A New Malaria Agent in African Hominids

Benjamin Ollomo1,3*, Patrick Durand2,3, Franck Prugnolle2,4, Emmanuel Douzery3, Céline Arnathau2, Dieudonné Nkoghe1, Eric Leroy1,4, François Renaud2

1 Unité des Maladies Virales Émergentes, Centre International de Recherches Médicales de Franceville, Franceville, Gabon, 2 Laboratoire Génétique et Évolution des Maladies Infectieuses, UMR 2724 CNRS-IRD, IRD Montpellier, Montpellier, France, 3 Laboratoire de Paléontologie, Phylogénie & Paléobiologie, Institut des Sciences de l’Évolution (UMR 5554 CNRS), Université Montpellier II, Place E. Bataillon, Montpellier, France, 4 Unité Émergence des Pathologies Virales, UMR 190 IRD-Université de la Méditerranée, CIRMF, Franceville, Gabon

Abstract

Plasmodium falciparum is the single major human malaria agent responsible for 200 to 300 million infections and one to three million deaths annually, mainly among African infants. The origin and evolution of this pathogen within the human lineage is still unresolved. A single species, P. reichenowi, which infects chimpanzees, is known to be a close sister lineage of P. falciparum. Here we report the discovery of a new Plasmodium species infecting Hominids. This new species has been isolated in two chimpanzees (Pan troglodytes) kept as pets by villagers in Gabon (Africa). Analysis of its complete mitochondrial genome (5529 nucleotides including Cyt b, Cox I and Cox III genes) reveals an older divergence of this lineage from the clade that includes P. falciparum and P. reichenowi (~21 ± 9 Myrs ago using Bayesian methods and considering that the divergence between P. falciparum and P. reichenowi occurred 4 to 7 million years ago as generally considered in the literature). This time frame would be congruent with the radiation of hominoids, suggesting that this Plasmodium lineage might have been present in early hominoids and that they may both have experienced a simultaneous diversification. Investigation of the nuclear genome of this new species will further the understanding of the genetic adaptations of P. falciparum to humans. The risk of transfer and emergence of this new species in humans must be now seriously considered given that it was found in two chimpanzees living in contact with humans and its close relatedness to the most virulent agent of human malaria.

Introduction

Malaria is a major parasitic worldwide scourge, infecting and killing several million people each year [1]. Among the numerous Plasmodium species that infect reptiles, birds and mammals, four of them are human-specific: P. falciparum, P. vivax, P. malariae and P. ovale. The most virulent agent is P. falciparum, which kills up to three million people each year, mainly in Africa [1]. In spite of persistent control efforts set up since the end of the fifties, the disease is far from being under control. Even though numerous articles are published every year about the parasite and the disease, progress in controlling malaria has been limited. Resistance has evolved against virtually all drugs currently available [2], so that the disease frequently reemerges in different parts of the world [3,4].

The recent availability of complete Plasmodium genomes [5–9] has generated new hopes in the fight against this parasite. Thanks to their comparison we have now a far better understanding of their genomic architecture and of the genes that may help the parasite to escape the host immune response [5–10]. This approach remains unfortunately limited regarding the main malignant agent of malaria, P. falciparum. One problem is the lack of knowledge about other closely related apicomplexan models that can serve as reference and comparison [11]. At present, only one species, P. reichenowi, is known as a close sister lineage of P. falciparum [8,12,13]. Other Plasmodium species (P. rodhaini and P. schwetzi) were in the past described as parasites of the African great apes (i.e. chimpanzee and gorilla), but they were considered as closely related, for the first, to P. malariae and, for the second, to P. vivax [14] or P. ovale [15], which are very divergent from P. falciparum [12,13,16]. The development of comparative genomics for P. falciparum depends therefore on obtaining additional information about the diversity of P. reichenowi [17] and other Plasmodium species parasitic to the African Great Apes-Human lineage (the AGAH-lineage), currently represented by only two known species, P. falciparum and P. reichenowi.

In this manuscript, we report the discovery of a new Plasmodium species infecting Hominids in Africa. This new species was isolated from two chimpanzees and is a close relative of P. falciparum, the most virulent agent of human malaria.

