Evolutionary Origins of Human Malaria Parasites

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1. The Phylum Apicomplexa

The genus *Plasmodium* consists of nearly 200 described species that are parasitic to reptiles, birds, and mammals. *Plasmodium* belongs to the phylum Apicomplexa, which includes more than 5000 described species and many more still to be described. The Apicomplexa are all parasites, characterized by their eponym structure, the apical complex, which probably plays an important role in the parasite's penetration of host cells. Apicomplexa other than *Plasmodium* that cause disease in humans include *Toxoplasma*, *Cryptosporidium*, and *Babesia*, although the toll attributed to any one of these is small compared to *Plasmodium*. There is no fossil record of apicomplexans (Margulis *et al*., 1993); molecular phylogenetic investigations provide the best insight to the origins of the phylum. These studies have indicated that the Apicomplexa are very ancient, perhaps as old as the multicellular kingdoms of plants, fungi, and animals, and thus somewhat older than one billion years (Ayala *et al*., 1998). The *Plasmodium* lineage diverged from other Apicomplexa several hundred million years ago, perhaps earlier than the Cambrian and before the vertebrates (chordates) originated form their ancestral invertebrate lineage (Figure 6.1).

Some Apicomplexans are *monogenetic,* parasitic to one or several related species, without requiring an intermediate host; e.g., the coccidean *Cryptosporidium parvum*, which infects animal gut epitelia; although other coccideans, such as *Sarcocystis* and *Toxoplasma* are *digenetic* parasites, requiring two separate host species to complete their life cycle. The

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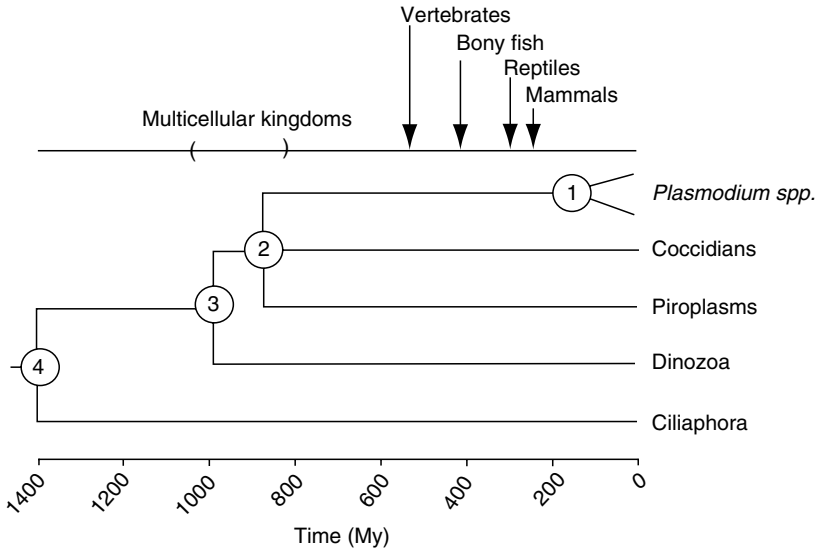

Figure 6.1. Simplified phylogeny of *Plasmodium* and related protozoa. The branching nodes refer to: 1, radiation of the *Plasmodium* genus; 2, radiation of the phylum Apicomplexa; 3 and 4, Apicomplexa divergence from two related phyla, Dinozoa and Ciliophora.

two host species of digenetic parasites are distinguished as being either definitive or intermediate, with the definitive host being the site in which sexual reproduction of the parasite occurs. All *Plasmodium* species are digenetic. An unsettled question is whether *Plasmodium* parasites evolved directly from monogenetic parasites of ancient marine invertebrates ancestors of modern chordates, or whether they descended from other digenetic parasites (Huff, 1938; Manwell, 1955; Garnham, 1966; Barta, 1989). Molecular phylogenetic comparisons have not resolved this issue, but it is apparent from analyses of ribosomal DNA and other genetic loci that the digenetic life style has multiple independent origins among apicomplexans (Barta, 1989; Escalante and Ayala, 1995; Fast *et al*., 2002).

Vertebrates are the typical intermediate hosts of *Plasmodium*, while invertebrate species are the definitive hosts or vectors. *Plasmodium* are intracellular parasites occupying the blood cells of the intermediate host for a large part of their life cycle; accordingly, their invertebrate vectors are blood-feeding organisms, most typically mosquitoes, although in the case of some of the reptilian malarias, sand flies serve as the vector (Kimsey, 1992).

Two phyla related to the Apicomplexa are the Ciliophora (ciliates), which includes *Paramecium,* and the Dinozoa, which includes the dinoflagellates. Two large classes of Apicomplexa are the Coccidea, already mentioned, and the Hematozoa, which includes the order

Haemosporida, to which *Plasmodium* belongs, and the Piroplasmida, which includes species parasitic to dogs, cats, horses, and cattle and are mostly transmitted by ticks (definitive hosts).

2. The Genus Plasmodium

Human malaria is caused by four *Plasmodium* species: *P. falciparum, P. vivax, P. malariae,* and *P. ovale.* The human toll of malaria is stunning, perhaps the greatest of all human afflictions (Sherman, 1998), although nowadays AIDS and tuberculosis may approach the number of fatalities attributed to malaria, between 1 and 3 million deaths per year, mostly children. The worldwide incidence of malaria is estimated at 300–500 million cases per year. Most morbidity and mortality occurs in subSaharan Africa, caused by *P. falciparum*, the most virulent species, accounting for nearly 85% of the total. *P. vivax* is the most geographically widespread of the human malarias, estimated to account for 70–80 million clinical cases across much of Asia, Central and South America, the Middle East, and parts of Africa.

