Evolutionary relationships between 15 *Plasmodium* species from New and Old World primates (including humans): a 18S rDNA cladistic analysis

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SUMMARY

We present a new phylogenetic analysis of 15 primate *Plasmodium* species based on 18S rDNA sequences including new sequences of *Plasmodium coatneyi*, *P. fieldi*, *P. gonderi*, *P. hylobati* and *P. simium*. The results are discussed in the context of the parasite host species and their geographical distribution. Contrary to other phylogenies constructed with this 18S rDNA molecule, we observed that the topology of phylogenetic trees was not affected either by the quality of the nucleotide matrices, or by the species present in the outgroup. This analysis showed the following. (1) The polyphyly of human *Plasmodium* is confirmed. (2) The monophyly of *Plasmodium* from Old World monkeys is confirmed by the new added sequences and *P. gonderi*, an African species, possibly could be at the root of this group. (3) The most parsimonious biogeographical hypothesis is that *P. vivax* originated in Asia; thus, its related species *P. simium* appears to be derived through a transfer from the human *P. vivax* to New World monkey species in South America. (4) Sampling efforts of non-human primate *Plasmodium* could permit improvement of the knowledge of primate *Plasmodium* phylogeny and also consideration of the risks of malaria emergence from monkey reservoirs.

Key words: primate *Plasmodium*, 18S rDNA, cladistic analysis.

INTRODUCTION

Environmental disturbances are creating the opportunities for microbes and parasites to colonize new ecological niches and thus increasing the risk for new pathogens to emerge. In this context, molecular phylogenies are important for understanding the evolution of pathogenicity since they permit determination of whether virulence in a given taxa is genetically inherited. Although sequences of the whole genomes are available for *Homo sapiens*, *Anopheles gambiae* and *Plasmodium falciparum* (Holt *et al*. 2002; Garner *et al*. 2002), all involved in the life-cycle of 1 of the 4 human malaria species, the origin of the 4 human *Plasmodium* spp. is still far from being solved and is controversial (Waters, Higgins & McCutchan, 1991, 1993; Escalante & Ayala, 1994; Quari *et al*. 1996; Escalante *et al*. 1997; Rathore *et al*. 2001). This group of malaria parasites includes at least 172 known species, parasites of birds, reptiles, rodents and primates. Four are human parasites. *P. falciparum*, which causes acute infections and is responsible for clinical infections in 500 million people and 1·5 million deaths per year, mainly in Sub-Saharan Africa (WHO, 1998). *P. vivax* and *P. ovale* cause acute infections and are also implicated in relapsing infections. *P. vivax* is responsible for 75 million acute episodes per year mainly in Asia and South America (Sina, 2002), while *P. ovale* is rare. *P. malariae* is involved in chronic infections that may persist with low parasitaemia for many years without causing true relapses (Carnevale *et al*. 1984).

Many descriptions of malaria parasites in African and Asian monkeys made during the first half of the 20th century were re-examined. In his book on malaria parasites and other Haemosporidia, Garnham (1966) gave details on primate *Plasmodium* but, since this work, very few observations have been added. Poirriez *et al*. (1995) noted that before 1993 only 1 species of *Plasmodium* (*P. gonderi*) was known for African monkeys in the Cercopithecidae family; these authors described 2 more species (*P. georgesi* and *P. petersi*) in the same primate family.

Currently, 22 species are recognized as non-human primate *Plasmodium* (Gysin, 1998): 2 species

