enabled by the *Volvox* genome sequence, allowing a more complete understanding of the transformation from a cellularly complex *Chlamydomonas*-like ancestor to a morphologically and developmentally complex "fierce roller."

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A Molecular Clock for Malaria Parasites

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The evolutionary origins of new lineages of pathogens are fundamental to understanding emerging diseases. Phylogenetic reconstruction based on DNA sequences has revealed the sister taxa of human pathogens, but the timing of host-switching events, including the human malaria pathogen *Plasmodium falciparum*, remains controversial. Here, we establish a rate for cytochrome b evolution in avian malaria parasites relative to its rate in birds. We found that the parasite cytochrome b gene evolves about 60% as rapidly as that of host cytochrome b, corresponding to ~1.2% sequence divergence per million years. This calibration puts the origin of *P. falciparum* at 2.5 million years ago (Ma), the initial radiation of mammalian *Plasmodium* at 12.8 Ma, and the contemporary global diversity of the Haemosporida across terrestrial vertebrates at 16.2 Ma.

The rate of nucleotide substitution in DNA sequences can provide a molecular clock useful for inferring absolute times in phylogenetic trees (*1*). This rate can be estimated by direct observation over reasonable time periods, as with several viral parasites of humans (*2*, *3*) and experimental populations of *Drosophila* (*4*). Relatively slow nucleotide substitution precludes this approach for malaria parasites, for which calibration is indirect. For some specialized parasites, phylogenetic analyses have revealed codivergence of host and parasite evolutionary lineages, which permits calibration of genetic distance in one relative to the other (*5*–*7*). In contrast, *Plasmodium* and other haemosporidian parasites of terrestrial vertebrates exhibit widespread host switching, often across considerable host taxonomic distance (*8*–*11*). Cospeciation cannot, therefore, provide a means of clock calibration.

In spite of evident host switching, biologists have used the ages of host phylogenetic ancestral nodes to calibrate the rate of nucleotide substitution in *Plasmodium* and to estimate the ages of *Plasmodium* lineages. For example, Ollomo *et al.* (*12*) suggested that a *Plasmodium* lineage newly discovered in chimpanzees diverged from another chimpanzee pathogen, *Plasmodium reichenowi*, 21 ± 9 million years ago (Ma) on the basis of placing the *P. reichenowi*–*P. falciparum* divergence coincident with the human-chimp divergence 4 to 7 Ma. In another analysis, Hayakawa *et al.* (*13*) calibrated amino acid substitutions in three mitochondrial genes based on host-parasite codivergence of *P. gonderi* (a parasite of African primates) and a clade of malaria parasites of southeast Asian primates, including humans. This calibration yielded a divergence time of either 2.5 ± 0.6 million years (My) or 4.0 ± 0.9 My for *P. falciparum* and *P. reichenowi*, depending on the dating of the split between lineages of Asian and African macaques, on one hand, and Asian and African colobine monkeys, on the other hand. However, as Rich *et al.* (*14*) point out, humans could have acquired *P. falciparum* any time after the split of the human-chimpanzee lineage "by a single host transfer, which may have

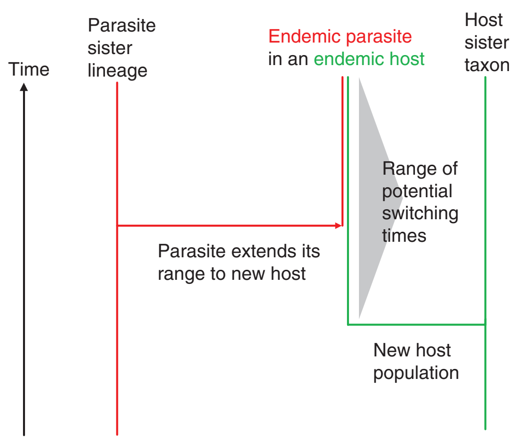

Fig. 1. An approach to estimating a calibration for the rate of haemosporidian nucleotide substitution. We assume that a parasite can switch to a new host at any point with equal probability during the host's independent evolutionary history. Although the range of switching times corresponds to the age of the contemporary host taxon, the range of genetic distances relative to the host is equal to the ratio of the parasite-to-host nucleotide substitution rate. Endemic parasites limited to a single host are suitable for analysis because their divergence from their sister taxon in a different host represents the historical event of host switching (for alternatives, see appendix S1).

