and instead revealed that both *P. falciparum* and *P. vivax* evolved from parasites infecting wild-living African apes. It is now clear that *P. falciparum* resulted from a recent cross-species transmission of a parasite from a gorilla, whilst *P. vivax* emerged from an ancestral stock of parasites that infected chimpanzees, gorillas and humans in Africa, until the spread of the protective Duffy-negative mutation eliminated *P. vivax* from human populations there. Although many questions remain concerning the biology and zoonotic potential of the *P. falciparum*- and *P. vivax*-like parasites infecting apes, comparative genomics, coupled with functional parasite and vector studies, are likely to yield new insights into ape *Plasmodium* transmission and pathogenesis that are relevant to the treatment and prevention of human malaria.

1. Introduction

Of the *Plasmodium* spp. known to commonly infect humans, *Plasmodium falciparum* and *Plasmodium vivax* cause the vast majority of malaria morbidity and mortality, and are the principal targets of malaria prevention and eradication efforts. *Plasmodium falciparum* is highly prevalent in sub-Saharan Africa where it is responsible for nearly 200 million clinical cases (Bhatt et al., 2015) and over 300,000 malaria-related deaths annually, predominantly in children under 5 years of age (World Health Organization, 2015). *Plasmodium vivax* is rare in sub-Saharan Africa, but endemic in many parts of Asia, Oceania, as well as Central and South America where it causes an estimated 16 million cases of clinical malaria, which represents approximately half of all malaria cases outside Africa (World Health Organization, 2015).

Given the devastating effects of malaria, the origins of the *Plasmodium* parasites infecting humans have long been a subject of interest. Descriptions of malaria-like illness can be found in ancient texts from China, India, the Middle East, Africa and Europe, indicating that humans have been combatting *Plasmodium* infections throughout much of our recorded history (Carter and Mendis, 2002). Indeed, variants in the human genome that are associated with resistance to *Plasmodium* infection and malaria-associated disease are estimated to be thousands of years old (Hedrick, 2011). One such variant is the sickle cell trait, which is common in African populations and protects against fatal *P. falciparum* malaria (Taylor et al., 2012). Similarly, a mutation that abolishes the expression of the Duffy antigen receptor of chemokines on the surface of red blood cells (the so-called "Duffy-negative phenotype") approaches fixation in western and central Africa, and confers almost complete protection from *P. vivax* parasitemia (Miller et al., 1976; Howes et al., 2011). Together, these findings indicate that *Plasmodium* infections have impacted human health for millennia, but the prevailing view has been that this history goes back much further.

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http://dx.doi.org/10.1016/j.ijpara.2016.05.008
0020-7519/

One long-standing hypothesis suggested that humans and chimpanzees each inherited *P. falciparum*-like infections from their common ancestor, and that these parasites co-evolved with their respective host species for millions of years (Escalante and Ayala, 1994). In contrast, *P. vivax* was believed to have arisen several hundred thousand years ago, following the cross-species transmission of a parasite from a macaque in southeastern Asia (Escalante et al., 2005; Jongwutiwes et al., 2005; Mu et al., 2005; Neafsey et al., 2012). However, both of these theories have recently been refuted following the characterisation of a large number of additional *Plasmodium* parasites from African apes. Specifically, it is now clear that *P. falciparum* infection is relatively new for humans and arose after the acquisition of a parasite from a gorilla, likely within the past 10,000 years (Liu et al., 2010a; Sundararaman et al., 2016). Similarly, *P. vivax* did not emerge in Asia, but represents a bottlenecked lineage that escaped out of Africa before the spread of Duffy negativity rendered African humans resistant to *P. vivax* (Liu et al., 2014). In this review, we describe the findings that led to this new understanding and summarise what is known about the epidemiology, vector tropism, zoonotic potential and pathogenicity of the ape precursors of the human parasites.

2. Early studies of ape *Plasmodium* infections

The first indication that African apes harbour *Plasmodium* infections was the finding of three morphologically distinct forms of parasites in the blood of wild-caught chimpanzees (*Pan troglodytes*) and western gorillas (*Gorilla gorilla*) in Cameroon (Reichenow, 1920). Microscopic characterisation identified parasites from apes that resembled *P. falciparum*, *Plasmodium malariae*, and either *Plasmodium ovale* or (the similar) *P. vivax* in humans, suggesting the existence of distinct *Plasmodium* spp. which were classified as *Plasmodium reichenowi*, *Plasmodium rhodaini* and *Plasmodium schwetzi*, respectively (Sluiter et al., 1922; Brumpt, 1939). Moreover, *P. falciparum* and *P. reichenowi* were found to differ substantially from the other *Plasmodium* spp. in both life cycle and gametocyte morphology, prompting their placement in a separate subgenus, termed *Laverania* (Bray, 1958; Coatney et al., 1971). The existence of two divergent clades of malaria parasites infecting primates was subsequently confirmed when the various *Plasmodium* spp. were first molecularly characterised (Fig. 1). Comparing rRNA small subunit gene sequences, Escalante and Ayala (1994) showed that amongst the known species, *P. falciparum* and *P. reichenowi* were the closest relatives of each other, and that both were only distantly related to other *Plasmodium* spp. Assuming that rRNA gene sequences in *Plasmodium* spp. evolved at the same rate as had been estimated for some bacteria, it was inferred that *P. falciparum* and *P. reichenowi* had diverged ~10 million years ago, close to the time of the human-chimpanzee common ancestor. This led to the conclusion that parasites infecting humans and chimpanzees had co-diverged with their respective hosts (Escalante and Ayala, 1994). Due to a lack of preserved material, gene sequences from *P. schwetzi* and *P. rhodaini* were never determined, thus leaving their relationship to other malaria parasites open to question.

Interest in ape *Laverania* infections was rekindled in 2009 when Ollomo and colleagues found parasites morphologically similar to *P. reichenowi* in the blood of two pet chimpanzees from Gabon (Ollomo et al., 2009). Analysis of mitochondrial DNA (mtDNA) sequences revealed that these parasites were related to, but divergent from, *P. falciparum* and *P. reichenowi*, suggesting the existence of a third *Laverania* sp. which they named *Plasmodium gaboni* (Ollomo et al., 2009). Follow-up studies of additional captive and wild apes confirmed a greater diversity of *Laverania* parasites, but interpretations differed as to the number of species and their