Various molecular phylogenetic analyses have revealed the relationships among the *Plasmodium* species. Herein, we show a representative phylogenetic tree based on the circumsporozoite protein (see, Figure 6.2) gene sequences. Table 6.1 provides information about the hosts and the geographic distribution of the species. The trees are obtained by the "neighbor-joining" (NJ) method (Saitou and Nei, 1987) based on genetic distances calculated according to Tamura's three-parameter method (Tamura, 1992). Trees obtained with other methods (such as maximum likelihood) and/or based on other measures of genetic distance have fundamentally identical topologies as those shown in Figure 6.2 (certainly with respect to the conclusions that will be formulated later). The root of the *Csp* tree has been determined by maximum likelihood. (Additional details can be found in Escalante and Ayala, 1995; Escalante *et al*., 1995; and Ayala *et al*., 1998). Estimates of divergence times based on two genes, *Csp* and *rRNA* trees are given in Table 6.2.

The phylogenies represented in Figure 6.2 include three human parasites, *P. falciparum, P. vivax,* and *P. malariae*. All four species (i.e., including *P. ovale*) have been included in phylogenies based on the mitochondrial gene encoding cytochrome-b (*cyt-B*) (Figure 6.3, after Perkins and Schall, 2002; Qari *et al*. 1996). These phylogenetic studies yield the following conclusions concerning the evolutionary history of the human malarial parasites.

1. The four human parasites, *P. falciparum, P. ovale, P. malariae,* and *P. vivax* are very remotely related to each other, so that the

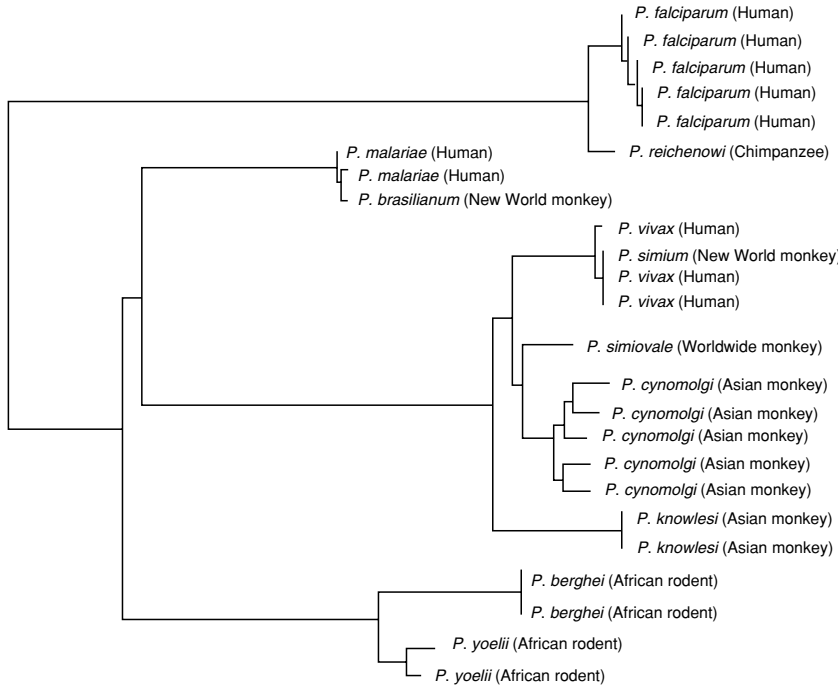

Figure 6.2. Phylogeny of 11 *Plasmodium* species (30 isolates) inferred from *Csp* gene sequences. Each parasite's host is given in parentheses. Some independent isolates have identical sequences of which only one is shown; thus, only five out of eight isolates are shown for *P. falciparum*, three out of four for *P. vivax,* and one out of two for *P. simium*.

evolutionary divergence of these four human parasites greatly predates the origin of the hominids. It follows that their parasitic associations with humans are phylogenetically independent (i.e., all but one – at the most) of these species have been laterally

Table 6.1. *Plasmodium* Species Used for Construction of Phylogeny Based on *Csp* Sequences

Species	Number of strains	Host	Geographic distribution
P. falciparum	8	Human	Tropics worldwide
P. malariae	2	Human	Tropics worldwide
P. vivax	4	Human	Tropics worldwide
P. reichenowi	1	Chimpanzee	African tropics
P. brasilianum	1	Monkey	New World tropics
P. simiovale	1	Monkey	Tropics worldwide
P. cynomolgi	5	Monkey	Asian tropics
P. simium	2	Monkey	New World tropics
P. knowlesi	2	Monkey	Asian tropics
P. berghei	2	Rodent	African tropics
P. yoelii	2	Rodent	Africa

Table 6.2. Time (in Million Years) of Divergence Between *Plasmodium* Species, Based on Genetic Distances at Two Loci (Ayala *et. al*., 1999)

	rRNA	*Csp*
falciparum versus *reichenowi*	11.2 ± 2.5	8.9 ± 0.4
vivax versus monkey*	20.9 ± 3.8	25.2 ± 2.1
vivax versus *malariae*	75.7 ± 8.8	103.5 ± 0.6
falciparum versus v*ivax/malariae*	75.7 ± 8.8	165.4 ± 1.6