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occurred as early as 2 to 3 million years ago, or as recently as 10,000 years ago."

Here, we calibrate the mitochondrial cytochrome b nucleotide substitution rate in haemosporidian parasites of birds, relating it to the rate of cytochrome b evolution in avian hosts (*15*). We assume that nucleotide substitution is clocklike, so that the number of substitutions is binomially distributed, and that the distribution of switching times is uniform over the age of the endemic host taxon (Fig. 1) (supporting online text, appendix S1). Our approach requires identification of endemic parasite lineages, which in turn depends on thorough sampling. In our survey of avian haemosporidians in the West Indies (*16*–*19*), we have screened extensive samples of small land birds from all the major islands, except Cuba. Moreover, many host species are endemic to individual islands. We identified seven endemic parasite lineages (appendix S2) and measured the genetic distances, based on cytochrome b, between these parasite lineages and their sister lineages, and between their hosts and the sister taxon of each host (Fig. 1). The variable of interest is the ratio (k) of the rates of nucleotide substitution between the pairs of parasite and host sequences.

The probability (p) of a nucleotide substitution at a single position over time is equal to the rate of substitution (r) × time (t), or $p = rt$. The mean number of substitutions is the number of nucleotides (n) times the probability, or np, and the variance is $np(1 - p)$; for small numbers of nucleotide substitutions (p near 0), this approaches a Poisson distribution with variance np; the probability of multiple events is low enough to be ignored (*20*).

Because we assume that a parasite can switch to a new host any time after the ancestral host lineage splits, the expected mean for the number of substitutions separating the parasite sequences (N) is

$$N = \frac{1}{t}\int_{i=0}^{t} knri\,di \qquad (1)$$

where k is the ratio of the rate of substitution (parasite/host). Accordingly, $N = knrt/2$ and $k = 2N/nrt$.

For the seven comparisons of host and parasite genetic divergence considered in this analysis, nrt is the estimated number of host substitutions [average 48.94 base pairs (bp), with correction for within-species variation]; the corrected parasite distances (N) averaged 15.23 bp; thus, $k = 2 \times 15.2/48.9 = 0.62$ (appendix S3). According to this estimate, the rate of substitution in the parasite lineages is 62% of that in the host lineages. The average ratio of the parasite divergence to the host divergence for each of the seven comparisons was 0.292 ± 0.119 SD (0.048 SEM; 95% confidence limits, 0.197 to 0.387). The value

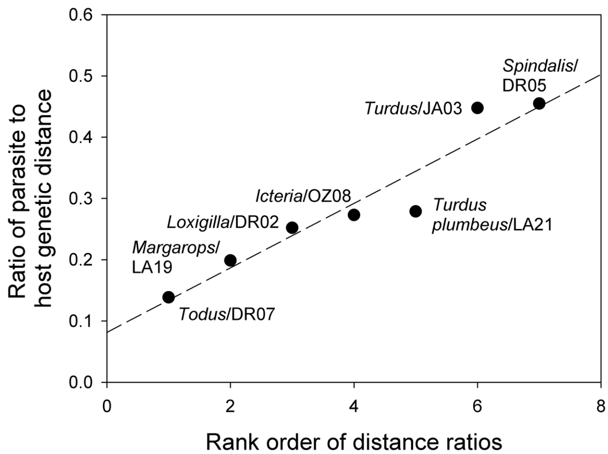

Fig. 2. Rank-ordered ratios of parasite-to-host genetic distances are consistent with a uniform distribution.