Results/Discussion

To explore the diversity of species belonging to the Plasmodium AGAH-lineage in Africa, we collected blood samples from 17 chimpanzees recently trapped from the wild and kept as pets in...
Author Summary

In 2002, the publication of the genome of Plasmodium falciparum, the most malignant agent of malaria, generated hopes in the fight against this deadly disease by the opportunities it offered to discover new drug targets. Since then results have not lived up to the expectations. The development of comparative genomics to further understanding of P. falciparum has indeed been hindered by a lack of knowledge of closely related species’ genomes. Only one species, P. reichenowi, infecting chimpanzees, was hitherto known as a sister lineage of P. falciparum. Here we describe a new Plasmodium species infecting chimpanzees in Africa. Based on its whole mitochondrial genome, we demonstrate that this species is a relative of P. falciparum and P. reichenowi. The analysis of its genome should thus offer the opportunity to explore P. falciparum specific adaptations to humans. Our results bring new elements to the debate surrounding the origin of this lineage. They suggest that it may have been present in early hominoids and may have experienced a radiation congruent with that of its hosts. Our discovery highlights the paucity of our knowledge on the richness of Plasmodium species infecting primates and calls for more research in this direction.

villages of Gabon by hunters and their families [see Figure S1]. Considering that only the subspecies Pan troglodytes troglodytes has been found in Gabon, these 17 animals are likely to belong to this chimpanzee subspecies.

Among them, two were found to be infected with Plasmodium by means of PCR assay or microscopy. The other 15 animals were found negative both by microscopy and PCR assay. For these two chimpanzees (named B and K), observed parasites under microscopy were falciparum-like (ring stages with two chromatin dots and presence of multiply-infected red blood cells [15]). Thick blood smears revealed low parasitemia in both individuals, approximately 300 parasites/μl for chimpanzee B and 2000 parasites/μl for chimpanzee K.

For both, we amplified and sequenced the parasite’s Cytochrome b (Cyt b) gene. The Cyt b sequences obtained were similar between the two samples (identity of 99.8% based on 866 nucleotides (nt)), but different from all other Plasmodium Cyt b sequences known to date. The most similar sequences obtained using BLAST were Cyt b sequences from P. reichenowi and P. falciparum, which show 92% and 91% identity, respectively.

Because the Cyt b sequences were partial, we studied the whole mitochondrial DNA (mtDNA) of these two new isolates (named P. sp_K and P. sp_B). For isolate K, we amplified 5529 nt including three main genes: Cytochrome oxidase I (Cox I), Cytochrome oxidase III (Cox III) and Cytochrome b (Cyt b). Apart from short missing segments amounting to 420 nt, the mtDNA sequenced corresponds to the whole P. falciparum 3D7 mtDNA (5949 nt). For technical reasons (certainly due to the very low parasitemia and degraded DNA), we were unable to accomplish this sequencing for isolate B.

In order to determine the evolutionary relationships of this new Plasmodium relative to other species, we compared its sequence to 17 known complete Plasmodium mitochondrial genome sequences, with the bird apicomplexan parasite Leucocytozoon caulleryi as an outgroup [see Table S1]. Maximum likelihood (ML) phylogenetic trees were reconstructed at both the nucleotide and amino acid levels on the whole mitochondrial genome sequence, considered as a single genetic unit [18].

DNA and protein analyses provided identical results; the parasite collected in chimpanzee K belongs to the AGAH-lineage but is more divergent from P. falciparum than is P. reichenowi (Figure 1; see also Figure S3 for the tree reconstructed from the partial Cyt b sequence (866 nt) and including both P. sp_K and P. sp_B). Over the entire mitochondrial genome, the genetic distance observed between the new taxon (P. sp_B) and P. falciparum (d = 0.213 substitutions per nucleotide site on the ML phylogram) or P. reichenowi (d = 0.215) is almost four times higher than the distance observed between P. falciparum and P. reichenowi (d = 0.058).

To estimate the divergence time of the plasmodium AGAH-lineage, we used a calibration chosen within the hominid hosts. The age of the P. falciparum/P. reichenowi split is generally considered to be similar to the one separating humans from chimpanzees [12,13,19], that is, between four and seven million years [20–22]. Because of pervasive variations of mitochondrial substitution rates among malaria parasite lineages (Figure 1), a Bayesian relaxed molecular clock was used, which revealed a divergence time of 21±9 Myrs between the new Plasmodium species and the clade constituted by P. falciparum and P. reichenowi. Interestingly, this estimated time frame fits with the radiation of hominoids during the Miocene [23]. Our results suggest therefore that the plasmodium AGAH-lineage may have been present in early hominoids [23] and that this lineage may have also experienced a diversification during the early Miocene period as it occurred for their hosts [23]. Obviously, this estimated time of divergence is dependent on the calibration used. Recently, Martin and colleagues [24] suggested that the split between P. falciparum/ P. reichenowi might have occurred far earlier than previously considered. They propose that P. falciparum originated from a recent transfer of P. reichenowi to humans during the last 2.8 Myrs [25]. Under this hypothesis, the new species would have diverged from P. falciparum/P. reichenowi about 10 Myrs ago. As divergence data are lacking for those parasites from the fossil record, we are unable to distinguish between these two hypotheses. Further data on the diversity of Plasmodium infecting great apes in Africa will certainly help resolve this particular aspect of the evolution of P. falciparum.