**brasilianum* and *simium* not included

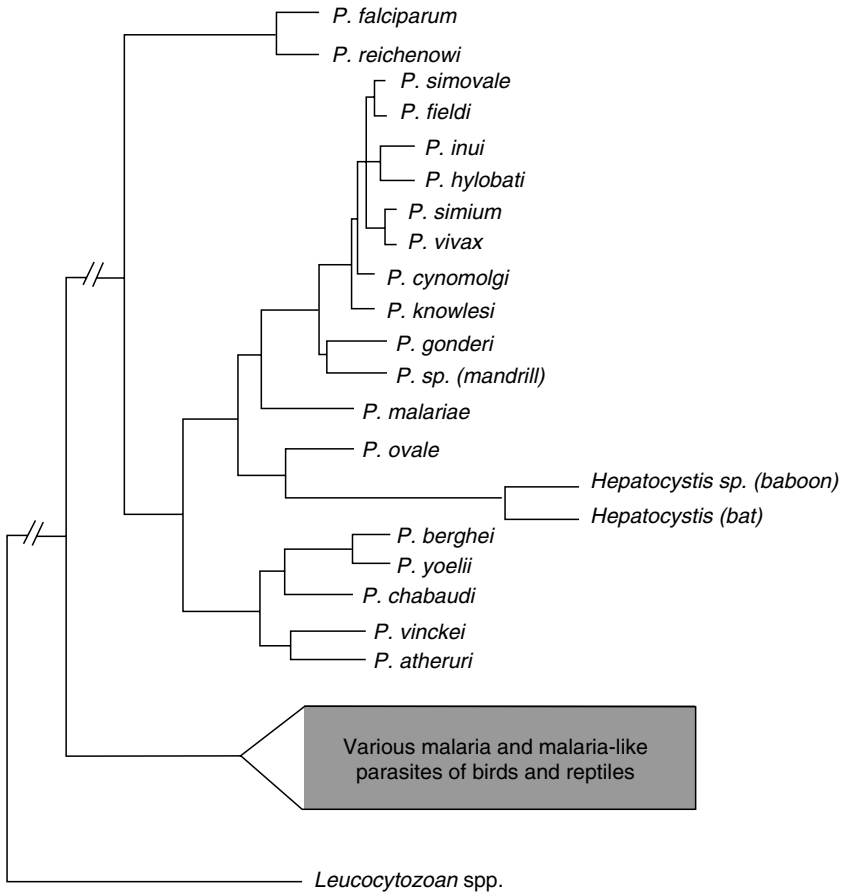

Figure 6.3. Phylogeny of 19 *Plasmodium* species (+2 *Hepatocystis* spp.) inferred from mitochondrial cytochrome-b gene sequences. "*Plasmodium sp*." is an undescribed species isolated from a mandrill, *Mandrillus leucophaeus*, in Gabon. Maximum likelihood analyses give strong support for monophyly (i.e., a single common ancestor) of the mammalian malaria species. The inclusion of two *Hepatocytsis* species within the mammalian malaria clade may indicate that the *Plasmodium* genus is paraphyletic (i.e., its phylogeny includes species of a different genus), a situation that would call for taxonomic revision. From Perkins and Schall, 2002.

transmitted to the human ancestral lineage from other, nonprimate hosts. These results are consistent with the diversity of physiological and epidemiological characteristics of these four *Plasmodium* species (Coatney *et al*., 1971; López-Antuñano and Schumumis, 1993).

2. *Plasmodium falciparum* is more closely related to *P. reichenowi*, the chimpanzee parasite, than to any other *Plasmodium* species. On the basis of the *Csp* and *rRNA* genes, the time of divergence between these two *Plasmodium* species is estimated at 8–11 million years (My) ago, which is consistent with the time of divergence between the two host species, human and chimpanzee. (The divergence time of parasitic species is likely to predate the divergence of their host species, similarly as the divergence times of ancestral gene lineages are likely to predate the divergence of their species; alternative polymorphic states may become fixed in one or the other carrying species.) A parsimonious interpretation of this state of affairs is that *P. falciparum* is an ancient human parasite, associated with our ancestors since the divergence of the hominids from the great apes. The *Csp* tree confirms that *P. falciparum* is closely related to *P. reichenowi*, but distinct from it. Note that the cluster of the *P. falciparum* strains has a 92% bootstrap value, which is highly significant. (Figure 6.3 shows only five of the eight *falciparum* strains because others are identical to those shown.) The genetic distance between *falciparum* and *reichenowi* is 0.044 ± 0.002, five times as large as the average intraspecific distance among the eight *falciparum* strains, which is 0.009 ± 0.001 (Escalante *et al*., 1995). The cytochrome-b tree (Figure 6.2) also confirms that *falciparum* is most closely related to *reichenowi* than to any other *Plasmodium* species, but different from it (Ayala *et al*., 1998; Escalante *et al*., 1998).

McCutchan *et al*. (1996) failed to separate unambiguously *P. falciparum* and *P. reichenowi* when they analyzed amino acid rather than nucleotide sequences. This ambiguity can be attributed to the difficulty of aligning, for several *Plasmodium* species, amino acid sequences that are quite different and variable in length (Escalante *et al*., 1995; see also Rich *et al*., 1997), with the consequence that only the more conserved amino acids can be reliably aligned. When the comparison is made between *P. reichenowi* and all available sequences of *P. falciparum*, the difference between the two species is unambiguous (see Rich *et al*., 1997 for the distinct composition of the central *Csp* repeat region).

3. *Plasmodium malariae*, a human parasite and *P. brasilianum*, a New World monkey parasite, are genetically indistinguishable at the *Csp* gene. We infer that a lateral transfer between hosts has occurred in recent times, either from monkeys to humans or vice versa.

Moreover, *Plasmodium vivax* is genetically indistinguishable from *P. simium* at the *Csp* gene (and also at the *18SSUrRNA*; see Escalante *et al*., 1997). *P. simium* is, like *P. brasilianum*, a parasite of New World monkeys. We infer, again, a recent lateral transfer between human and monkey hosts.