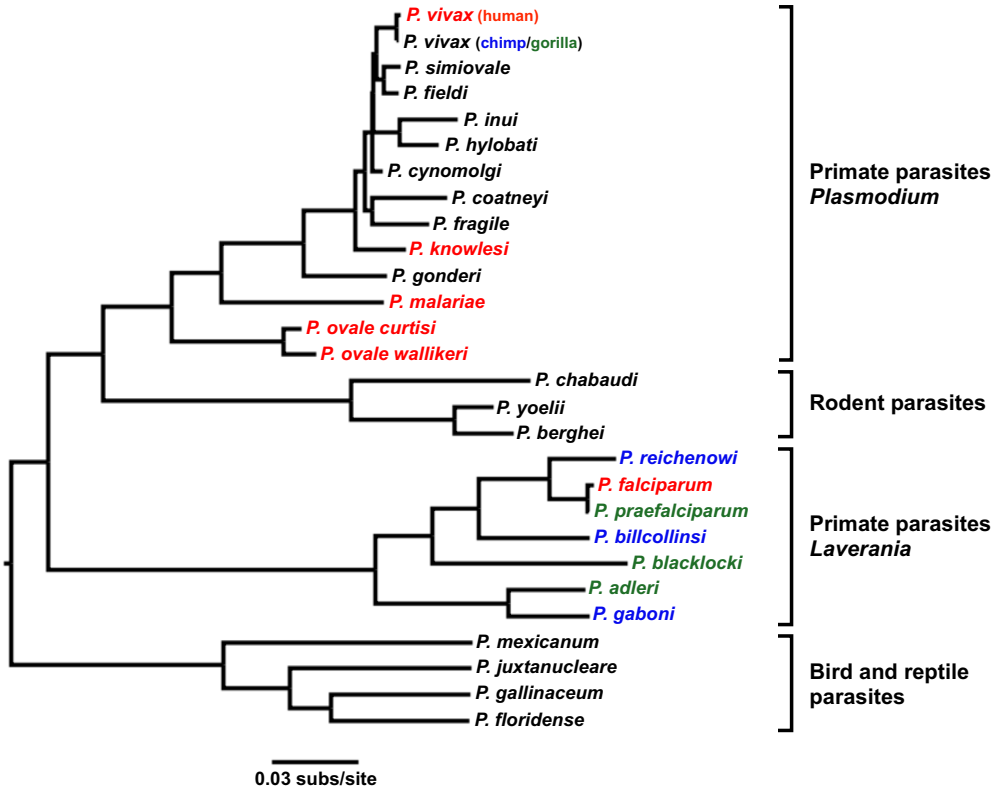

Fig. 1. Evolutionary relationships of *Plasmodium* spp. Colours highlight *Plasmodium* spp. that infect humans (red), chimpanzees (blue) and gorillas (green). Four groups of *Plasmodium* spp. are shown, with subgenus designations indicated for primate parasites. The phylogeny was estimated by maximum likelihood analysis of 2.4 kb of the mitochondrial genome; the scale bar indicates 0.03 substitutions per site.

host associations. Amplifying mtDNA and nuclear gene sequences of parasites from members of two chimpanzee subspecies, Rich and colleagues (2009) identified several distinct *Laverania* lineages, but chose to consider all of them as "*P. reichenowi*", even though one of these new lineages corresponded to *P. gaboni* (Rich et al., 2009). In contrast, Krief and colleagues (2010) classified a similar diversity of chimpanzee parasites into three species, termed *P. reichenowi*, *Plasmodium billcollinsi* and *Plasmodium billbrayi*, where the latter corresponded to *P. gaboni* (Krief et al., 2010). These investigators also amplified *P. falciparum* mtDNA from the blood of captive bonobos (*Pan paniscus*), concluding that this ape species served as the likely source of the human infection (Krief et al., 2010). Finally, Prugnolle and colleagues developed non-invasive methods that permitted parasite detection in ape faecal samples, which identified diverse *Laverania* lineages not only in chimpanzees but also in western gorillas (Prugnolle et al., 2010). However, these investigators classified all chimpanzee parasites as either *P. reichenowi* or *P. gaboni*, and concluded that *P. falciparum*-like sequences found in faecal samples of wild-living western gorillas indicated ongoing transmission from humans to gorillas (Prugnolle et al., 2010). The consensus of these studies was that wild-living apes harbour a much greater diversity of *Laverania* parasites than previously recognised. However, there was disagreement concerning the number of ape *Laverania* spp. as well as the origin of *P. falciparum*, with some investigators implicating chimpanzees (Rich et al., 2009; Duval et al., 2010; Prugnolle et al., 2010) and others bonobos (Krief et al., 2010) as the original source of the parasites now infecting humans.

3. Six *Laverania* spp. in wild-living chimpanzees and gorillas

The seemingly discrepant results from these early studies were reconciled by comprehensive studies of *Laverania* infections in wild-living apes, which employed improved faeces-based detection methods and targeted different regions of both organelle and nuclear parasite genomes (Liu et al., 2010a). One technical advance was the use of limiting dilution PCR (termed single genome amplification or SGA), which in contrast to standard (bulk) PCR precludes the generation of in vitro recombinants that confound phylogenetic analyses (Liu et al., 2010b). Using this approach to characterise the molecular epidemiology of ape malaria, *Plasmodium* infections were found to be widespread in both chimpanzees and western gorillas, including parasites that were closely related to human *P. malariae*, *P. ovale* and *P. vivax* (Liu et al., 2010a). However, the great majority of parasite sequences grouped within one of three chimpanzee-specific or three gorilla-specific parasite lineages, with each clade being well supported and quite distinct from the others, pointing to the existence of six ape *Laverania* spp. (Fig. 1). Subsequent surveys of wild-living apes in Gabon (Boundenga et al., 2015) and Cote d'Ivoire (Kaiser et al., 2010) confirmed these findings, demonstrating that chimpanzees and western gorillas represent a substantial *Laverania* reservoir.

Fig. 2A summarises the current knowledge concerning the geographic distribution and host species association of ape *Laverania* infections at over 100 field sites across sub-Saharan Africa (Kaiser et al., 2010; Liu et al., 2010a, 2016; De Nys et al., 2013; Boundenga et al., 2015). All chimpanzee subspecies, including western (*Pan troglodytes verus*), Nigeria-Cameroon (*Pan troglodytes ellioti*), central (*Pan troglodytes troglodytes*) and eastern (*Pan troglodytes schweinfurthii*) chimpanzees, as well as western lowland gorillas (*G. g. gorilla*) are endemically infected with *Laverania* parasites, with faecal detection rates ranging from 24% to 40% (Table 1). The true prevalence rates are likely to be considerably higher, since the amount of *Laverania* DNA that is shed in faecal samples is substantially less than that from replicating parasites in the blood (Liu et al., 2010a; Sundararaman et al., 2016). Although Cross River gorillas (*Gorilla gorilla diehli*), eastern lowland gorillas (*Gorilla beringei graueri*), and bonobos (in the wild) have appeared to be free of *Laverania* infections, the numbers of individuals tested from these potential hosts are still too small to draw definitive conclusions (Liu et al., 2010a).