In conclusion, we bring to light the existence of a new Plasmodium species that infects chimpanzees in Gabon. We propose to name this new species Plasmodium gaboni sp. nov. in reference to the country where we obtained it. Our discovery suggests that great apes and perhaps simian primates may host a far higher diversity of Plasmodium species in Africa than previously recognised. Beyond the interest of this new species in the understanding of the evolution of this group of parasites, its position in the AGAH-lineage as the sister-group of P. falciparum/P. reichenowi opens up the possibility of exploring lineage-specific evolution using comparative genomics, and hence, to look for the genes responsible for the adaptation of these parasites to their specific hosts. Comparison between genomes will advance understanding of the differences in pathology and the processes at work in the interaction with the vertebrate or the mosquito hosts [9,17]. It is thus essential to complete the nuclear genome sequence of this new species of phylogenetic importance within the AGAH-lineage, in order to enhance our knowledge of the functional genomics of human malaria parasites.

Finally, this new species was discovered in two chimpanzees conserved as pets by villagers in Gabon. Given the recent history of primate to human shifts in several pathogens (e.g. HIV [26]; Ebola [27]; for Plasmodium, the most recent involved P. knowlesi and occurred from macaques to humans in Asia [28]) and the close proximity between P. gaboni and the most virulent agent of malaria,
P. falciparum, we think that the risk of transfer of this species to humans must be seriously considered.

Materials and Methods

Origin of parasite samples, microscopic analyses and DNA extraction

Blood aliquots of 17 chimpanzees were collected from different parts of Gabon (Figure S1). The samples were collected from wild-born animals kept as pets by hunters and their families. The investigation was approved by the Government of the Republic of Gabon and by the Animal Life Administration of Libreville, Gabon (no. CITES 00956). All animal work has been conducted according to relevant national and international guidelines. Blood samples were collected in 7 ml EDTA vacutainers from chimpanzees under ketamine anaesthesia. Clots and plasma were obtained by centrifugation and stored at $-20\degree C$ until they were transported to the Centre International de Recherches Médicales de Franceville (CIRMF), Gabon, where they were stored at $-80\degree C$ until processed for testing. Total DNA (Plasmodium and host) was isolated and purified using the DNeasy blood kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The DNA was eluted in 100 µl of sterile water.

Microscopic analyses of the blood samples by thin blood smears revealed that two chimpanzees were infected by falciparum-like parasites (ring stages with two chromatin dots and presence of multiply-infected red blood cells [15]). The two infected individuals were respectively a young chimpanzee (Pan troglodytes, male of 3 years old) from the area of Bakoumba ($1^\circ28'0" S/13^\circ0'0" E$, Haut-Ogooué Province) and a young chimpanzee (Pan troglodytes, male of 2–3 years old) from the village of Koulamoutou ($1^\circ23'59" S/12^\circ13'0" E$, Ogooué-Lolo Province) (Figure S1). The two Plasmodium isolates were named P. sp_B and P. sp_K, respectively.

In both chimpanzees (named B and K), parasitemia were estimated using thick blood smears. The number of parasites per microliter was estimated using the ratio of the number of observed parasites by the number of observed lymphocytes. To extrapolate to the number of parasites per microliter, we considered that there were about 8000 lymphocytes per microliter of whole blood. The infection status of the two chimpanzees was then confirmed by PCR of the Cytochrome b gene (see below for PCR conditions).

PCR conditions

To obtain whole mitochondrial genome sequences from P. sp_B and P. sp_K, seven primer pairs were designed based on the mitochondrial genome sequence of Plasmodium falciparum (GenBank accession number AY282930) using Primer 3 (v. 0.4.0) [29] and eight primer pairs already published [30] (Table S2). PCR amplification and sequencing were performed on seven and eight overlapping regions covering a complete linear copy of the mtDNA genome, respectively. The Cytochrome oxydase I (Cox I), the
Alignment and analysis of mitochondrial genomes

For P. sp_B, we were only able to amplify and sequence a part of Cyt b (866 nt) (deposited in the GenBankTM Database under the accession number FJ895308). For P. sp_K, sequences obtained from all primer datasets were aligned and compared using ClustalW [v 1.8.1 in BioEdit v.7.0.9.0 software [33]] and a mtDNA consensus sequence of P. sp_K was created (GenBank Accession number FJ895307; see Figure S2).