The average intraspecific distances for each of the three human parasites (and for *P. simium*; only one strain of *P. brasilianum* was investigated) are shown in Table 6.3. The Table also gives the genetic distance among the three human and two primate parasites. The genetic distance between *malariae* and *brasilianum* is 0.002 ± 0.002, not greater than the distance among the two *malariae*, or the four *vivax,* or the eight *falciparum* sequences available. Although *P. malariae* and *P. brasilianum* are isolated from very different hosts—*P. malariae* from humans, *P. brasilianum* from New World monkeys—the question arises whether they are different species, since they are genetically indistinguishable. *P. malariae* and *P. brasilianum* might be considered either two distinct species or a single species exhibiting "host polymorphism" (Escalante and Ayala, 1994) (i.e., able to parasitize more than one host species). But this is a question of taxonomy and convenience, rather than biologically substantive. Whether or not *malariae* and *brasilianum* are considered one or two distinct species, it would be the case that either *brasilianum* is a recent platyrrhine parasite acquired from humans, or *malariae* has only recently become a human parasite by a host–switch from New World monkeys. In the latter case, *malariae* would have become a human parasite within the last 15,000 years, after the first human colonization

Table 6.3. Average Genetic Distance Within and Between Various *Plasmodium* Species, Based on the *Csp* Gene

Species	Number of strains	Intraspecific	*malariae*	*vivax*	*simium*	*brasilianum*
falciparum	8	.009±.001	.697±.003	.581±.003	.837±.002	.687±.004
malariae	2	.004±.003		.517±.006	.513±.004	.002±.002
vivax	4	.004±.001			.004±.001	.517±.000
simium	2	.000±.000				.508±.187

of the Americas, and perhaps only within the last several hundred years, after the expansion of human populations that followed the European colonizations of South America.

3. Transfers Between Human and Monkey Hosts

The same issue, whether they should be considered only one or two species, arises with respect to *vivax* versus *simium*. Three considerations favor a lateral transfer from human to monkey hosts:

(i) *Plasmodium vivax* has a worldwide distribution, in contrast to the limited geographic range of *simium*, restricted to a few South American monkey species, *Alouatta fusca, Brachyteles arachnoides,* and *Ateles* sp. (Gysin, 1998). The counterpoint can be made, however, that humans are exceedingly mobile. Infected humans could readily have carried the parasite from South America to other world continents.

(ii) There are no records of malaria in South America (or elsewhere in the New World) before the arrival of the European colonizers within the last 500 years. This would be consistent with the interpretation that *P. vivax* (as well as *P. malariae* and *P. falciparum*) was introduced to the New World by the European colonizers and their African slaves. The weakness of this argument is that it consists of negative evidence, which is particularly unreliable when there are no extensive observations, experiments, or studies that would have likely manifested the presence of malaria in the New World before the year 1500, even if malaria had indeed been present.

(iii) Historical records suggest that nonmalignant malaria has occurred in the Old World for several thousand years. Chinese medical writings, dated 2700 BC, cuneiform clay tablets form Mesopotamia, dated about 2000 BC, the Ebere Egyptian Papyrus (ca. 1570 BC), and Vedic period Indian writings (1500–800 BC), mention severe periodic fevers, spleen enlargement and other symptoms suggestive of malaria (Sherman, 1998). Spleen enlargement and the malaria antigen have been detected in Egyptian mummies, some more than 3000 years old (Miller *et al*., 1994; Sherman, 1998). Hippocrates' (460–370 BC) discussion of tertian and quartan fevers, "leaves little doubt that by the fifth century BC *Plasmodium malariae* and *P. vivax* were present in Greece" (Sherman, 1998, p. 3). If this interpretation is correct, the association of *malariae* and *vivax* with humans

could not be attributed to a host–switch from monkeys to humans that would have occurred after the European colonizations of the Americas. This seems definitive evidence, so long as one accepts the interpretation that the fevers described by Hippocrates were indeed caused by the two particular species *P. vivax* and *P. malariae*.

Three considerations favor the alternative hypothesis, namely, that the host "invasion" has occurred from monkeys to humans:

(a) Humans are biologically (evolutionarily) more closely related to Old World monkeys (catharrhines) than to New World monkeys (platyrrhines). If lateral host–switch from humans to monkeys were likely, it would be more likely that the natural transfer would have been to our closer, rather than to our more remote relatives. This argument would be much weakened if the chimpanzee/gorilla parasite *P. rodhaini,* which is thought to be quite similar to *P. vivax*, or *P. schwetzi*, also a chimpanzee/gorilla parasite, which is similar to *P. malariae* (Gysin, 1998), were shown to be genetically identical (or very similar) to the corresponding human parasites, so that they might have been recently acquired by the apes from humans. We note that, according to Gysin (1998), "it has been shown" that *P. schwetzi* is "closely related" to *P. malariae*, but is "homologous" to *P. vivax*. This is a strange claim considering the considerable phylogenetic distance between *P. malariae* and *P. vivax* (see Figs. 6.2 and 6.3). We would point out, moreover, that *P. reichenowi*, and *P. falciparum*, which are "closely related" and "homologous" (Gysin, 1998) are evolutionarily as distant as their hosts, chimpanzees/gorillas and humans (see earlier section). The catharrhine parasite *P. inui*, widely thought to be closely related to *P. malariae*, has been shown to be quite different (Escalante *et al*., 1998).

(b) Humans and their ancestors have been geographically associated with catarrhine monkeys for millions of years, but only for several thousand with platyrrhine monkeys. If the natural transfer from humans to monkeys were likely, it would have been much more likely that the transfer would have occurred to species with which humans have been in geographic association for a much longer period.

(c) *Plasmodium simium* is parasitic to several platyrrhine species. A lateral host transfer from humans to monkeys would require several host–switches, either from human to each monkey species, or from human to one monkey species and then from one to other monkey species, all in a short time interval (a few

thousand, or even a few hundred years). This state of affairs is more extreme when we consider the case of *P. malariae* and *P. brasilianum*, since this parasite's hosts include numerous platyrrhine species (26 taxa are listed by Gysin, 1998, p. 420).