Analyses of nearly 3,500 SGA-derived mt, apicoplast and nuclear DNA sequences from ape faecal and blood samples have confirmed the existence of six *Laverania* spp. (Figs. 1 and 3). Of these, *P. reichenowi*, *P. gaboni* and *P. billcollinsi* have thus far only been identified in chimpanzees, whilst *Plasmodium praefalciparum*, *Plasmodium blacklocki* and *Plasmodium adleri* have only been found in western gorillas. All six *Laverania* spp. have been classified based on numerous SGA-derived organelle and nuclear gene sequences from many different field isolates (Liu et al., 2010a, 2016). In addition, whole genome sequencing of *P. reichenowi* and *P. gaboni* parasites confirmed that they represent distinct species, with no evidence of interspecific hybridization (Otto et al., 2014; Sundararaman et al., 2016). Whilst it has been argued that detection of parasite DNA in either faeces or blood, in itself, is not proof of productive *Plasmodium* infection (Valkiunas et al., 2011), the high prevalence rates of *Laverania* infections (Table 1) and their widespread distribution (Fig. 2A) provide compelling evidence for significant ongoing transmission. Of note, *Laverania* parasites exhibit strict host specificity when infecting wild-living apes, including at field sites where all six species are co-circulating in sympatric chimpanzee and gorilla populations (octagons in Fig. 2A). SGA, which yields a proportional representation of all parasites present in a sample, failed to detect even minor fractions of parasites from the "wrong" species in more than 100 *Laverania*-infected apes (Liu et al., 2016). In contrast, host species specificity in captivity is not absolute (Duval et al., 2010; Pacheco et al., 2013), and so it will be of great interest to decipher to what extent host and/or vector biology contribute to host species restriction in the wild.

4. Origin of *P. falciparum* in western gorillas

Characterisation of the various ape *Laverania* spp. identified one lineage in western gorillas that was comprised of parasites that were nearly identical to *P. falciparum* (Liu et al., 2010a; Prugnolle et al., 2010). This was initially interpreted as indicating that human parasites can infect gorillas (Prugnolle et al., 2010). However, with the characterisation of mtDNA sequences from large numbers of additional wild-living gorillas, it became apparent that all extant *P. falciparum* strains from humans fall within the radiation of these gorilla parasites (Liu et al., 2010a). Analyses of both mt (Fig. 3A) and nuclear (Fig. 3B) sequences confirmed these relationships, indicating that human *P. falciparum* resulted from the cross-species transmission of a parasite that had previously diversified in gorillas. This gorilla parasite lineage has been named *P. praefalciparum* to indicate its role in the origin of *P. falciparum*. To investigate how often *P. praefalciparum* crossed the species barrier to humans, we constructed a phylogenetic tree from concatenated mt protein sequences of these and closely related *P. reichenowi* parasites, which yielded evidence for only a single transmission event (Fig. 3C). These findings are consistent with results from epidemiological surveys in Cameroon and Gabon, which demonstrated that humans living in the immediate vicinity of wild-living chimpanzees and gorillas do not harbour ape *Laverania* parasites (Sundararaman et al., 2013; Delicat-Loembet et al., 2015). Thus, *P. praefalciparum* parasites appear incapable of infecting humans, suggesting that the particular gorilla parasite strain that was able to cross the host species barrier must have carried one or more