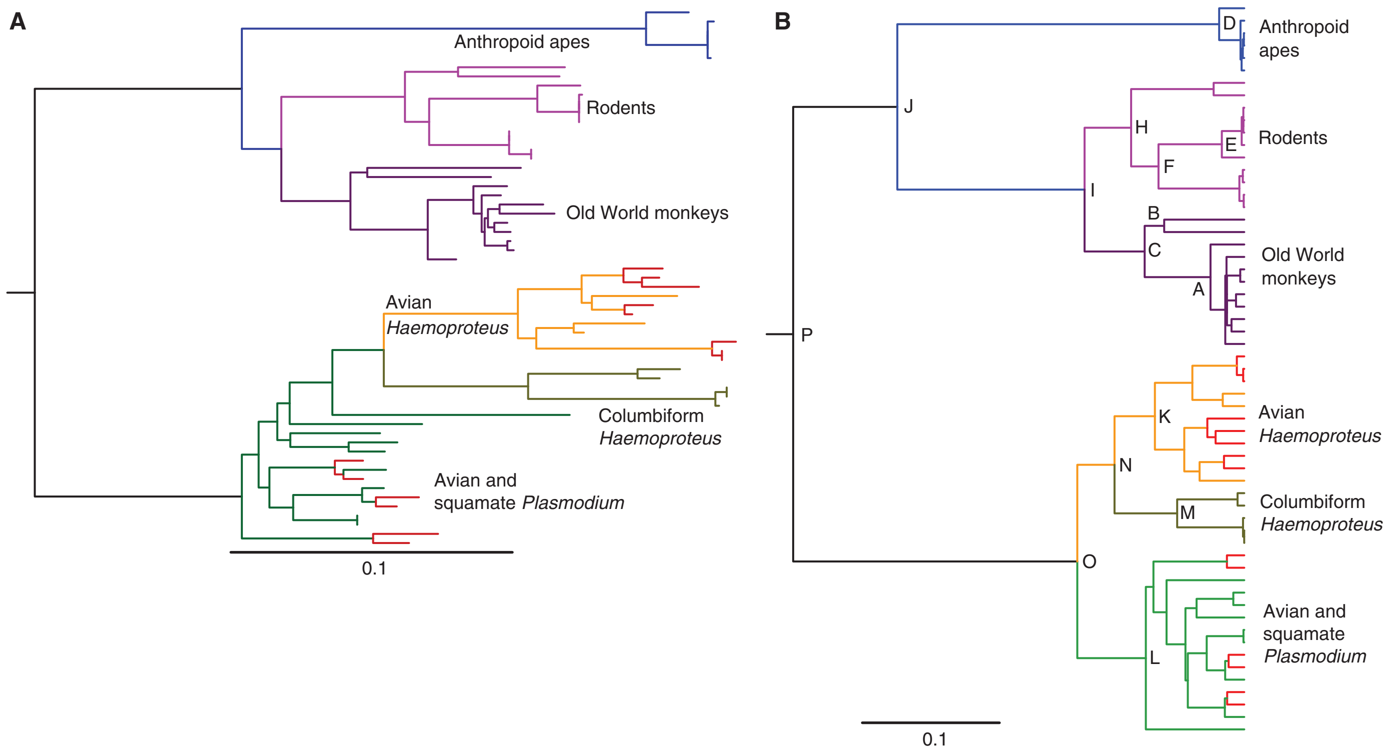

Fig. 3. Phylogenetic trees for representative haemosporidian cytochrome b sequences. (**A**) Tree produced by maximum likelihood optimization under a GTR + Γ model of nucleotide evolution. (**B**) Tree produced under a GTR + Γ model of nucleotide evolution using a strict clock. Letters in (B) indicate aged nodes (see Table 1). Calibration pairs in both panels are indicated in red.

of k estimated from these parasite/host ratios was $2 \times 0.292 = 0.584 \pm 0.096$ SEM [95% confidence interval (CI), 0.394 to 0.774]. Thus, in seven comparisons of genetic distance in avian haemosporidian cytochrome b sequences, ranging from 1 to 3% sequence divergence, the estimated rate of nucleotide substitution was close to 60% of the host rate. (See appendix S4 for an analysis of the variance in genetic distances among pairs of host and parasite taxa.)