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(*P. brasilianum*† and *P. simium*†) occur in the New World monkeys of the Cebidae family; 11 (*P. coatneyi*†, *P. cynomolgi*†, *P. fieldi*†, *P. fragile*†, *P. gonderi*†, *P. georgesi*, *P. inui*†, *P. knowlesi*†, *P. petersi*, *P. shortti* and *P. simiovale*†) in the Old World monkeys of the Cercopithecidae family; 4 (*P. eylesi*, *P. hylobati*†, *P. jefferyi*, *P. youngi*) in gibbons of the Hylobatidae family (localized in South East Asia), and 5 in the great apes *Pan troglodytes* and *Gorilla gorilla* in West and Central Africa (*P. reichenowi*†, *P. rodhaini*, *P. schwetzi*) and *Pongo pygmaeus* in South Asia (*P. pitheci* and *P. silvaticum*). Unfortunately, only 12 of these taxa are available from infected blood samples in the American Type Culture Collection (ATCC). Another *Plasmodium* sp. found in *Mandrillus* (African Cercopithecidae) was not available and has never been taxonomically described. Consequently, genotyping characterization and molecular phylogenetic studies are unfortunately restricted to these taxa†. This could be the main reason why our knowledge of primate *Plasmodium* evolutionary relationships is so limited. Another reason could be the choice of the molecular markers used. Phylogenies established from cytochrome b (Escalante *et al*. 1998; Rathore *et al*. 2001; Ricklefs & Fallon, 2002; Perkins & Schall, 2002) are constructed from only a few informative sites, as discussed by Perkins & Schall (2002). The phylogenies obtained from 18S rDNA (Waters *et al*. 1991, 1993; Escalante & Ayala, 1994; Quari *et al*. 1996; Escalante *et al*. 1997; Rathore *et al*. 2001) depend not only on the species included in the ingroup and the outgroup, but also on the sequence alignment.

In this paper, using a parsimonious cladistic phylogenetic analysis from 18S rDNA sequences, we present a phylogeny of primate *Plasmodium* including the 10 primate *Plasmodium* sequences available in GenBank and 5 new primate *Plasmodium* sequences, *P. coatneyi*, *P. fieldi*, *P. gonderi*, *P. hylobati*, *P. simium*. We tested the impact of two matrices of alignment data and of several outgroup species on the phylogenetic results. We also discuss the biogeography and the possible evolution of these parasite taxa as a function of the geographical distribution of their hosts.

MATERIALS AND METHODS

Parasite samples

Several *Plasmodium* species (*P. berghei*, *P. falciparum*, *P. vivax*) have been found to contain 2 or 3 distinct copies of 18S rDNA genes, expressed in a specific stage of the life-cycle (Gunderson *et al*. 1987; McCutchan *et al*. 1988; Li *et al*. 1997). The A ribosome type occurs in infected erythrocytes and corresponds to the trophozoite stage of the parasite; the S ribosome type occurs in the sporozoite stage, while the O ribosome type occurs in the oocyst stage of the parasite. The 18S rDNA A type (trophozoite stage) sequences are the most available in GenBank and phylogenies were built from this sequence type.

The GenBank accession numbers of 15 sequences (18S rDNA type A) and isolate origins are presented in Table 1. The *P. simiovale* species (from *Macaca sinica*), a proximate species to *P. vivax* could not be supplied by ATCC because of the risk of human infection.

Although 3 sequences of *P. knowlesi* have already been published, we sequenced a new one because there were notable differences between the two A type sequences referenced in GenBank (Accession numbers U83876 and U72542). Sequence U83876 was close to 3 sequences of the sporozoite stage (i.e. S type) of *P. vivax* and may correspond to a sequence of the sporozoite stage of *P. knowlesi*. For the same reason, we sequenced a new isolate of *P. simium*. The U69605 sequence referenced in GenBank as a sequence of the trophozoite stage of *P. simium* was also close to the 3 sequences of the sporozoite stage of *P. vivax*. We also included the sequence of *P. vivax* Belem type, which is a reference strain.

DNA isolation and PCR amplification

For the 7 isolates (*P. coatneyi*, *P. fieldi*, *P. gonderi*, *P. hylobati*, *P. knowlesi*, *P. simium* and *P. vivax* Belem type), DNA was isolated and purified using the QIAamp DNA Blood Kit following the manufacturer's instructions (Qiagen, CA). Amplification of 18S rDNA was performed using 2 genus-specific primers employed by Snounou *et al*. (1993), which give PCR products around 1050 bp long: rPLU6 5′TTAAAATTGTTGCAGTTAAAACG-3′ and rPLU5 5′CCTGTTTGTTGCCTTAAACTTC-3′. PCR was performed in a reaction mixture of 20 μl containing around 20 ng of genomic DNA, 1X reaction buffer, 2·5 mM $MgCl_2$, 80 μM of each deoxynucleotide triphosphate, 6 pmol of each primer and 1·3U of *Taq* polymerase (Promega). The amplification cycle involved a denaturation step of 2 min at 94 °C, followed by 35 cycles of denaturation 1 min at 94 °C, 1 min of annealing at 48 °C and 1 min of extension at 70 °C and a final elongation of 2 min. The PCR products were sequenced using an ABI PRISM 377 sequencer (Genaxis, Nîmes, France). The direct sequencing of *P. gonderi* PCR product produced a mixed sequence of the 2 types of 18S rDNA (trophozoite and sporozoite types). The *P. gonderi* PCR product was thus cloned into pGEM-Teasy vector (Promega) (Biofidal, Vaulx en Velin, France), previous to sequencing. The *P. gonderi* sequence was not fully obtained due to the likely presence of a recombinant site causing the loss of the first 300 bp at