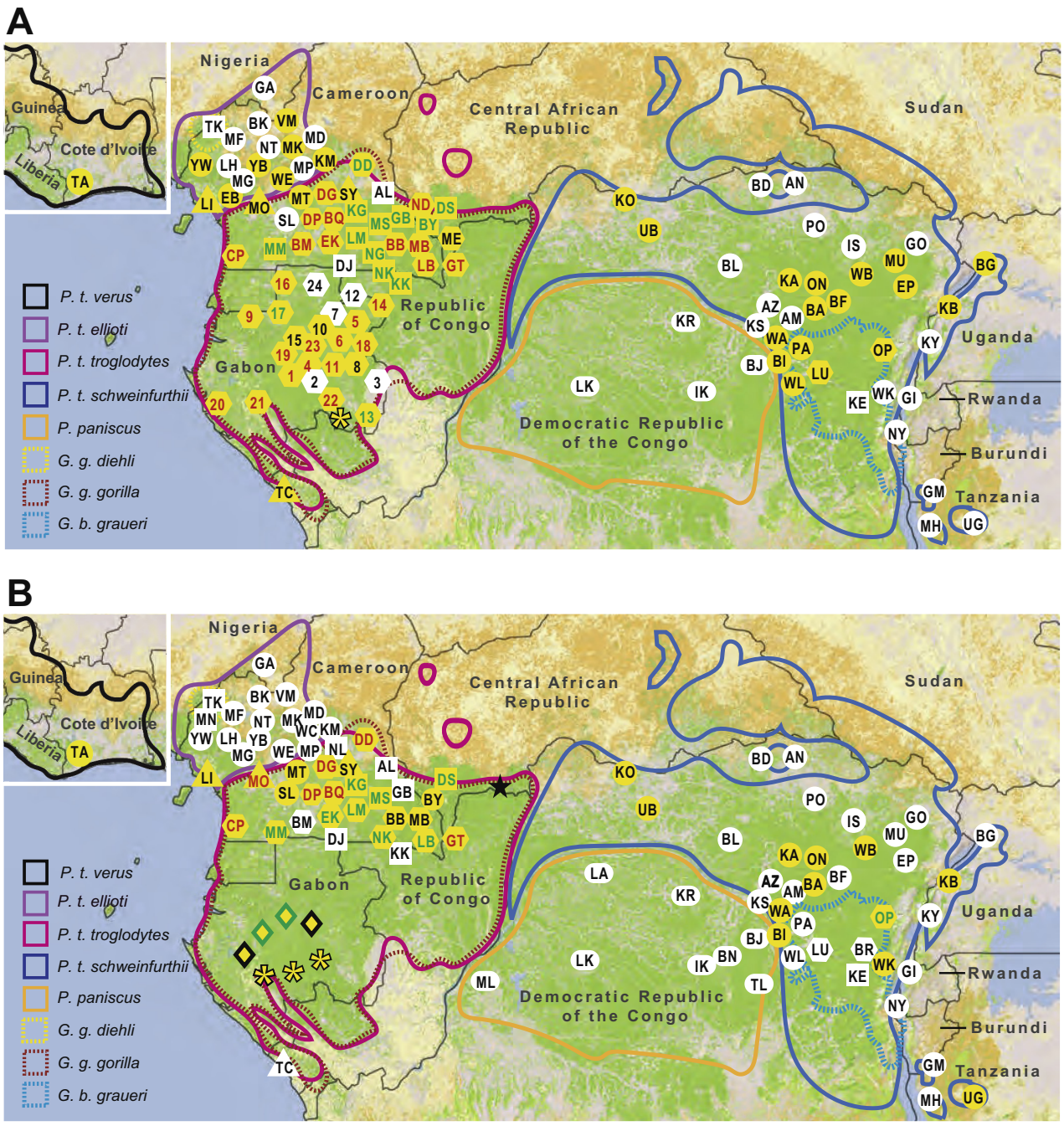

Fig. 2. Geographic distribution of (A) *Laverania* and (B) *Plasmodium vivax* infections in wild-living apes. Field sites are shown in relation to the ranges of the four subspecies of the common chimpanzee (inset: *Pan troglodytes verus*, black; *Pan troglodytes ellioti*, purple; *Pan troglodytes troglodytes*, magenta; *Pan troglodytes schweinfurthii*, blue), the Cross River (*Gorilla gorilla diehli*, yellow stripe), western lowland (*Gorilla gorilla gorilla*, red stripe), and eastern lowland (*Gorilla beringei graueri*, cyan stripe) gorilla, as well as the bonobo (*Pan paniscus*, orange) in sub-Saharan Africa (Caldecott and Miles, 2005). Field sites are labelled by a two-letter code as previously reported (Liu et al., 2010a, 2014) or numbers (Boundenga et al., 2015), and those where ape malaria was detected are highlighted in yellow, with black, green or red lettering indicating that chimpanzees, gorillas, or both were infected. Triangles denote ape rescue centres and asterisks mosquito collection sites. Circles, squares and hexagons identify locations where faecal samples were collected from chimpanzees, gorillas or both species, respectively. Ovals indicate bonobo sites. At the TA and KB sites, blood and tissue samples were obtained from injured or deceased habituated chimpanzees (Kaiser et al., 2010; Krief et al., 2010; De Nys et al., 2013). Diamonds in (B) indicate the capture sites of ape *P. vivax* infected sanctuary chimpanzees (black border) and gorillas (green border), respectively, and a star denotes the location where a European forester became infected with ape *P. vivax* (Prugnolle et al., 2013). Data were compiled from published (Kaiser et al., 2010; Liu et al., 2010a, 2014, 2016; De Nys et al., 2013; Paupy et al., 2013; Prugnolle et al., 2013; Boundenga et al., 2015) and unpublished studies (Table 1). The full names and locations of all sites are provided in Supplementary Table S1.

highly unusual mutations that conferred an ability to colonise humans.

Although alternative hypotheses concerning the origin of *P. falciparum* have been proposed, none has stood the test of time. For example, the finding of a *P. praefalciparum* infection in a greater spot-nosed monkey (*Cercopithecus nictitans*) (Fig. 3C) was taken to indicate that *P. falciparum* could have originated in monkeys (Prugnolle et al., 2011). However, this theory ignored the fact that *P. praefalciparum* sequences had been amplified from numerous wild-living gorillas at 11 different field sites up to 750 km apart, whereas only a single captive infected monkey was reported (Sharp et al., 2011). Indeed, subsequent testing of nearly 300

Table 1
Feces-based prevalence estimates of *Laverania* and *Plasmodium vivax* infections in wild-living African apes.

Species/subspecies	*Laverania*[a]					*P. vivax*[b]				
	Field sites tested[c]	Field sites positive	Faecal samples tested	Faecal samples positive	% Infection rate (CI)[d]	Field sites tested[c]	Field sites positive	Faecal samples tested	Faecal samples positive	% Infection rate (CI)[d]
Western chimpanzee (*Pan troglodytes verus*)[e]	1	1	171	34	40 (31–50)	1	1	171	2	4 (2–11)
Nigeria-Cameroon chimpanzee (*Pan troglodytes ellioti*)	15	7	148	21	29 (20–39)	15	0	149	0	0 (0–4)
Central chimpanzee (*Pan troglodytes troglodytes*)	47	31	1412	271	39 (36–42)	25	11	1130	25	8 (6–10)
Eastern chimpanzee (*Pan troglodytes schweinfurthii*)	33	17	1676	199	24 (20–28)	34	10	1784	20	4 (3–6)
Cross River gorilla (*Gorilla gorilla diehli*)	1	0	9	0	0 (0–53)	2	0	80	0	0 (0–8)
Western lowland gorilla (*Gorilla gorilla gorilla*)	49	38	1584	256	33 (30–36)	22	13	1575	30	7 (5–9)
Eastern lowland gorilla (*Gorilla beringei graueri*)	3	0	146	0	0 (0–4)	4	1	189	2	4 (1–9)
Bonobo (*Pan paniscus*)	4	0	161	0	0 (0–4)	8	0	754	0	0 (0–1)

[a] *Laverania* infection results were compiled from five studies (Kaiser et al., 2010; Liu et al., 2010; De Nys et al., 2013; Boundenga et al., 2015; Liu et al., 2016) as well as recently obtained unpublished data from additional field sites, including BJ, BK, DJ, GA, GI, GM, GO, IK, KB, KY, MD, MG, MH, MK, MP, NY, SL, TK, UG (the full names and locations of these sites are provided in Supplementary Table S1).

[b] Ape *P. vivax* infection results were compiled from four studies (Kaiser et al., 2010; Liu et al., 2010; De Nys et al., 2013; Liu et al., 2014) as well as recently obtained unpublished data from additional field sites, including BG, GA, GI, KB, KY, MH, NY (the full names and locations of these sites are provided in Supplementary Table S1).

[c] The location of field sites is shown in Fig. 2.

[d] Infection rates were estimated for each ape species or subspecies based on the combined numbers of PCR-positive faecal samples per total number of faecal samples screened, but assuming similar levels of specimen degradation, redundant sampling and diagnostic test sensitivities across all studies (Liu et al., 2010, 2014). Since there is less *Plasmodium* DNA shed into faecal samples than can be detected in the blood, the values represent minimum estimates. Parentheses indicate 95% confidence intervals (CI). Results from chimpanzee blood samples collected in the Tai Forest (TA) and Kibale National Park (KB) are not included (Kaiser et al., 2010; Krief et al., 2010).

[e] Faecal samples from *P. t. verus* were screened using pan-*Plasmodium cytB* primers, not *Laverania*- or *P. vivax*-specific PCR primers (Kaiser et al., 2010; De Nys et al., 2013).

wild-caught greater spot-nosed monkeys failed to identify a single *P. praefalciparum* infection, indicating that this monkey species is not a natural reservoir for this parasite (Ayouba et al., 2012). Similarly, amplification of *P. falciparum* sequences from captive bonobos was taken to indicate that the human malaria parasite originated in this ape species (Krief et al., 2010). However, phylogenetic analysis of these sequences revealed that they were completely interspersed with human *P. falciparum* (Fig. 3C), which together with the finding of drug resistance mutations in the bonobo parasites (Krief et al., 2010), indicated that these apes had acquired parasites from the local human population. This is not without precedent, since human *P. falciparum* has on occasion been found to infect chimpanzees in captivity (Duval et al., 2010; Pacheco et al., 2013).

5. Emergence of *P. falciparum* in humans

Plasmodium falciparum has long been suspected to exhibit unusually low levels of genetic diversity (Rich et al., 1998), but the underlying causes have remained unclear. Recent genome-wide comparisons of the chimpanzee parasites *P. gaboni* and *P. reichenowi* have shown that their within-species genetic diversity is approximately 10-fold higher than that seen in *P. falciparum* (Sundararaman et al., 2016). Thus, the extremely low diversity amongst extant *P. falciparum* strains is not a general characteristic of *Laverania* parasites. Recent selective sweeps of drug resistance mutations have reduced levels of polymorphism in *P. falciparum*, but because resistant and sensitive strains continue to recombine in mosquitoes, diversity has only been reduced in the immediate vicinity of the selected loci (Nair et al., 2003; Volkman et al., 2007). Instead, the greatly reduced level of diversity across the entire *P. falciparum* genome most likely resulted from a recent severe population bottleneck, which is most plausibly explained by the gorilla-to-human cross-species transmission event.