We have conjectured in the past (Escalante *et al*., 1995; Ayala *et al*., 1998), on the grounds of evolutionary parsimony, that the host–switch between *vivax* and *simium* and also between *malariae* and *brasilianum*—may have been from primates to humans so that *vivax* and *malariae* would have become human parasites only recently, perhaps only a few hundred years ago. The historical record (iii, see earlier section) is the strongest evidence against this conjecture. The matter can, in any case, be resolved by comparing the genetic diversity of the human and primate parasites. If the transfer has been from human to monkeys, the amount of genetic diversity in silent nucleotide sites (and other neutral polymorphisms) will be much greater in *P. vivax* than in *P. simium*, and in *P. malariae* than in *P. brasilianum* (including in each comparison the polymorphisms present in the several monkey host species). A transfer from monkey to humans would be evinced by much lesser polymorphism in the human than in the monkey parasites.

The genetic indistinguishability between *P. vivax* and *P. simium* has recently been confirmed by an investigation of 13 microsatellite loci and eight tandem-repeat (TR) loci, which includes 108 *P. vivax* individual samples broadly representative of the distribution of this parasite (Leclerc *et al*., 2004). Microsatellite polymorphisms arise at high rates by replication slippage, yielding new alleles with different numbers of the repeating unit. The genetic near-identity between *P. vivax* and *P. simium* is evinced, first, by the fact that all 13 microsatellite loci could be amplified in *P. simium*. The number of microsatellite loci that could be amplified in any one of eight other Old World (catarrhyne) monkey parasites range from zero (*P. gonderi*) to ten (*P. cynomolgi*). Moreover, *P. simium* carries the same allele as *P. vivax* at the nine loci that are monomorphic in this species, the most common *P. vivax* allele at the three slightly polymorphic loci, and one of the *P. vivax* alleles at the only locus that is polymorphic in this species. A parallel situation occurs at the TR loci which evolve also rapidly. First, all eight TR loci could be amplified in *P. simium*, but only between zero and three in any of the other catarrhyne parasites. Second, *P. simium* alleles are identical to those of *P. vivax* at all loci but one, at which *P. simium* presents one private allele. This, however, is also the case for several local populations of *P. vivax*, which each display at least one private allele at one TR locus. A neighbor-joining tree based on TR genetic distances between populations includes *P. simium* within the *P. vivax* polymorphism (Leclerc *et al*., 2004).

Lateral transmission of *Plasmodium* parasites from monkey hosts to humans is known for several species, including *P. simium* (Deane *et al*., 1966), *P. brasilianum* (Contacos *et al*., 1963), *P. cynomolgi* (Eyles *et al*., 1960), *P. knowlesi* (Chin *et al*., 1965) and, perhaps, *Plasmodium simiovale* (Qari *et al*., 1993). Transmission from humans to monkeys can be accomplished experimentally (Chin *et al*., 1965) and may also occur naturally (Collins, 1974). Among avian and reptilian malaria parasites, host–shifts have been a common occurrence (Bensch *et al*., 2000; Ricklefs and Fallon, 2002).

The direction of host transfer between *P. vivax* and *P. simium* can be settled genetically. If the transfer has occurred from platyrrhine to human, *P. simium* is expected to have greater nucleotide polymorphism at neutral sites than *P. vivax*. Ascertaining this will require the investigation of numerous independent samples of *P. simium*, which are not currently available. Moreover, the issue may not simply be resolved, because the genetic impoverishment of *P. vivax* may be due to a recent demographic (or selective) sweep, which for now is postulated as the likely explanation for this impoverishment (Leclerc *et al*., 2004; Rich 2004).

4. Population Structure of *Plasmodium falciparum*

As shown earlier, the lineage of *P. falciparum* has been associated with the human lineage throughout the evolution of the hominids (i.e., since the separation of the human and chimpanzee lineages), 6–8 million years (My) ago. The question we now raise is whether the population dynamics of *P. falciparum* has or not followed that of its hominid hosts. A possibility, for example, is that *P. falciparum* may have been restricted to some small locality from which it might have spread throughout other human populations as a consequence of increased virulence, environmental changes, or some other factor.

One way to approach this question is to investigate the distribution of genetic polymorphisms in *P. falciparum* populations. Numerous epidemiological studies have indicated that populations of *P. falciparum* are remarkably variable. Extensive genetic polymorphisms have been identified with respect to antigenic determinants, drug resistance, allozymes, and chromosome sizes (e.g., Sinnis and Wellems, 1988; Creasy *et al*., 1990; Kemp and Cowan, 1990; McConkey *et al*., 1990; Hughes and Hughes, 1995; Babiker and Walliker, 1997). Antigenic and drug resistance polymorphisms respond to natural selection, which is most effective in large populations—millions of humans are infected by *P. falciparum* and one single patient may harbor 10^{10} parasites (McConkey *et al*., 1990). The replacement of one allele by another, or the rise of polymorphism

with two or more alleles at high frequency may occur even in one generation. If the selection pressure is strong enough, all individuals exposed to the selective agent may die, except those carrying a resistant mutation. With populations as large as those of *P. falciparum*, any particular mutation is expected to arise in any one generation; and the same mutation may arise—and rise to large frequency—independently in separate populations. On the contrary, silent (i.e., synonymous) nucleotide polymorphisms are often adaptively neutral (or very nearly so) and not directly subject to natural selection. Thus, silent nucleotide polymorphisms reflect the mutation rate and the time elapsed since their divergence from a common ancestor. The population structure of *P. falciparum* is, consequently, best investigated by examining the incidence of synonymous polymorphisms.

The coalescence theory of population genetics assumes that the allele sequences of any given gene present in populations of an organism can be genealogically traced back to a single ancestral sequence ("cenancestor"). If one ignores the possibility of multiple hits (which is reasonable, so long as the sequences are not extremely polymorphic), the number of neutral polymorphisms observed in a sample of multiple strains will be a function of the neutral mutation rate, the time elapsed, and the number of lineages examined (and follow a Poisson distribution). If the neutral mutation rate can be established, the time elapsed since the cenancestor is simply determined by dividing the number of polymorphisms observed by the mutation rate times the number of neutral nucleotide sites observed in the full sample (Appendix).