† Taxa available from ATCC (American Type Culture Collection).

Table 1. Origin and reference numbers (ATCC and GenBank Accession number) of the *Plasmodium* species

(*From, Garnham (1966) and Gysin (1998).)

Plasmodium spp.	18S GenBank Accession number	Isolate source	Hosts (species and family)*
Plasmodium of primates			
P. brasilianum	AF130735		*Alouatta sp.* (4 taxa), *Ateles sp.* (7 taxa), *Aotus sp.*, *Brachyteles arachnoides*, *Callicebus sp.* (2 taxa), *Cebus sp.* (5 taxa), *Chiropetes chiropetes*, *Lagothrix sp.* (3 taxa), *Saimiri sp.* (2 taxa), **Cebidae**
P. coatneyi	AY579420	ATCC 30128	*Macaca fascicularis*, **Cercopithecidae**
P. cynomolgi	L07559		*Macaca arctoides*, *M. cyclopis*, *M. fascicularis*, *M. nemestrina*, *M. mulatta*, *M. radiata*, *M. sinica*, *Presbytis cristatus*, *P. entellus*, **Cercopithecidae**
P. falciparum	M19172		*Homo sapiens*, **Hominidae**
P. fieldi	AY579419	ATCC 30163**T**	*Macaca fascicularis*, *M. Nemestrina*, **Cercopithecidae**
P. fragile	M61722		*Macaca radiata*, *M. sinica*, **Cercopithecidae**
P. hylobati	AY579421	ATCC 30154	*Hylobates moloch*, **Hylobatidae**
P. gonderi	AY579416	ATCC 30045	*Cercocebus atterimus*, C. atys, C. galeritus agilus, Mandrillus leucophaeus, **Cercopithecidae**
P. inui	U72541		*Cynopithecus niger*, *Macaca cyclopis*, *M. fascicularis*, *M. mulatta*, *M. nemestrina*, *M. radiata*, *Presbytis cristatus*, *P. obscurus*, **Cercopithecidae**
P. knowlesi	AY579417	ATCC 30191	*Macaca Fascicularis*, *M. Nemestrina*, *Presbytis malalophus*, **Cercopithecidae**
P. malariae	M54897		*Homo sapiens*, **Hominidae**
P. ovale	L48987		*Homo sapiens*, **Hominidae**
P. simium	AY579415	ATCC 30130	*Alouatta fusca*, *Ateles sp.*, *Brachyteles arachnoides*, **Cebidae**
P. vivax	AY579418	I. Pasteur, Paris	*Homo sapiens*, **Hominidae**
P. reichenowi	Z25819		*Pan troglodytes*, *Gorilla gorilla*, **Great apes**, **Hominidae**
Plasmodium of birds			
P. gallinaceum	M61723		*Gallus gallus* and jungle fowl, **Phasianidae**
P. juxtanucleare	AF159790		*Gallus lafayettei* and jungle fowl, **Phasianidae**
P. lophurae	X13706		*Lophura ignita*, **Phasianidae**
Plasmodium of lizards			
P. mexicanum	L11716		*Scleroporus ferraripezi*, *S. horridus*, *S. microlepidotus*, *S. pyrocephalus*, *S. variabilis*, *S. torquatus torquatus*, ***Ignanidae***
Plasmodium of rodents			
P. berghei	M14599		*Thamnomys surdaster*, *Praomys jacksoni*, *Leggada bella*, **Muridae**
P. yoelii	AF180727		*Thamnonys rutilans*, **Muridae**

the 5′ end during the PCR product cloning step. Consequently, the sequences's length used for the phylogenetic analyses was adjusted according to the *P. gonderi* sequence (689 base pairs).