Previous attempts to date the last common ancestor of *P. falciparum* strains have yielded estimates of up to several hundred thousand years ago (Hughes and Verra, 2001; Pacheco et al., 2011; Neafsey et al., 2012), but all made assumptions concerning the *Plasmodium* molecular clock that are difficult to justify. In contrast, others have proposed a much shorter time scale, arguing that the low probability of maintaining endemic *P. falciparum* infections in human hunter-gatherer populations (Livingstone, 1958), together with estimates of the age of *P. falciparum* resistance mutations in Africa (Hedrick, 2011), favour a much more recent emergence (Carter and Mendis, 2002). From a comparison of 12 strains from different countries in Africa and Asia, the average diversity of *P. falciparum* at fourfold degenerate sites (which should be neutral and thus reflect mutation rates) was estimated to be 8×10^{-4} per site (Sundararaman et al., 2016). Published mutation rates for *P. falciparum* are in the range $1–10 \times 10^{-9}$ mutations per site per replication cycle (Paget-McNicol and Saul, 2001; Lynch, 2010; Bopp et al., 2013), and it can be deduced from the *P. falciparum* life cycle that parasites are likely to undergo at least 200 replication cycles per year, even assuming varying lengths of time that the parasites spend in either the vector or the mammalian host. This suggests that the observed level of genetic diversity in *P. falciparum* could have readily accumulated within the past 10,000 years.

6. Allelic dimorphism in ape *Laverania* spp.

Nearly 30 years ago, it was recognised that a gene (now called *msp-1*) encoding a merozoite surface protein exists as two highly divergent alleles in *P. falciparum*, with recombination suppressed over much of the length of the gene (Tanabe et al., 1987). Similar allelic dimorphism was subsequently found for other genes encoding merozoite surface proteins including *msp-2*, *msp-3* and *msp-6* (Roy et al., 2008). The extent of divergence is extreme: for example, large regions of the two *msp-1* alleles in *P. falciparum* are more different from each other than one of those alleles is from the one *msp-1* allele so far found in *P. gaboni*, suggesting that the *P. falciparum* alleles diverged prior to the last common ancestor of extant *Laverania* spp. (Roy, 2015). This suggests some form of selection that maintains divergent allelic types over very long periods of time, analogous, for example, to the trans-specific polymorphism of self-incompatibility alleles in a family of flowering plants (Solanaceae), which may date to more than 30 million years (Myr) ago (Ioerger et al., 1990). Although the mechanism(s) that maintain the dimorphic *msp* alleles in *P. falciparum* remain largely unknown (Roy and Ferreira, 2015), some genes appear to be under balancing selection (Ochola et al., 2010; Amambua-Ngwa et al., 2012) and represent targets of allele type-specific antibody responses that

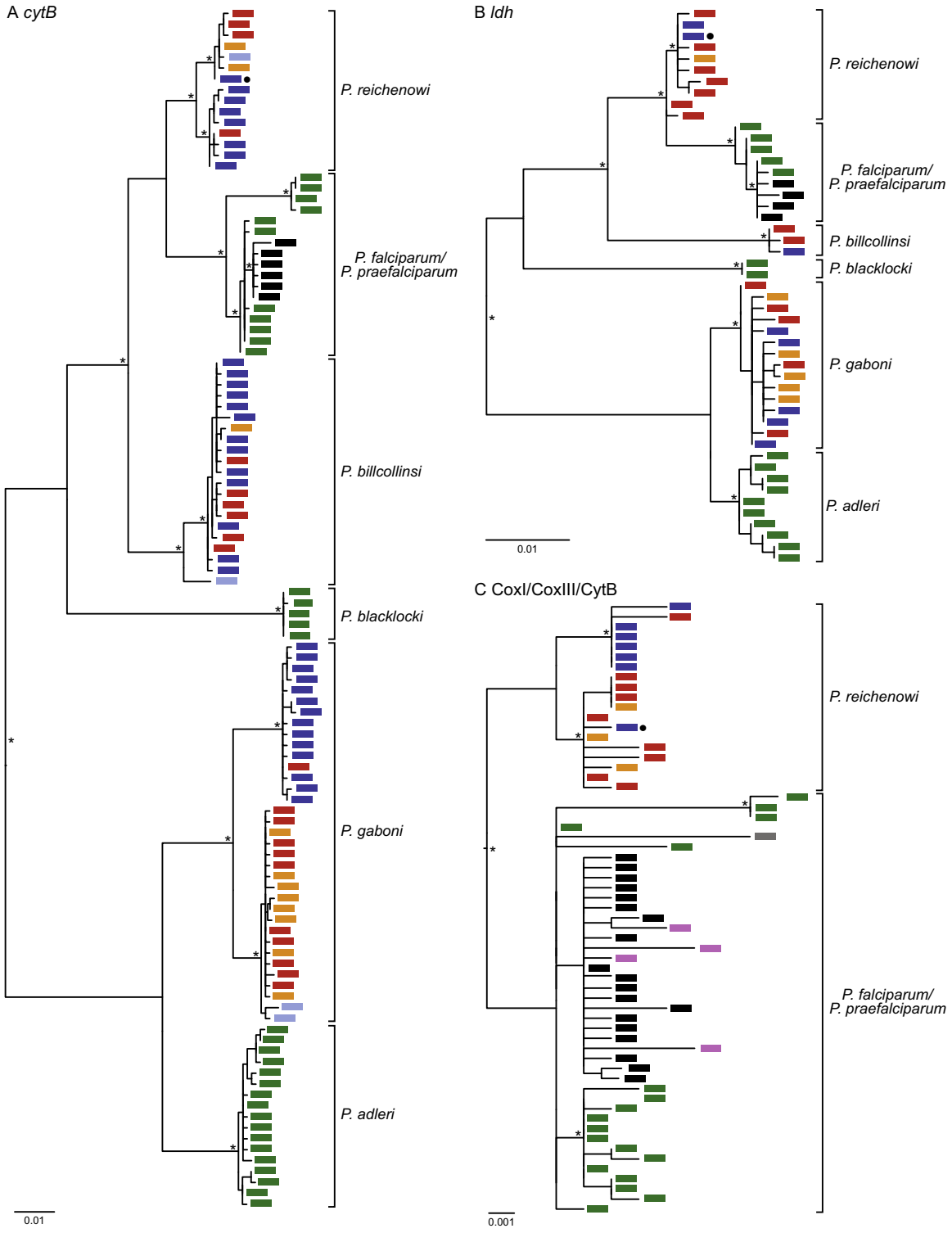

Fig. 3. Evolutionary relationships of ape and human *Laverania* parasites. The phylogenetic relationships of (A) mitochondrial cytochrome B (*cytB*; 956 bp) and (B) nuclear lactate dehydrogenase (*ldh*; 772 bp) gene sequences, as well as (C) concatenated mitochondrial protein (CoxI/CoxIII/CytB; 981 amino acids) sequences are shown. Ape parasite sequences are coloured according to their host species (*Pan troglodytes verus*, light blue; *Pan troglodytes troglodytes*, red; *Pan troglodytes schweinfurthii*, dark blue; *Pan troglodytes ellioti*, orange; *Gorilla gorilla gorilla*, green), and human parasite reference sequences are shown in black. A black circle denotes the *Plasmodium reichenowi* PrCDC reference sequence (Otto et al., 2014) derived from a chimpanzee captured in the Belgian Congo (now the Democratic Republic of the Congo) (*Pan troglodytes schweinfurthii*) (Coatney et al., 1971). (C) Four *Plasmodium falciparum* sequences from captive bonobos (Krief et al., 2010) and one *Plasmodium praefalciparum* sequence from a captive greater spot-nosed monkey (Prugnolle et al., 2011) are shown in magenta and grey, respectively. Brackets indicate *Laverania* spp. Phylogenies were generated using maximum likelihood methods. Asterisks at major nodes indicate bootstrap values ⩾65%, and the scale bars represent (A, B) 0.01 nucleotide substitutions per site, or (C) 0.001 amino acid replacements per site, respectively. Sequences were combined from multiple studies (Kaiser et al., 2010; Krief et al., 2010; Liu et al., 2010a, 2016; Prugnolle et al., 2011).

confer protective immunity against malaria (Polley et al., 2007; Tetteh et al., 2013).