An important assumption in our calibration is that switching times are distributed uniformly over the age of the host lineage. Alternatively, new host lineages might be available immediately for "colonization," and parasites switch quickly to the new host. In this case, the ages of endemic parasite lineages would be skewed toward the host age, with few host/parasite genetic distance ratios at low values. In our data, the ratios of parasite to host genetic distances are broadly spread between 0.14 and 0.45 (Fig. 2) and are consistent with an even distribution of switching times (appendix S5).

Assuming the rate of nucleotide substitution in Haemosporida is 0.584 times that of their avian hosts, the parasite rate can be obtained directly from calibrations of the host rate. In the case of birds, a generally agreed-upon average value for the rate of nucleotide divergence in cytochrome b is ~0.021 My^{-1} (*21*, *22*). Fifty-eight percent of this rate is ~0.012 (1.2%) genetic divergence My^{-1} (0.002 SEM, 0.0079 to 0.0155 CI), which is equivalent to 0.83 My per 0.01 (1%) sequence divergence.

To calculate absolute divergence times for the major clades of the Haemosporida, we used two estimates of the depths of the major nodes. First, we produced DNA distance matrices for subsets of lineages using the F84 model of nucleotide substitution (appendices S6 and S7). Clades were identified on the basis of a maximum likelihood (ML) phylogenetic tree of 54 species, or lineages, of representative haemosporidian parasites, including the sister pairs analyzed here (Fig. 3A). Ages of the basal nodes within designated clades were calculated as the means of the distances between all pairs of species or lineages descending from the two branches emanating from the node. Second, we produced a phylogenetic tree under a strict clock assumption, which resulted in branch lengths proportional to time. The resulting phylogeny is rooted between the clades of mammalian and avian-reptilian parasites and has a topology similar to that of the ML tree rooted at this point (Fig. 3B).

The estimated node depths in the clock-enforced (ultrametric) tree match reasonably well those calculated independently from sequence distances (Table 1), particularly with respect to the origins of the major clades: rodent versus Old World monkey (OWM) (ultrametric, 9.3 My, and F84, 8.7 My); basal node in mammalian *Plasmodium* (11.9 and 12.8, respectively); avian *Haemoproteus* versus *Plasmodium* (9.3 and 9.0); avian-reptilian versus mammalian Haemosporida (16.9 and 16.2). Although the estimated age of the split between *P. gonderi* and the *Plasmodium* parasites of Asian Old World monkeys was similar (2.8 and 2.8), estimated ages for many of the other mammalian nodes were younger in the clock-enforced tree, including nodes within the rodent malarias and the node ancestral to *P. falciparum* and *P. reichenowi* (1.2 and 2.5) (appendix S7).

The divergence of the human *P. falciparum* from the chimpanzee *P. reichenowi* dates to either 2.5 Ma (F84 distance) or 1.2 Ma (ultrametric distance), which is considerably more recent than the estimated divergence time of their hosts, on the basis of fossil evidence and corroborated by molecular dating (4 to 7 Ma) (*23*). The divergence times between the parasites of African and Asian Old World monkeys, estimated—if we assume codivergence—from primate fossil evidence at 6 and 10 My (*24*) and used by Hayakawa *et al*. (*13*) to calibrate age on their parasite phylogenetic tree, date to 2.8 Ma with both F84 and ultrametric distances.