In comparative analyses, we used 17 previously published mitochondrial genome sequences from P. falciparum, P. reichenowi, P. gallinaceum, P. falciparum, P. kraziele, P. simiovale, P. simiovale, P. vivax, P. cynomolgus, P. yoelii, P. berghei, P. chabaudi, P. malariae, P. gonderi, and P.sp. DAJ-2004, with Leucocytozoon caulleryi, an avian malaria parasite used here as outgroup. Hosts and GenBank accession numbers for these taxa are given in the Table S1. The multiple alignment of the 18 sequences was conducted using ClustalW (see e.g. Figure S2).

Phylogenetic relationships between mtDNA haplotypes (for whole mitochondrial genome) were inferred from all codon positions and non-coding regions. Non-sequenced sites and sites with gaps (or missing sites) (when gaps were present in more than 5% of the species) were removed, yielding a total of 5,805 sites available for subsequent inferences.

Maximum Likelihood (ML) tree reconstruction was conducted from the whole mitochondrial genome. For this, Cyt b, Cox I, Cox III, and non-coding sequences were concatenated and analysed under a single model of nucleotide or amino acid evolution. The best-fitting ML model under the Akaike Information Criterion was GTR (General Time Reversible) + F (Gamma distribution) + I (Invariable sites’s distribution) for nucleotides as identified by ModelTest [34] and mtART (replacement matrix developed for arthropod mitochondrial proteins) + F + I for amino acids as identified by ProtTest [35]. The highest-likelihood DNA and protein trees and corresponding bootstrap support values were obtained by PhyML (freely available at the ATGC bioinformatics platform http://www.atgc-montpellier.fr/) using NNI (Nearest Neighbor Interchange)+SPR (Subtree Pruning Regrafting) branch swapping and 100 bootstrap replicates [36].

Estimation of divergence time

The ML analysis evidenced pervasive variations of mitochondrial DNA substitution rates among malaria parasite lineages. In this context, we used a Bayesian relaxed molecular clock approach to estimate the divergence times of Plasmodium species. The log-normal rate-autocorrelated model [37] was adopted to relax the molecular clock hypothesis as it has been shown to reasonably fit various data sets [38]. We assumed a calibration interval of 4–7 Myrs for the split between P. falciparum and P. reichenowi [12,19] to reflect the one among their human and chimpanzee hosts [20–22]. Dating estimates were computed by the Bayesian procedure implemented in the PhyloBayes software [38,39], version 3.0 (http://www.atgc-montpellier.fr/phylobayes/), with a uniform prior on root age and divergence times. We used the CAT Dirichlet process with the number of components, weights and profiles all inferred from the ML topology, with a general time reversible (GTR) matrix of exchangeability among nucleotides, and a 4-category discrete Gamma (Γ) distribution of substitution rates across sites. Two independent MCMC runs were conducted for 1,000,000 generations, with sampling every 10 cycles. After a burn-in of 100 cycles, divergence times were computed, and were virtually identical for the two chains.

Supporting Information

Figure S1 Location of the 17 sampled chimpanzees (Pan troglodytes) in Gabon. Each circle represents a unique sample. The fifteen uninfected chimpanzees are shown in yellow and the two infected ones in red. These latter two were collected in the villages of Koulamoutou (Ogooué-Lolo province) and Bakombou (Haut Ogooué province), respectively. Found at: doi:10.1371/journal.ppat.1000446.s001 (4.88 MB TIF)

Figure S2 Multiple sequence alignment of the whole mitochondrial DNA of the three species P. falciparum (3D7 strain), P. reichenowi and P. sp_K using CLUSTAL W (v. 1.81). A dot indicates an identical nucleotide and a dash indicates a gap compared to the P. falciparum reference sequence. Degenerate nucleotides as follows: W = AT, Y = CT, K = GT, M = AC. Found at: doi:10.1371/journal.ppat.1000446.s002 (7.36 MB TIF)