Table 6.4 summarizes the polymorphisms that we found in a sample of 10 genes for which several sequences were available in DNA data banks (Rich *et al*., 1998). The gene sequences analyzed derive form isolates of *P. falciparum* representative of the global malaria endemic regions. The *Dhfr* and *Ts* genes are found directly adjacent to one another on the parasite's fourth chromosome and encode the bifunctional dihydrofolate reductase–thymidylate synthetase (DHFR–TS) domain. Certain mutations in the *Dhfr* gene have been widely associated with *P. falciparum* resistance to antifolate drugs, including pyrimethamine. Two other genes in Table 6.4 have been implicated with drug-resistant phenotypes of *P. falciparum*: the gene coding for dihydropteroate synthetase (*Dhps*) and the gene for multidrug resistance (*Mdr1*). The circumsporozoite protein (encoded by *Csp1*) is antigenic, and the rhoptry-associated protein (encoded by *Rap1*) may also be immunogenic. The other four genes in Table 6.4 are not known to be immunogenic or associated with resistance to any antimalarial drug currently in use. They code for calmodulin (*Calm*), glucose-6-phosphate dehydrogenase (*G6pd*), heat-

Table 6.4. Polymorphisms in 10 Gene Loci of *Plasmodium falciparum*

Gene	Chromosome location	Length (bp)	Sample size	Polymorphic sites		Number of synonymous sites	
				nonsynonymous	synonymous	Fourfold	Twofold
Dhfr	4	609	32	4	0	2144	4128
Ts	4	1215	10	0	0	1250	2640
Dhps	8	1269	12	5	0	1536	2724
Mdr1	5	4758	3	1	0	1350	2088
Rap1	—	2349	9	8	0	1092	1668
Calm	14	441	7	0	0	364	602
G6pd	14	2205	3	9	0	726	1404
Hsp86	7	2241	2	0	0	532	910
Tpi	—	597	2	0	0	180	262
Csp1 5′ end	3	387	25	7	0	688	2010
Csp1 3′ end	3	378	25	17	0	1050	1625
Total	—	—	—	51	0	10,912	20,061

shock protein 86 (*Hsp86*), and triose phosphate isomerase (*Tpi*). Six of the ten loci exhibit amino acid polymorphisms, including the drug-resistance genes *Dhfr*, *Dhps,* and *Mdr-1*, as well as the antigenic *Cps* and *Rap1*. The significant result is that no silent polymorphisms are observed in any of the 10 genes. An independent study of ten gene loci, most encoding antigenic determinants, has shown a similar scarcity of silent polymorphisms (Escalante *et al*., 1998).

5. Malaria's Eve Hypothesis

Estimating time of divergence from neutral polymorphism requires that the neutral mutation rate (of third position in synonymous codons) be known. We have estimated the neutral mutation rate by comparing the *P. falciparum* gene sequences with *P. reichenowi* (the chimpanzee parasite) and also with a set of rodent *Plasmodium* parasites (Rich et al., 1998). The number of neutral polymorphisms in Table 6.4 is zero and, thus, at face value, the time elapsed since the cenancestor (t) would be zero, although it would become positive as soon as some neutral polymorphisms are observed. In any case, t is expected to follow a Poisson distribution, which allows calculating the upper confidence limit for the time since the cenancestor. As shown in Table 6.5, the 95% upper confidence level is between 25,000–50,000 years ago, depending on which mutation rate estimate is used; the 50% upper confidence limit is between 6000–13,000 years. We have referred to this conclusion, that the world expansion of *P. falciparum* is recent, as the Malaria's Eve hypothesis.

Table 6.5. Estimated Upper-boundary Times (t_{95} and t_{50}, in Years) to the Cenancestor of the World Populations of *P. falciparum*

Assumption	Mutation rate × 10^{-9}		t_{95}	t_{50}
	Fourfold	Twofold		
Plasmodium radiation				
55 My	7.12	2.22	24,511	5,670
129 My	3.03	0.95	57,481	13,296
falciparum-reichenowi				
5 My	3.78	1.86	38,136	8,821
7 My	2.70	1.33	53,363	12,342

Mutation rates are estimated based on two sets of assumptions, concerning either the origin of the *Plasmodium* genus or the time of divergence between *P. falciparum* and *P. reichenowi*. The *P. falciparum* cenancestor lived more recently than 24,511 or 57,481 years ago, with a 95% probability; and more recently than 5670 or 13,296 years with a probability of 50%.

In the few years since we first proposed the Malaria's Eve hypothesis (1998), the issue has been subject to a contentious debate. Our initial conclusion was based on sequences that were then available from GenBank, and the only criteria for inclusion of genes in our dataset was that they had to be void of repetitive DNA sequences and show no evidence of being under positive selection impacting synonymous codon substitutions. In 1998, the amount of sequence data available for the species was rather limited, but since that time the dataset has grown enormously, including the complete genome sequence of *P. falciparum* published in 2002 (Gardner *et al*., 2002).

One of the new studies entails a large-scale sequencing survey of 25 introns, located on the second chromosome, from eight *P. falciparum* isolates collected among global sites (Volkman *et al*., 2001). The findings of this study confirm our previous result: there is an extreme scarcity of silent-site polymorphism among extant populations of *P. falciparum*. Among some 32,000 nucleotide sites examined, Volkman *et al*. (2001) found only three silent single-nucleotide polymorphisms (SNPs). Combining their data with ours, these authors estimated that the age of Malaria's Eve was somewhere between 3200 and 7700 years, depending on the calibration of the substitution clock.