The basal node in the phylogeny of contemporary haemosporidian parasites in the genera *Plasmodium* and *Haemoproteus* can be dated to between 16 and 17 Ma, well after the evolutionary diversification of their hosts, as well as fossil evidence of the parasites in dipteran vectors preserved in amber (appendix S8). Thus, the history of the contemporary Haemosporida is one of rapid diversification and spread through the terrestrial vertebrate classes (*10*, *25*). In addition, both the avian-reptilian and mammalian parasite clades have long stems, indicative of considerable pruning (extinction) of lineages since their origin. With respect to cytochrome b [but not other genes such as the mitochondrial cytochrome oxidase I and the apicoplast caseinolytic protease C (ClpC)], the divergence between bird-reptile and mammal parasite clades involved substantial protein evolution (i.e., nonsynonymous nucleotide substitution) possibly associated with the shift between nucleated and nonnucleated erythrocytes (*26*). Shifts between avian and reptilian hosts have occurred more recently and likely several times (*27*, *28*); birds and reptiles both have nucleated erythrocytes.

The age estimates for nodes in the parasite phylogeny emphasize that a new disease might emerge in a host soon after its origin and at any time thereafter. The lineage of *Plasmodium falciparum* evidently has infected the ancestors of humans for several million years and likely was relatively benign through much of that period, as is

Table 1. Genetic distances based on the mitochondrial cytochrome b gene and estimated ages of principal nodes in a phylogenetic tree of the Haemosporida. Genetic distances were obtained in Phylip-3.69 (program dnadist.exe) using the following default settings: F84 model, Ts/Tv = 2.0, homogeneous substitution rate. The depth of each node was calculated as the average pairwise distance between sequences on either side of the node. Upper and lower confidence limits are based on the 95% CI calculated for the ratio of parasite-to-host nucleotide substitution. No standard deviation (SD) is available for (B) and (G) because only a single sequence from each side of the split was used to estimate the genetic distance.

Comparison	Genetic distance			Age (My), 95% CI		
	Sample	Mean	SD	Mean	Lower	Upper
A. African (*P. gonderi*) versus Asian Old World monkeys (OWM)	1,8	0.033	0.005	2.76	2.14	4.19
B. Between *P. ovale* and *P. malariae*	1,1	0.072		6.03	4.67	9.16
C. Between *P. ovale* or *P. malariae* and sister OWM clade	2,9	0.074	0.006	6.19	4.79	9.41
D. *P. falciparum* versus *P. reichenowi* (human-chimp)	3,1	0.030	0.001	2.49	1.93	3.79
E. *P. berghii* versus *P. yoellii* (rodent pathogens)	1,3	0.032	0.001	2.67	2.07	4.06
F. *P. chabaudi* versus (D) (rodent pathogens)	3,4	0.066	0.007	5.51	4.27	8.38
G. *P. vinckii* versus *P. atheruri* (rodent pathogens)	1,1	0.061		5.07	3.93	7.71
H. Basal split in rodent *Plasmodium*, (G) versus (F)	2,9	0.081	0.006	6.77	5.25	10.29
I. Rodent versus OWM	4,6	0.105	0.008	8.73	6.76	13.26
J. Basal split in mammal *Plasmodium*	2,6	0.154	0.011	12.82	9.93	19.49
K. Basal split in avian *Haemoproteus*	5,6	0.076	0.015	6.37	4.93	9.68
L. Basal split in avian P*lasmodium*	3,11	0.085	0.011	7.06	5.47	10.74
M. Basal split within dove *Haemoproteus*	2,2	0.094	0.005	7.85	6.08	11.92
N. *Haemoproteus* versus *Parahaemoproteus*	5,10	0.131	0.007	10.93	8.46	16.60
O. Avian *Haemoproteus* versus *Plasmodium*	10,6	0.108	0.015	9.01	6.98	13.70
P. Avian versus mammalian haemosporidians	7,7	0.195	0.018	16.21	12.56	24.64

the case of most haemosporidian parasites (*29*, *30*). The recent expansion of the *P. falciparum* population, evidenced by its low genetic diversity (*31*), and the emergence of malaria as a major disease in humans, almost certainly was associated with the origins of agriculture and increasing population density, as well as large-scale movements of humans and introduction of the parasite to susceptible human populations (*29*, *30*, *32*).