For *msp-1*, *msp-3* and *msp-6*, we have found evidence for both allelic types in *P. praefalciparum* (Liu, W., Sundararaman, S.A., Loy, D.E., Learn, G.H., Li, Y., Plenderleith, L.J., Ndjango, J.B., Speede, S., Rayner, J.C., Peeters, M., Hahn, B.H., Sharp, P.M., 2015. On the origins of allelic dimorphism of the *Plasmodium falciparum* msp-1 and msp-6 genes. Am. Soc. Trop. Med. Hyg. (ASTMH), 64th Annual Meeting, Philadelphia, PA, USA). However, it is unclear how two highly divergent alleles, at a number of different loci, survived the genetic bottleneck at the origin of *P. falciparum*. Sporozoites, which are injected into the human host by the mosquito, are haploid. Transmission from gorilla to human of two dimorphic alleles could occur in a single event, if heterozygous oocytes in the mosquito generated two types of sporozoites present in the same inoculum. However, the transmission of divergent alleles of multiple genes would require that oocytes were simultaneously heterozygous at all loci that are now dimorphic in *P. falciparum*. Alternatively, backcrossing of human parasites to gorilla parasites, in the immediate aftermath of the initial cross-species transmission event, but before they became isolated, could lead to the transfer of additional alleles to humans. Regardless of the mechanism, however, any proposed scenario involving the transfer of multiple alleles at multiple *msp* loci must at the same time explain the paucity of genetic variation seen elsewhere in the genome (Sundararaman et al., 2016). It would require there to have been very strong selection retaining both dimorphic alleles at the various loci, in the face of extreme random genetic drift (perhaps due to a very small number of initial human hosts) affecting all other loci. Characterisation of the extent of allelic dimorphism across other *Laverania* spp., together with the corresponding host immune responses, may shed more light on this puzzle.

7. A sylvatic reservoir of *P. vivax*

Although early studies of ape blood and faecal samples indicated that chimpanzees and gorillas harbour *P. vivax*-like parasites, the number of sequences recovered was too limited to draw definitive conclusions (Kaiser et al., 2010; Krief et al., 2010; Liu et al., 2010a; Prugnolle et al., 2013). As for *Laverania* parasites, elucidation of the molecular epidemiology of *P. vivax* in apes required a comprehensive analysis of wild-living populations across central Africa (Liu et al., 2014). Table 1 and Fig. 2B summarise available data from 97 field sites, showing that *P. vivax* is relatively common amongst central and eastern chimpanzees as well as western lowland gorillas, which together represent a considerable sylvatic *P. vivax* reservoir (Kaiser et al., 2010; Liu et al., 2010a, 2014; De Nys et al., 2013). However, amplification of *P. vivax* DNA sequences from faecal samples was considerably less efficient than from blood samples, most likely reflecting much lower parasite loads in faecal samples compared with blood (Liu et al., 2014). Thus, the observed faeces-based infection rates, which ranged from 4% to 8% for the various ape species and subspecies (Table 1), are expected to greatly underestimate the actual prevalence rates, perhaps by as much as an order of magnitude. The low sensitivity of faecal parasite detection may also explain why *P. vivax* has not yet been detected in wild-living Nigeria-Cameroon chimpanzees, Cross River gorillas or bonobos. Indeed, *P. vivax*-like sequences were readily amplified from the blood of captive Nigeria-Cameroon chimpanzees, indicating that this subspecies is susceptible to *P. vivax* infection (Fig. 4).

Phylogenetic analyses of SGA-derived sequences showed that ape and human *P. vivax* were very closely related. However, chimpanzee- and gorilla-derived parasites exhibited greater genetic diversity than even the most geographically diverse human *P. vivax* strains. In phylogenetic trees of mt (Fig. 4A), nuclear (Fig. 4B), and apicoplast (Fig. 4C) sequences, human *P. vivax* sequences formed a single lineage within the radiation of the ape parasites. In contrast, parasite sequences derived from chimpanzee and gorilla samples were interspersed, suggesting that *P. vivax* circulates freely between these two ape species. Of note, analysis of nearly 1,000 bushmeat samples failed to identify related sequences in samples from any of 16 different monkey species, strongly suggesting that *P. vivax* is restricted to African apes (Liu et al., 2014).

8. African origin of human *P. vivax*

Until recently, the closest known relative of *P. vivax* was a parasite, *Plasmodium cynomolgi*, which infects macaques in Asia (Tachibana et al., 2012). In phylogenetic trees, *P. vivax* and *P. cynomolgi* fall within a clade of parasites that includes at least eight other *Plasmodium* spp. infecting southeast Asian primates (Fig. 1). The consensus view has thus been that *P. vivax* emerged in southeastern Asia following the cross-species transmission of a macaque parasite (Escalante et al., 2005; Jongwutiwes et al., 2005; Mu et al., 2005; Neafsey et al., 2012). However, this hypothesis has always been at odds with two other observations. First, the high prevalence of the Duffy-negative phenotype in sub-Saharan Africans, which suggested that this mutation arose in response to prolonged selection pressure from *P. vivax* (Carter, 2003) rather than another unidentified pathogen (Livingstone, 1984). Second, modern humans did not arrive in Asia until approximately 60,000 years ago (Mellars, 2006); yet, *P. vivax* has likely diverged from macaque parasites much earlier than this (Escalante et al., 2005; Jongwutiwes et al., 2005; Mu et al., 2005; Neafsey et al., 2012). Thus, *P. vivax* would have had a rather convoluted history, requiring transmission from macaques to an earlier hominin such as *Homo erectus*, followed by its diversification in that host before numerous lineages were transmitted to modern humans after they emerged from Africa. The discovery of *P. vivax* in large numbers of chimpanzees and gorillas now resolves these inconsistencies, providing compelling evidence for an African, rather than an Asian, origin of human *P. vivax*.

The phylogenetic relationships of organelle as well as nuclear gene sequences indicate that all extant human *P. vivax* strains form a monophyletic clade within the radiation of ape parasites (Fig. 4). This could be interpreted to mean that *P. vivax* originated in humans following a single transmission event. However, the lack of host specificity of ape *P. vivax* in natural settings (Liu et al., 2014), together with the finding of a human infection with ape *P. vivax* (Prugnolle et al., 2013), argues against this theory. Instead, it is much more likely that extant human *P. vivax* represents a lineage that survived after spreading out of Africa. This scenario explains the reduced diversity of the human parasites as resulting from an out-of-Africa bottleneck, as seen in *P. falciparum* (Conway et al., 2000; Tanabe et al., 2010) and in humans themselves (Ramachandran et al., 2005). Human *P. vivax* strains that are currently found in Madagascar and parts of Africa are likely the result of a reintroduction of this parasite from Asia (Culleton and Carter, 2012).