Phylogenetic analyses

Sequences were aligned using the CLUSTAL X program (Thompson *et al*. 1997) with editing and management using the MUST package (Philippe, 1993). Sequence alignment was manually improved according to criteria proposed by Barriel (1994) in order to minimize the mutation number and reduce the phylogenetic effects. For the analyses, 2 sequence data sets were taken into account, the whole sequences and only sequences without ambiguous alignments. Alignments are available from the authors on request. The most parsimonious trees

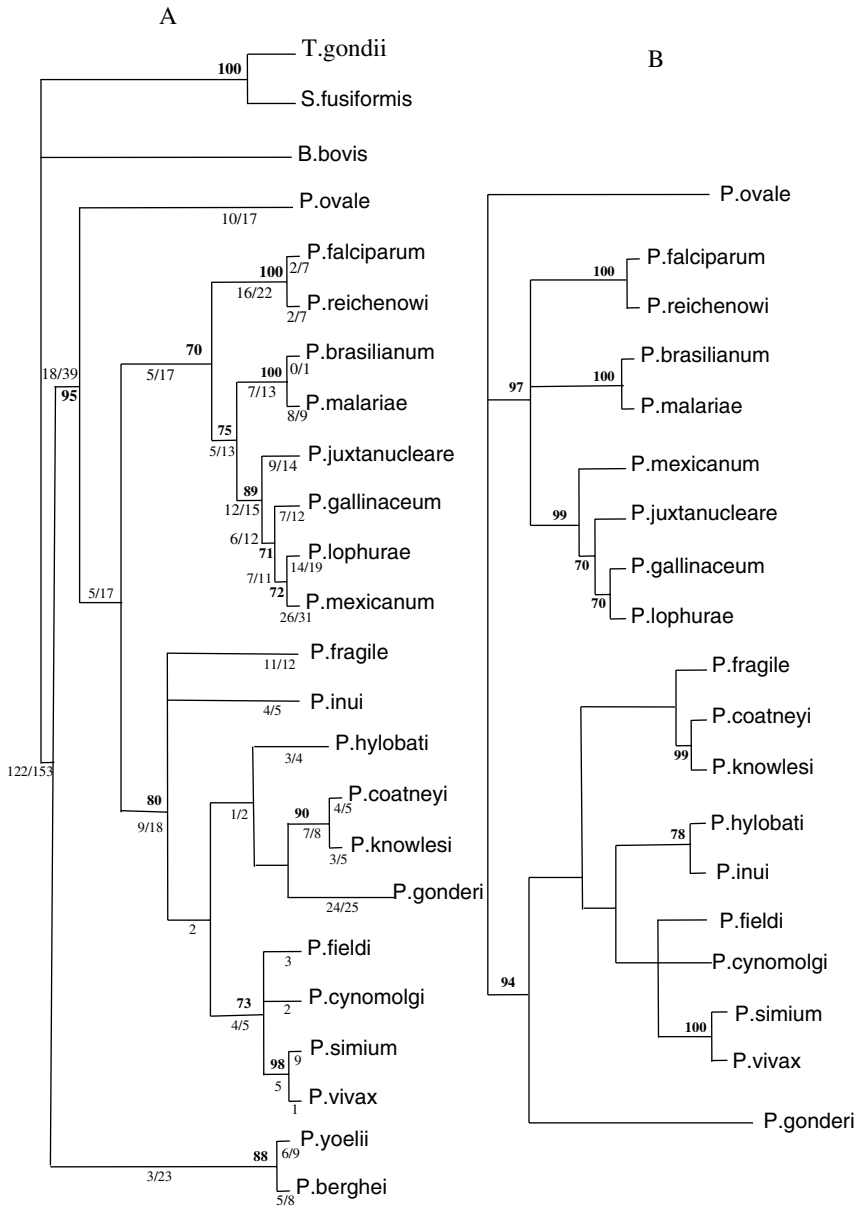

Fig. 1. (A) Strict consensus tree of the 3 most parsimonious trees obtained from 584 aligned positions of the SSU rDNA of 21 *Plasmodium* species. In bold the bootstrap values over 70% (based on 10 000 replicates). Other numbers on the branches indicate the minimal/maximal number of synapomorphies and autapomorphies according to the optimization of informative sites. (B) Branch topologies characterizing the strict consensus tree obtained with 705 aligned sequences, only topologies that differ from (A) are shown.