Conway *et al*. (2000) have presented further evidence in support of Malaria's Eve, based on analysis of the *P. falciparum* mitochondrial genome. They examined the entire mitochondrial DNA (mtDNA) sequence of four *P. falciparum* isolates originating from Africa, Brazil, and two from Thailand, as well as the chimpanzee parasite, *P. reichenowi*. Alignment of the four complete mtDNA sequences (5965 bp) showed

that 139 sites contain fixed differences between *falciparum* and *reichenowi*, whereas only four sites are polymorphic within *falciparum*. The corresponding estimates of divergence (K, between *P. reichenowi* and *P. falciparum*) and diversity (π, within *P. falciparum* strains), are 0.1201 and 0.0004, respectively (i.e., divergence in *mtDNA* sequence between the two species is 300-fold greater than the diversity within the global *P. falciparum* population). If we use the *rDNA*-derived estimate of 8 million years as divergence time between *P. falciparum* and *P. reichenowi*, then the estimated origin of the *P. falciparum* mtDNA lineages is 26,667 years (i.e., 8 million/300), which corresponds quite well with our estimate based on 10 nuclear genes (Rich *et al*., 1998). In a subsequent survey of a total of 104 isolates from Africa (n = 73), Southeast Asia (n = 11), and South America (n = 20), Conway *et al*. (2000) determined that the extant global population of *P. falciparum* is derived from three mitochondrial lineages that started in Africa, and migrated subsequently (and independently) to South America and Southeast Asia. Each mitochondrial lineage is identified by a unique arrangement of the four polymorphic *mtDNA* nucleotide sites.

More recently, the complete 6-kb mitochondrial genome has been sequenced from 100 geographically representative isolates, (Joy *et al*, 2003). The sequences support a rapid expansion of *P. falciparum* in Africa, starting approximately 10,000 years ago. The data indicate, however, that some lineages are five to ten times more ancient and that *P. falciparum* populations have existed in Southeast Asia and South America perhaps earlier than 50,000 years ago. This is unexpected, because if *falciparum* originated in Africa, the oldest polymorphisms should occur in that continent, as is the case for human mitochondrial lineages. Moreover, the presence of *P. falciparum* in South America more than 50,000 years ago is most unlikely, since humans colonized America 15,000–18,000 years ago (or, less likely, around 30,000 years ago) (Cavalli-Sforza *et al*., 1994) and, moreover, it is generally accepted that *falciparum* malaria was introduced in America by the slave trade (Watts, 1997). Rather, it seems likely that the high differentiation of some mitochondrial sequences may be a consequence of the large variation expected in a sample of nonrecombinant neutral sequences, as is the case for the mitochondrial genome, or a consequence of natural selection increasing the frequency of some favorable sequences, which then would appear to be much older than they actually are. Some mitochondrial genes are likely to be under selection, such as the gene encoding cytochrome b, which seems to underlie susceptibility to some antibiotics (Vaidya *et al*., 1993).

A recent investigation of 20 protein-coding genes has confirmed the low level of synonymous polymorphisms found in earlier studies

(Table 6.4; see also Escalante *et al*., 1998; Rich *et al*., 1998). Many of the 20 loci were chosen so as to be different from those previously analyzed, and are located on 11 different chromosomes. The 20 genes were sequenced in 5–7 reference isolates. Among the 22,611 nucleotides sequenced for each strain, there were 21 nonsynonymous polymorphisms, but only one synonymous polymorphism (Table 6.2 in Hartl, 2004), strongly supporting the Malaria's Eve hypothesis.

6. Malaria's Eve Counterarguments

Arguments against the Malaria's Eve hypothesis come in two forms. The first argument is that the loci chosen in the studies supporting the recent world expansion of *P. falciparum* are a biased sample and do not reflect the levels of polymorphism in the genome as a whole. The second argument concedes that nucleotide polymorphisms are scarce and proposes that this is not attributable to recent origins, but rather reflects strong selection pressure against the occurrence of synonymous substitutions.

Some studies have estimated that antigenic polymorphisms in *P. falciparum* may be 40 or more million years old, older than the origin of the hominids (Hughes, 1993; Hughes and Hughes, 1995). An analysis of nucleotide sequences for 23 gene loci has concluded that the cenancestor of extant populations of *P. falciparum* must be 300,000–400,000 years old (Hughes and Verra, 2001). As we have previously explained, these inferences ignore natural selection promoting rapid evolution of antigenic determinants; rely on erroneous sequence alignments that fail to recognize the presence of repetitive sequences; cannot account for large excess of nonsynonymous over synonymous substitutions; and depend on poorly scrutinized sequences obtained from data banks (Rich and Ayala, 1999, 2000, 2003). Some alleged polymorphisms are sequencing errors since they come from a single gene derived from a single clone, but sequenced in different laboratories (see Rich and Ayala, 2003; Hartl, 2004). Similar problems plague the survey by Mu *et al*. (2002) of more than 200 kb from the complete chromosome 3 and their conclusion that *P. falciparum* has maintained large populations for at least 300,000 years (Ayala and Rich, 2003; Hartl, 2004).

The argument that selection pressure against synonymous substitutions, rather than a recent cenancestor, can account for the scarcity of synonymous polymorphisms has also been answered in detail (Rich and Ayala, 1998, 2003). This argument is largely based on the predominance of AT pairs over GC pairs in the genome of *P. falciparum*. Indeed, the AT content of *P. falciparum* is 71.7% overall, and 83.6% in the third position

(Nakamura *et al*., 1997). In response, it may suffice here to say that: (1) AT excess lowers the rate of synonymous substitution but does not altogether eliminate it; (2) in fourfold redundant codons, the bias favors codons ending in A or T but it does not impact A↔T mutations; (3) other *Plasmodium* species are also AT rich and should have the same mutational constraints as *P. falciparum*, yet they exhibit abundant synonymous intraspecific polymorphisms as well as interspecific differentiation; (4) comparisons between *P. falciparum* and *P. reichenowi* at five genes for which data are available in both species yield high numbers of synonymous substitution (average $K_s = 0.072$ versus $K_n = 0.046$ for nonsynonymous substitutions) (Rich and Ayala, 1998).