Whilst it could be argued that the ape *P. vivax* was brought to Africa by humans who migrated from Asia (Prugnolle et al., 2013), this hypothesis has been refuted by sequences indicating the existence of a related, but distinct, *Plasmodium* sp. that also infects African apes. This *Plasmodium* sp. which is apparent in trees of mt, nuclear and apicoplast sequences (Fig. 4), has been found in chimpanzees from two different locations in Cameroon (the BQ and DG field sites in Fig. 2) and represents the closest known relative of *P. vivax*. The most parsimonious interpretation of this finding is that the common ancestor of these two species was in

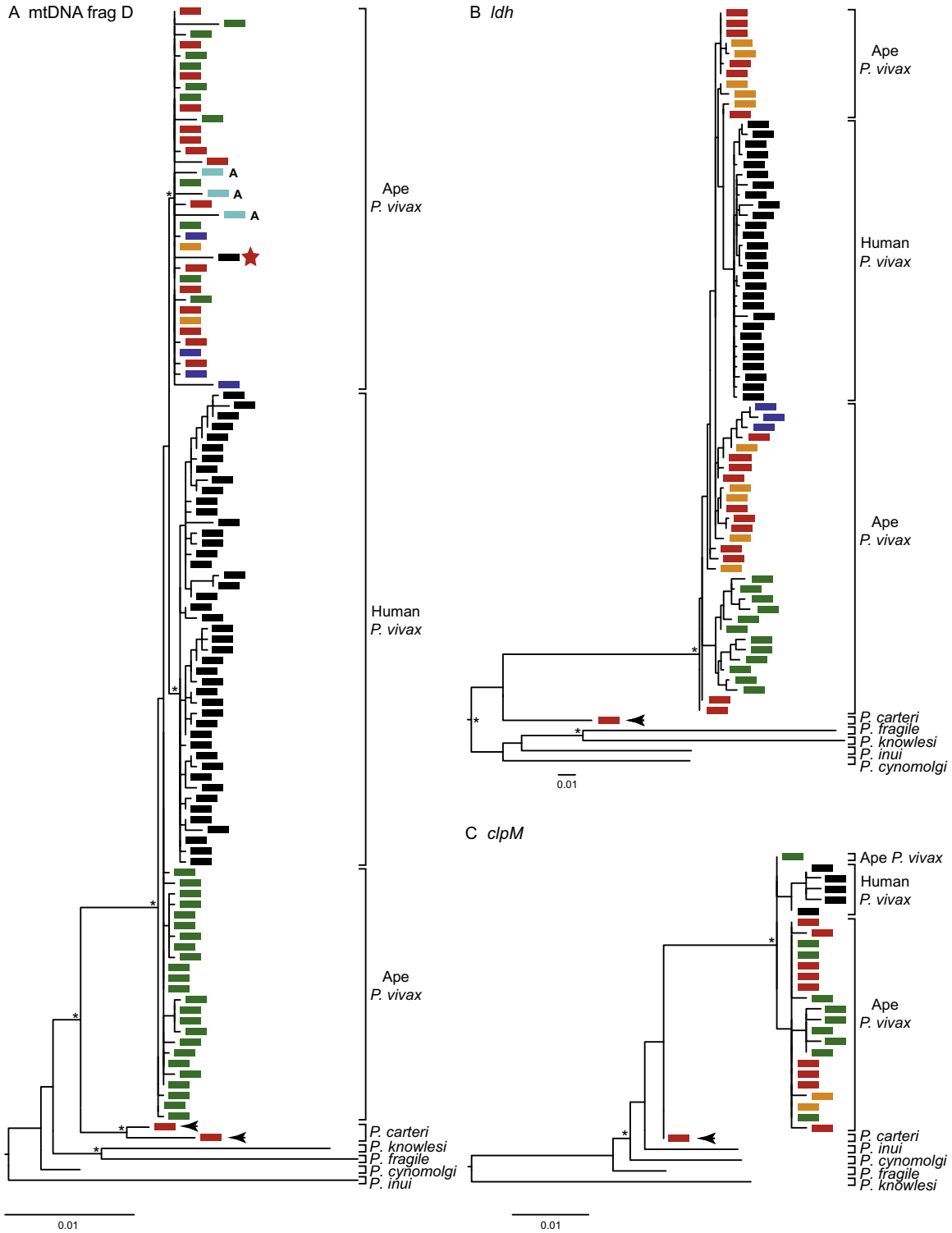

Fig. 4. Evolutionary relationships of ape and human *Plasmodium vivax* parasites. Phylogenies were derived from (A) mitochondrial (mt)DNA fragment D (2,539 bp), (B) nuclear DNA (*ldh* gene; 711 bp), and (C) apicoplast DNA (*clpM* gene; 574 bp). Parasite sequences are coloured according to their host species (*Pan troglodytes troglodytes*, red; *Pan troglodytes schweinfurthii*, dark blue; *Pan troglodytes ellioti*, orange; *Gorilla gorilla gorilla*, green; human, black); the red star denotes a parasite from a European person who worked in an African forest. Mosquito (*Anopheles moucheti*) derived sequences are shown in cyan (and denoted with 'A'). Reference sequences for *Plasmodium cynomolgi*, *Plasmodium inui*, *Plasmodium fragile* and *Plasmodium knowlesi* are indicated. A lineage of parasite sequences from wild chimpanzees, which is related to ape and human *P. vivax*, likely represents a new *Plasmodium* sp. which has been designated *Plasmodium carteri* (black arrows). Phylogenies were generated using maximum likelihood methods. Asterisks at major nodes indicate bootstrap values ⩾65%, and the scale bars represent 0.01 nucleotide substitutions per site. Sequences were combined from multiple studies (Krief et al., 2010; Paupy et al., 2013; Prugnolle et al., 2013; Liu et al., 2014).

Africa, indicating that the lineage existed there for a long time before *P. vivax* arose as a distinct species (Fig. 4). We propose to designate this newly described species *Plasmodium carteri*, in honour of Richard Carter, who has long championed the hypothesis that *P. vivax* originated in Africa (Carter, 2003; Culleton and Carter, 2012).