were calculated using the heuristic search of PAUP 4 (Swofford, 1999) with simple stepwise addition, TBR (tree bisection-reconnection) branch swapping and branches with maximum length zero collapsed to yield polytomies. Tree robustness was determined in terms of bootstrap (BS) proportions (Felsenstein, 1985) with 10 000 replicates. The 3 outgroup species used in the analysis belong to the Apicomplexa phylum to which *Plasmodium* also belongs: *Babesia bovis* in the class Hematozoa, the same class as *Plasmodium* and *Sarcocystis fusiformis* and *Toxoplasma gondii* in the class Coccidia. The GenBank Accession numbers of their 18S rDNA sequences are L19077, U03071, U87145 respectively. The data concerning the primate *Plasmodium* host species and their geographical distribution were obtained from Garnham (1966), Collins & Aikawa (1993), Poirriez *et al*. (1993) and Gysin (1998).

RESULTS

The total length of the aligned sequences without ambiguous alignments was 584 positions of which 212 were informative for parsimony. The PAUP analysis yielded 3 most parsimonious trees 561 steps in length, with a consistency index of 0·7219 and a retention index of 0·7997. The strict consensus tree is presented in Fig. 1A. The total length of the aligned

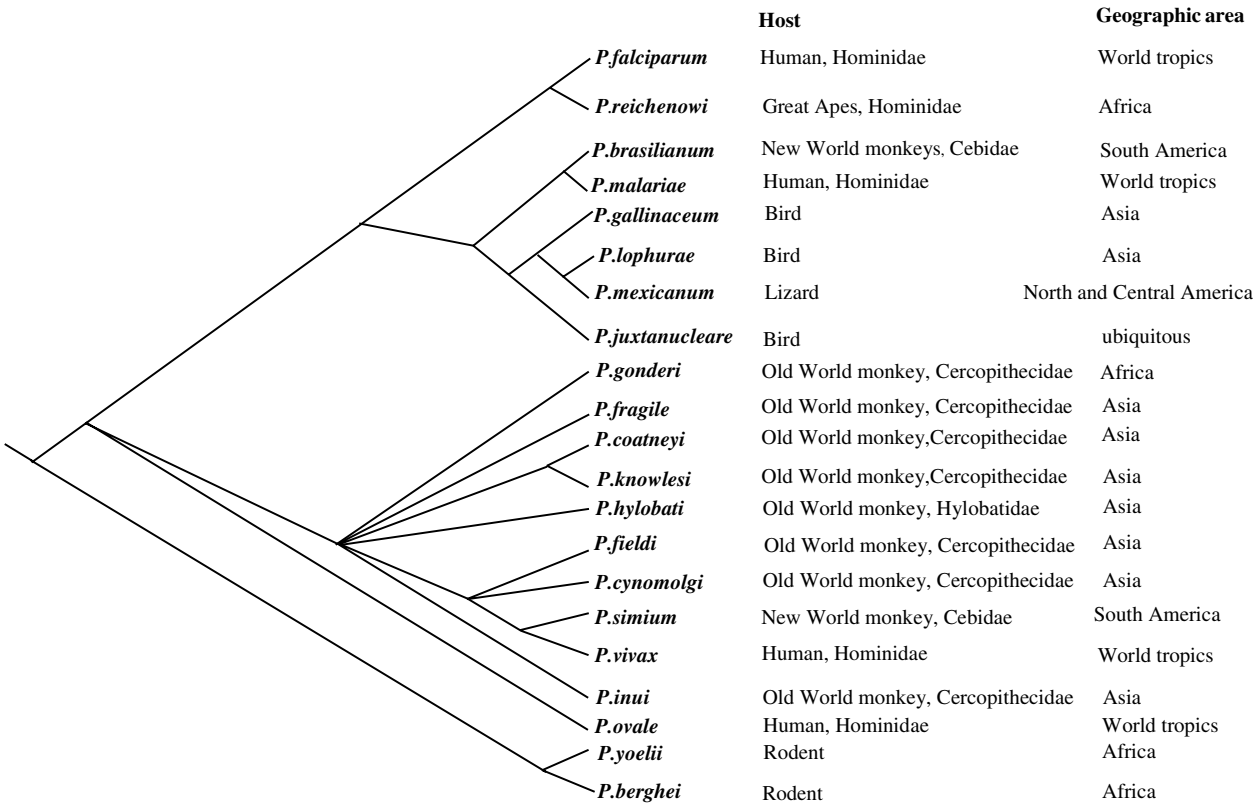

Fig. 2. Phylogenetic relationships of 21 *Plasmodium* lineages. For each *Plasmodium* species, their host species (primates, birds, lizard or rodents) and their geographical distribution are shown. In the tree, only nodes that were sustained by a BS value over 70% in Fig. 1A are shown.

sequences with all positions was 705 positions of which 319 were relevant for a cladistic analysis. The PAUP analysis yielded 8 most parsimonious trees, 991 steps in length, with a consistency index of 0·6697 and a retention index of 0·7588. No illustration is provided of the whole tree but only of the branch topology (Fig. 1B), which differs from the consensus tree obtained previously (Fig. 1A). Whatever the number of taxa included in the outgroup, the phylogenies obtained were the same.