Figure S3 Phylogenetic relationships among Plasmodium species (including P. sp_K and P. sp_B). The phylogram presented here was reconstructed by a Maximum Likelihood approach from partial Cyt b DNA sequence data (866 nt). Bootstrap values obtained are only shown (in blue) for the nodes inside the African Great Apes - Human lineage [represented in red]. Leucocytozoon caulleryi was used as outgroup. Scale bar shows 0.09 substitutions per site. Found at: doi:10.1371/journal.ppat.1000446.s003 (1.87 MB TIF)

Table S1 Parasite species used in this study with GenBank accession numbers and a description of their natural hosts.
Table S2  Amplification primers of the mitochondrial genome. Primers used in this study for the amplification of the whole mitochondrial genome and the three genes (Cox I, III and Cyb) separately for P. falciparum and P. vivax based on the mitochondrial genome sequence of P. falciparum 3D7 strain (GenBank acc. no. AY282930). The fragment sizes are also estimated from the P. falciparum 3D7 sequence.

Author Contributions
Conceived and designed the experiments: BO PD CA. Analyzed the data: PD FP ED FR. Contributed reagents/materials/analysis tools: BO DN EL. Wrote the paper: PD FP FR.

Acknowledgments
The authors thank Alison Ducan, Francisco Ayala, Ana Rivero, Yannis Michalakis, Fred Andayi and Anne-Laure Bañuls for critical reading of a previous version of the manuscript and Jean-Pierre Hugot and Michel Brunet for helpful discussions. Authors also thank Xavier Pourrut, Philippe Yaba, André Délicat, Paul Ngari and Philippe Enguendja for technical support in the field. The contribution of EJPD is the one N° 2009-046 of the Institut des Sciences de l’Evolution de Montpellier (UMR 5554 - CNRS).

References
4. Jeffares DC, Pain A, Berry A, Cox AV, Stalker J, et al. (2007) Genome variation in malaria parasites: evidence from the circumsporozoite protein gene. Mol Biol Evol 24: 12819–12824.
5. Conway DJ, Fanello C, Lloyd JM, Al-Joubori BMAS, Baloch AH, et al. (2000) A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. J Parasitol 86: 972–978.
6. Schwobel B, Alifrangs M, Salanti A, Jelinek T (2003) Different mutation patterns of atovaquone resistance to Plasmodium falciparum in vitro and in vivo: rapid detection of codon 268 polymorphisms in the cytochrome b gene sequences. J Parasitol 89: 792–797.
7. Thorne JL, Kishino H, Painter IS (1998) Estimating the rate of evolution of the human immunodeficiency viruses: SIV infection in wild gorillas. Nature 444: 164–164.
8. Leroy EM, Rouquet P, Formenty P, Souquiere S, Kilbourne A, et al. (2004) Multiple Ebola virus transmission events and rapid decline of central African wildlife. Science 303: 397–399.
9. Vythilingam I, NoorAzian YM, Huat TC, Jram AI, Yusri YM, et al. (2008) Plasmodium knowlesi in humans, macaques and mosquitoes in peninsular Malaysia. Parasit Vectors 1: 26.
10. Rozen S, Skaltsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa, NJ: Humana Press. pp 363–386.
11. Conway DJ, Fanello C, Lloyd JM, Al-Joubori BMAS, Baloch AH, et al. (2000) Origin of Plasmodium falciparum malaria is traced by mitochondrial DNA. Mol Biochem Parasitol 111: 163–171.
12. Perkins SL, Schall JJ (2002) A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. J Parasitol 88: 972–978.
13. Schwobel B, Alifrangs M, Salanti A, Jelinek T (2005) Different mutation patterns of atovaquone resistance to Plasmodium falciparum in vitro and in vivo: rapid detection of codon 268 polymorphisms in the cytochrome b gene sequences. J Parasitol 86: 972–978.
14. Hall N, Karras M, Raine JD, Carlson JM, Kooij TWA, et al. (2005) A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. Science 307: 82–86.
15. Weedall GD, Polley SD, Conway DJ (2000) Gene-specific signatures of elevated non-synonymous substitution rates correlate poorly across the Plasmodium genus. PLoS ONE 5: e2281. doi:10.1371/journal.pone.0002281.
16. Escalante AA, Barrio E, Ayala FJ (1993) Evolutionary origin of human and primate malaria: evidence from the circumsporozoite protein gene. Mol Biol Evol 12: 616–626.
17. Garnham PGC (1966) Malaria Parasites and Other Haemoparasita. Oxford: Blackwell Scientific Publications. 1114 p.
18. Goonetilleke N, Skaltsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa, NJ: Humana Press. pp 363–386.
19. Rozen S, Skaltsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa, NJ: Humana Press. pp 363–386.