9. Mosquito vectors of ape *Plasmodium* spp.

Identifying the mosquito vectors that transmit ape *Plasmodium* parasites is critical for understanding their host specificity and zoonotic potential. Initial studies of whole mosquito DNA identified *P. praefalciparum* in *Anopheles moucheti*, whilst ape *P. vivax* was found in both *A. moucheti* and *Anopheles vinckei*, although these mosquitoes were analysed with molecular tools that were unsuitable to differentiate between parasite stages (Paupy et al., 2013; Prugnolle et al., 2013). A subsequent study screened dissected salivary glands for parasites, and demonstrated that *A. vinckei*, *A. moucheti* and *Anopheles marshallii* are transmitting vectors of ape *Plasmodium* species (Makanga et al., 2016). *Anopheles vinckei* was found to be most frequently infected, harbouring *P. vivax*-like, *P. malariae*-like, and *Laverania* spp., and carried gorilla as well as chimpanzee parasites (Makanga et al., 2016). Moreover, human landing catches showed that all three *Anopheles* spp. had the propensity to bite humans, indicating that they could serve as bridge vectors for human infection (Makanga et al., 2016). Although these three *Anopheles* spp. may not represent the entirety of vectors capable of transmitting ape *Plasmodium* parasites, it seems clear that the strict host specificity of *Laverania* spp. in wild-living ape populations cannot be explained by mosquitoes that exclusively feed on chimpanzees or gorillas. It is likely that *P. praefalciparum* was first transmitted to humans by one of these sylvatic vectors, but the subsequent dispersal of the newly created *P. falciparum* required adaptation to *Anopheles gambiae*, which is the main human transmission vector (Molina-Cruz and Barillas-Mury, 2016). It will thus be important to determine to what extent ape *Laverania* parasites can productively infect *Anopheles* spp. that are more domesticated (Molina-Cruz and Barillas-Mury, 2016).

10. Zoonotic potential of ape parasites

Despite the identification of suitable bridge vectors, both experimental transmission and molecular epidemiological studies indicate that ape *Laverania* parasites do not normally cause blood stage infections in humans. Attempts to inoculate humans with a parasite identified as "*P. reichenowi*" over 100 years ago did not result in parasitemia (Blacklock and Adler, 1922). More importantly, two recent field studies conducted in rural Cameroon and Gabon failed to identify ape *Laverania* parasites in the blood of humans living in close proximity to infected chimpanzees and gorillas (Sundararaman et al., 2013; Delicat-Loembet et al., 2015). In contrast, ape *P. vivax* has been shown to cause clinical malaria in Duffy-positive humans, as exemplified by the case of a Caucasian male who acquired this infection after working for 18 days in a forest in the Central African Republic (Fig. 2B). Parasite sequences amplified from this individual's blood did not fall within the human *P. vivax* lineage, but instead clustered with parasites obtained from wild-living chimpanzees and gorillas (Fig. 4A), confirming acquisition by cross-species transmission from a wild ape (Prugnolle et al., 2013). Similarly, "*P. schwetzi*" was experimentally transmitted from apes to humans and must have represented ape *P. vivax* in at least some cases, since only Caucasians, but not African-Americans, became blood-stage infected, likely because the latter were Duffy-negative (Contacos et al., 1970). Together, these data indicate that ape *Laverania* parasites do not switch between host species, except under highly unusual circumstances, whilst ape *P. vivax* is much less host-specific and has the potential to infect Duffy-positive humans, suggesting that human and ape *P. vivax* parasites represent a single species. Although *P. praefalciparum* apparently crossed the species barrier to humans only once, it will be important to elucidate the host, vector and/or ecological barriers that have prevented additional ape *Laverania* transmissions. Since ape *P. vivax* is substantially more diverse than human *P. vivax*, it will be important to determine whether ape parasites are biologically more versatile. Moreover, emergence of ape *P. vivax* should be monitored in areas of Africa where an influx of Duffy-positive humans through commerce and travel coincides with forest encroachment and ape habitat destruction.

11. Natural history of ape *Plasmodium* infections

Although *P. falciparum* and *P. vivax* are highly pathogenic in humans, the disease causing potential of their ape relatives remains largely unknown. Given the high prevalence (Table 1) and wide distribution of both *Laverania* and *P. vivax* infections amongst chimpanzees and gorillas (Fig. 2), it is highly unlikely that they cause severe disease and malaria-related deaths in many animals. However, studies of habituated chimpanzees in the Tai National Forest in Cote d'Ivoire revealed higher faecal parasite burdens in both young (De Nys et al., 2013) and pregnant (De Nys et al., 2014) animals, similar to what has been described in humans in malaria endemic regions. Moreover, a recent report of an initially *Plasmodium* naive chimpanzee, who was introduced into a sanctuary where ape *Laverania* infections were endemic, showed that *P. reichenowi* can cause high parasitemia associated with fever and anaemia (Herbert et al., 2015). In contrast, other captive chimpanzees in African sanctuaries that tested positive for *Laverania* or *P. vivax* sequences in their blood or faecal samples, were asymptomatic and blood smear negative (Herbert et al., 2015; Sundararaman et al., 2016). Thus, it seems clear that *Plasmodium* infections can be pathogenic in apes; however, like humans, apes seem to develop resistance to life threatening malaria in areas of intense parasite transmission.

12. Conclusions and perspectives

Although ape *Plasmodium* parasites were first identified nearly 100 years ago, it is only very recently that the complexities of their evolutionary relationships, geographic distribution, prevalence rates, and mammalian host and vector associations have been elucidated. Whilst the evolutionary origins of human *P. falciparum* and *P. vivax* have now been clarified, nothing is known about the mechanistic processes that led to their emergence; yet, such information is critical to understanding how ape parasites crossed the species barrier and whether such events are likely to occur again. The lack of in vitro culture systems poses a significant challenge to the functional analysis of ape *Plasmodium* parasites, but whole genome sequencing, even from suboptimal specimens such as subpatently infected unprocessed blood, represents a critical first step towards understanding their biology (Otto et al., 2014; Sundararaman et al., 2016). Such analyses have already revealed a number of unexpected findings such as horizontal transfer of invasion genes amongst ape *Laverania* parasite species (Sundararaman et al., 2016). The genome sequences of additional parasites, in particular *P. praefalciparum* and ape *P. vivax*, will provide templates for mechanistic studies and in vitro genome manipulations to compare the function of key proteins amongst the various ape and human *Plasmodium* spp. Population genomic studies of ape *Laverania* parasites may also inform ongoing malaria vaccine development efforts by identifying antigens that are under strong immune selection pressure in apes as well as humans (Ochola et al., 2010; Amambua-Ngwa et al., 2012; Tetteh et al., 2013). In this context, it will be important to further characterise the transmitting vectors of ape *Plasmodium* parasites and assess to what extent humans are exposed to these parasites through the bites of infected mosquitoes. A careful analysis of antibody responses to preerythrocytic parasite stages could address this question. Finally, *P. ovale*- and

P. malariae-like sequences have been detected in African great apes (Duval et al., 2010; Kaiser et al., 2010; Krief et al., 2010; Liu et al., 2010a; Boundenga et al., 2015), and additional work is required to ascertain the relationship of these parasites to their human counterparts. In general, knowledge gained from comparative population and genomic studies of ape parasites will provide new insight into the biology and pathogenesis of human *P. falciparum* and *P. vivax*, and will inform malaria eradication efforts by identifying potential zoonotic threats.

Acknowledgements

We thank Richard Carter for helpful discussions and Shivani Sethi for artwork and manuscript preparation. This work was supported by grants from the National Institutes of Health, USA, R01 AI 091595, R01 AI 058715, R01 AI 120810, R37 AI 050529, T32 AI 007532, P30 AI 045008.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2016.05.008.

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