Fig. 1A shows the topology of a cladistic tree (i.e. the most parsimonious tree among 3 solutions). Clearly, the rodent *Plasmodium* species (*P. berghei* and *P. yoelii*) are outside all other *Plasmodium* (with 95% of BS value and 18/39 synapomorphic characters). The primate *Plasmodium* are polyphyletic and split into 3 major groups. The relative positions of these 3 groups are not resolved (in term of BS value). *P. ovale* constitutes one of these groups. The second group (80% of BS, and 9/18 synapomorphic characters) includes 7 *Plasmodium* species (*P. coatneyi*, *P. cynomolgi*, *P. fieldi*, *P. gonderi*, *P. fragile*, *P. inui*, *P. knowlesi*) infecting Cercopithecidae from Asia except *P. gonderi* which parasitizes Cercopithecidae from Africa, one *Plasmodium* species (*P. hylobati*) infecting Hylobatidae from Asia, and *P. vivax* human *plasmodium* which is closely related to *P. simium*, a species of New World monkeys. Inside this second group, the relative positions of *P. fragile*, *P. gonderi*, *P. hylobati*, *P. inui*, (*P. coatneyi*, *P. knowlesi*), (*P. fieldi*, *P. cynomolgi* (*P. simium*, *P. vivax*)) are questionable (Fig. 1A and B). The third group (70% of BS and 5/17 synapomorphic characters) includes the 2 human parasites, *P. falciparum* and *P. malariae*, related respectively to *P. reichenowi* (with 100% of BS and 16/22 synapomorphic characters) infecting great apes from Africa, and to *P. brasilianum* (with 100% of BS and 7/13 synapomorphic characters) infecting New World monkeys. The bird *Plasmodium* species (i.e. *P. gallinaceum*, *P. juxtanucleare* and *P. lophurae*) and 1 lizard *Plasmodium* species (i.e. *P. mexicanum*) are also included in this third group where they constitute a subgroup sustained by 89% of BS value and 12/15 synapomorphic characters. The topology of Fig. 1B is different but the same main groups as in Fig. 1A are respectively resolved and unresolved in terms of BS values, except the positions of the 3 subgroups (*P. falciparum*-*P. reichenowi*), (*P. malariae*-*P. brasilianum*), (birds and lizard *Plasmodium*).

Fig. 2 shows the cladistic consensus tree obtained for *Plasmodium* species (i.e. only bootstrap values over 70% in Fig. 1A were considered), with their host species and their geographical distribution.

Fig. 3 shows the Cercopithecidae phylogeny (Purvis, 1995) and their known *Plasmodium* parasite species.