Beyond genetic inference, other considerations support the Malaria's Eve hypothesis. Sherman (1998) has noted the late introduction and low incidence of *falciparum* malaria in the Mediterranean region. Hippocrates (460–370 BC) describes quartan and tertian fevers, but there is no mention of severe malignant tertian fevers, which suggests that *P. falciparum* infections did not yet occur in classical Greece, as recently as 2400 years ago.

The expansion of *P. falciparum* across the globe after the Neolithic revolution, perhaps about 5,000–6,000 years ago, starting from a highly restricted geographic location, probably in tropical Africa, may have been made possible by (1) changes in human societies, (2) genetic changes in the host–parasite-vector association that have altered their compatibility, and (3) climatic changes that entailed demographic changes (migration, density, etc.) in the human host, the mosquito vectors, and/or the parasite.

One factor may have been changes in human living patterns, particularly the development of agricultural societies and urban centers that increased human population density (Livingston, 1958; Weisenfeld, 1967; De Zulueta *et al*., 1973; de Zulueta, 1994; Coluzzi, 1997, 1999; Sherman, 1998). Genetic changes that have increased the affinity within the parasite–vector–host system are also a possible explanation for a recent expansion, not mutually exclusive with the previous one. Coluzzi (1997, 1999) has cogently argued that the recent worldwide distribution of *P. falciparum* has come about, in part, as a consequence of a recent dramatic rise in vectorial capacity due to repeated speciation events in Africa of the most anthropophilic members of the species complexes of the *Anopheles gambiae* and *A. funestus* mosquito vectors. Biological processes implied by this account (2, above) may have been associated with, and even be dependent on the onset of agricultural societies in Africa (1, above) and climatic changes (3, above), specifically gradual increase in ambient temperatures after the Würm glaciation, so that about 6000 years ago climatic conditions

in the Mediterranean region and the Middle East made the spread of *P. falciparum* and its vectors beyond tropical Africa possible (De Zulueta *et al*., 1973; de Zulueta, 1994; Coluzzi, 1997, 1999). Once demographic and climatic conditions became suitable for propagation of *P. falciparum*, natural selection would have facilitated evolution of *Anopheles* species that were highly anthropophilic and effective *falciparum* vectors (De Zulueta *et al*. 1973; Coluzzi 1997, 1999).

The Malaria's Eve hypothesis is consistent with the evolutionary history of genetically determined immunity factors that confer resistance to *P. falciparum*. A glucose-6-phosphate dehydrogenase (*G6pd*) genetic deficiency occurs at high frequency in areas of malaria endemicity, particularly in subSaharan Africa. Tishkoff *et al*. (2001) have concluded that the *G6pd* mutants are only 3330 years old (95% confidence interval, 1600–6640 years), indicating that *P. falciparum* has only recently become a disease burden to humans. It has been known for decades that hemoglobin S (Hbs; βGglu→Val) heterozygosity confers protection against severe malaria (Allison, 1964; Hill and Weatherall, 1998). Current evidence suggests that the sickle cell mutation has arisen at least twice, once in India or the Middle East and once in Africa, although it is possible that the mutation may have arisen more than once in Africa (Hill and Weatherall, 1998). Analyses of the β globin gene haplotypes associated with a sickle-cell mutation in Africa suggest that it is recent in origin, perhaps no more than 2000 years (Currat *et al*., 2002; Weatherall, 2004). A hemoglobin C (Hbc; β6Glu→Lys) mutation seems to have arisen in Africa equally recently, or even more recently. HbC is associated with a 29% reduction in risk of clinical malaria in heterozygotes (HbCA) and of 93% reduction in HbCC homozygotes, which exhibit limited pathology compared to the severely disadvantaged HbSS (Modiano *et al*., 2001).

Appendix

If a population grows to a large size after a bottleneck, it is reasonable to assume that the genealogy of a sample of multiple strains collected from widely distributed localities would be a star-like phylogeny with the common ancestor at the vertex of the star, as in Figure 6.4 (Slatkin and Hudson, 1991). Under this assumption, and ignoring the possibility of multiple hits at individual sites, the number of neutral polymorphisms that we observe in a sample of multiple strains will have a Poisson distribution with a mean that depends on the neutral mutation rate, the time elapsed, and the number of lineages examined. The expected number of polymorphisms is

$$\lambda = \mu_a t \sum n_i l_i + \mu_b t \sum n_i m_i ,$$

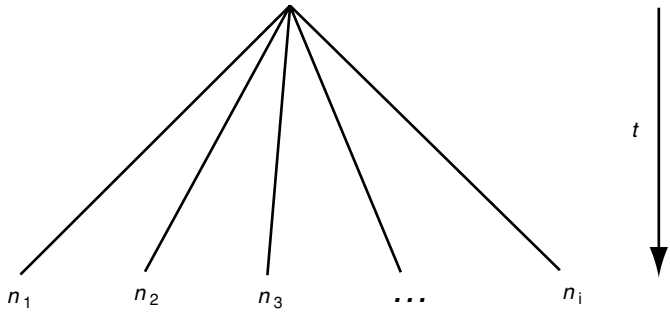

Figure 6.4. Schematic representation of a star phylogeny with the most recent common ancestor (MRCA) at the apex. The scale of *t* represents the time elapsed since this MRCA gave rise to all its descendents in the extant population (n_1, n_2, n_3, ... n_i).

where μ_a and μ_b are the neutral mutation rates at the third position of fourfold and twofold degenerate codons, respectively; *t* is the time since the bottleneck; n_i the number of lineages sampled at the *i*th locus; and l_i and m_i, respectively, the number of fourfold and twofold synonymous sites examined at the *i*th locus. This expression suggests an estimator of the time of the bottleneck, obtained by solving for *t* and replacing λ (the *expected* number of polymorphisms) by *S*, the *observed* number of polymorphisms:

$$\hat{t} = \frac{S}{\mu_a \sum n_i l_i + \mu_b \sum n_i m_i}.$$

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