The haemosporidian parasites of terrestrial vertebrates apparently began to diversify ~20 Ma, possibly displacing other types of parasites in the phylum Apicomplexa and, through host switching, bridging several hundred million years of vertebrate evolution. Because of their prevalence and broad distribution among terrestrial vertebrates, haemosporidian parasites make an excellent model system for investigating host-parasite coevolutionary relationships, host switching, and emerging diseases. A time calibration for the evolution of the group now provides a context for haemosporidian evolution with respect to host diversification, biogeographic distribution, and environmental change.

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An Autophagy-Enhancing Drug Promotes Degradation of Mutant α_1-Antitrypsin Z and Reduces Hepatic Fibrosis

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In the classical form of α_1-antitrypsin (AT) deficiency, a point mutation in AT alters the folding of a liver-derived secretory glycoprotein and renders it aggregation-prone. In addition to decreased serum concentrations of AT, the disorder is characterized by accumulation of the mutant α_1-antitrypsin Z (ATZ) variant inside cells, causing hepatic fibrosis and/or carcinogenesis by a gain–of–toxic function mechanism. The proteasomal and autophagic pathways are known to mediate degradation of ATZ. Here we show that the autophagy-enhancing drug carbamazepine (CBZ) decreased the hepatic load of ATZ and hepatic fibrosis in a mouse model of AT deficiency–associated liver disease. These results provide a basis for testing CBZ, which has an extensive clinical safety profile, in patients with AT deficiency and also provide a proof of principle for therapeutic use of autophagy enhancers.

The classical form of α_1-antitrypsin (AT) deficiency is caused by a point mutation (substitution of lysine for glutamate at residue 342) that alters the folding of an abundant liver-derived plasma glycoprotein during biogenesis and also renders it prone to polymerization (*1*). In addition to the formation of insoluble aggregates in the endoplasmic reticulum (ER) of liver cells, there is an 85 to 90% reduction in circulating concentrations of AT, the predominant physiologic inhibitor of neutrophil elastase. Liver fibrosis and carcinogenesis are caused by a gain–of–toxic function mechanism. Indeed, AT deficiency is the most common genetic cause of liver disease in childhood but can also present for the first time with cirrhosis and/or hepatocellular carcinoma in adulthood (*1*).

Genetic and/or environmental modifiers determine whether an affected homozygote is susceptible to liver disease (*2*). Two general explanations for the effects of such modifiers have been postulated: variation in the function of intracellular degradative mechanisms (*3*, *4*) and/or variation in the signal transduction pathways that are activated to protect the cell from protein mislocalization and/or aggregation. As for degradation, the proteasome is responsible for degrading soluble forms of α_1-antitrypsin Z (ATZ) (*5*), and macroautophagy is specialized for disposal of the insoluble polymers and aggregates (*6*, *7*). However, disposal of ATZ may involve other degradative mechanisms, as yet not well defined (*8*, *9*). In terms of cellular response pathways, accumulation of ATZ activates nuclear factor κB (NF-κB)

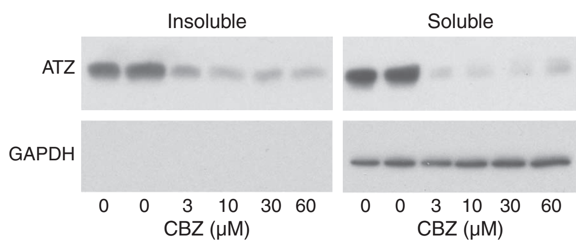

Fig. 1. Effect of CBZ on steady-state levels of ATZ in the HTO/Z cell line. Immunoblot analysis of HTO/Z cells treated with various concentrations of CBZ, separated into soluble and insoluble fractions, and then probed with antibodies to AT (**top**) and to GAPDH (**bottom**).

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