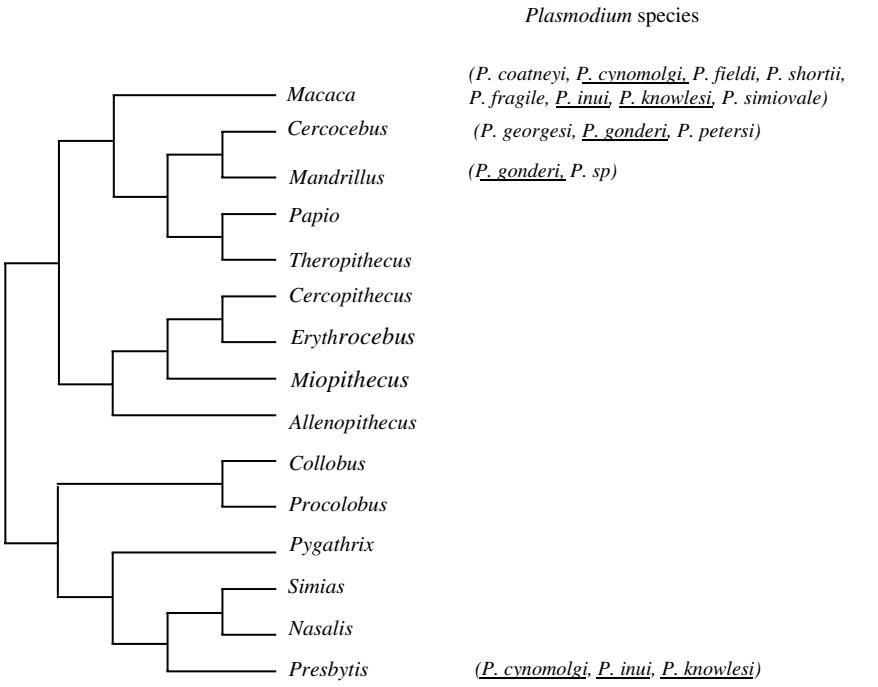

Fig. 3. Cercopithecidae phylogeny from Purvis (1995) and their known *Plasmodium* parasite species. Underlined parasite species found in more than one host genus.

DISCUSSION

Surprisingly, the rodent *Plasmodium* appear in the phylogenetic trees at the root of primate *Plasmodium*, whereas Quari *et al*. (1996) and Escalante & Ayala (1994) showed in their phylogenetic trees that these rodent parasites were inside the primate parasites. Indeed, the same results were found by Perkins & Schall (2002) using cytochrome b. The human *Plasmodium* species are not monophyletic. This result is in accordance with previous results from rDNA sequence phylogenies (Waters *et al*. 1993; Escalante & Ayala, 1994; Quari *et al*. 1996) and from cytochrome b phylogenies (Perkins & Schall, 2002; Ricklefs & Fallon, 2002).

From our analysis, *P. falciparum* and its counterpart species parasites of great apes (i.e. *P. reichenowi*) do not originate from avian *Plasmodium* as suggested by Waters *et al*. (1991, 1993), and the relative position of the 2 subgroups stays unresolved. Indeed, these authors proposed that *P. falciparum* shares a common ancestor with avian malarial parasites, and that a host switching from avian to human took place at the beginning of agricultural development, when the human habitat was settled 10000 years ago. Perkins & Schall (2002) and Ricklefs & Fallon (2002) in their study on cytochrome b recently showed that parasites of birds and lizards were clearly separated from mammalian parasites.

We produced 5 new sequences of primate *Plasmodium*. Three of them (*P. coatneyi*, *P. fieldi*, *P. hylobati*) originate from Asian monkeys, 1 of them (*P. gonderi*) from African monkeys, and *P. simium* is a New World monkey parasite. They cluster with the Old World monkey *Plasmodium*. These results are coherent with the results of Waters *et al*. (1993), Escalante & Ayala (1994), Quari *et al*. (1996), Perkins & Schall (2002) and Ricklefs & Fallon (2002). The position of *P. gonderi*, stays here unresolved, while from cytochrome b phylogenies, Escalante *et al*. (1998), Perkins & Schall (2002) and Ricklefs & Fallon (2002) found that its position was at the root of the Old World monkeys group. However, 24/25 autapomorphic characters distance *P. gonderi* from other species, which could mean an older origin. Otherwise, for 8 species (*P. coatneyi*, *P. cynomolgi*, *P. fieldi*, *P. fragile*, *P. hylobati*, *P. inui*, *P. knowlesi*, *P. vivax*), a recent radiation from a common ancestor could explain the small number of synapomorphic and autapomorphic characters displayed on the phylogenetic tree.

P. vivax appears to be the most recent parasite with *P. simium* inside a subgroup including *P. fieldi* and *P. cynomolgi*, two parasites of Asian Cercopithecidae. This observation argues that the origin of *P. vivax* would be from Asian Cercopothecidae. Thus, it seems that the most parsimonious hypothesis from a biogeographical point of view is to propose that *P. simium* (parasite of New World monkeys) derives from *P. vivax* rather than the reverse solution as previously discussed by Escalante, Barrio & Ayala (1995). However, we cannot exclude a molecular convergence of 18S rDNA sequences for geographically distant *Plasmodium*. Li *et al*. (2001) found differences in 18S rDNA sequences of sporozoite type between *P. vivax* originating from the Old and New World, but no difference between *P. simium*, *P. cynomolgi* and Old World *P. vivax*. Carter (2003)

supporting Li *et al*. (2001), proposed that *P. vivax* was introduced to the Americas twice on separate occasions: first by pre-Columbian human migration from Asia and secondly during the period of the European conquest, in which case *P. simium* would originate from the first entrance of *P. vivax* in the Americas while the New World *P. vivax* would date from the European arrival. In the same way, *P. brasilianum*, which displayed a sequence close to that of *P. malariae*, could also originate from a parasite transfer from humans to New World monkeys, but our phylogenetic analysis did not allow us to discuss this evolutionary scenario. Fandeur *et al*. (2000) proposed that monkeys of the rainforest in French Guiana are reservoirs for *P. brasilianum*/*P. malariae*. Generally, it is agreed that zoonose does exist and that Wild primates could represent a reservoir for human pathogens (Wolfe *et al*. 1998), but with the example of the pair *P. vivax*–*P. simium*, it seems that anthroponose should be considered.

Otherwise, it appears that only 4 Cercopithecidae genera are parasitized by *Plasmodium* species. Eight *Plasmodium* species parasitize the Asian genus *Macaca* (i.e. a genus including 16 species with very wide geographical distribution, Groves, 1993). The three Cercopithecidae genera from Asia (i.e. *Pygathrix*, *Simias* and *Nasalis*), were never found to be parasitized, but are comparatively less diversified in terms of species richness than the *Macaca* genus (Groves, 1993). Surprisingly, in Africa, the *Cercopithecus* genus, which includes 20 species with wide geographical distribution (Groves, 1993) appears never to have been parasitized whereas the *Cercocebus* genus (i.e. only 3 species) is parasitized by 3 *Plasmodium* species. Some species may have associated parasites, but we have not yet identified the parasites. Sampling efforts might improve our knowledge of primate *Plasmodium* phylogeny and by this way, the lemur *Plasmodium* species could show new light on this. Likewise, we have no information on the evolutionary relationships between the two type species found in *Pongo pygmaeus* of South Asia (i.e. *P. pitheci* and *P. silvaticum*) and other primate *Plasmodium* species.

In conclusion, three remarks could be made. (i) Once more, a phylogenetic study cannot permit any statement about an African origin of primate malaria. We only observe that the African rodent *Plasmodium* are located at the root of the primate parasite phylogeny. Inside the primate *Plasmodium*, the phylogenetic relationships remain unresolved for deep branching patterns. The *P. gonderi* position in the clade of Old World monkey *Plasmodium* could not be resolved but the high number of autapomorphic characters, another significant phylogenetic character, is in favour of a deep branching of this taxon in the clade. (ii) The investigation gap in the sampling of monkey *Plasmodium* appears to be one of the principal reasons why the phylogenetic knowledge of primate *Plasmodium* is so limited. (iii) Given the opportunity for lateral transfers in the *Plasmodium* genus as illustrated by *P. vivax*–*P. simium*, the emergence of new virulent *Plasmodium* taxa in humans from wildlife and *vice versa* constitutes a real possibility.

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