# Comparative Gene Evolution in Haemosporidian (Apicomplexa) Parasites of Birds and Mammals

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ABSTRACT: Haemosporidian parasites of birds and mammals reproduce asexually inside nucleated and nonnucleated host erythrocytes, respectively. Because of these different parasite environments and because bird parasites are paraphyletic, we evaluated whether patterns of parasite molecular evolution differ between host groups. We compared two mitochondrial (mt) genes and one apicoplast gene across mammal Plasmodium, bird Plasmodium, and bird Parahaemoproteus. Using a molecular phylogenetic approach, we show that the parasite mt cytochrome b (cyt b), mt cytochrome oxidase I (COI), and the apicoplast caseinolytic protease C (ClpC) exhibit similar levels of sequence divergence, yet each gene tree presents a strikingly different pattern of internal versus terminal branch lengths. In cyt b, the ratio of nonsynonymous (NS)-to-synonymous substitutions (dN/dS) is markedly elevated along the internal branch linking mammalian and avian parasites despite the sister relationship between mammal and bird Plasmodium. This is not the case for either COI or ClpC. When NS substitutions are excluded from the parasite cyt b alignment, the resulting phylogenetic tree resembles that of COI (both with and without NS substitutions). The high dN/dS ratio in the cyt b branch separating avian and mammalian parasites and a mammal-parasite codon bias suggest that adaptive evolution has distinguished mammal and bird parasites.

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## Introduction

Haemosporidian parasites of birds and mammals occupy different physiological environments inside the hosts' erythrocytes, which are nucleated in birds and reptiles, and nonnucleated in mammals. Phylogenetic studies indicate that haemosporidian parasites of mammals, birds, and lizards share a common ancestor with exclusively avian parasites of the genera Leucocytozoon and Haemoproteus. Derived avian parasites comprise the genera Parahaemoproteus, which are transmitted by ceratopogonid midges and do not multiply in the peripheral blood, and Plasmodium (shared with lizard hosts), which are transmitted by culicine mosquitoes and which undergo cycles of proliferation in the blood stream. Mammalian Plasmodium parasites have a sister relationship to avian/squamate Plasmodium but are transmitted by anopheline mosquitoes (Valkiunas 2005). The nucleated host erythrocytes that harbor avian parasites of both genera provide a strong contrast with the nonnucleated erythrocytes that harbor mammalian Plasmodium parasites. This contrast allows researchers to partition the influences of phylogenetic relationship and environment on molecular evolution, particularly with respect to the unique and highly simplified mitochondrial (mt) genome of the parasites, which is a 6-kb, concatentated genome with three protein-coding genes shared among all haemosporidians examined to date. The two genera of avian parasites share the environment of a nucleated erythrocyte, but differ with respect to their life cycles within the vertebrate hosts and with respect to their vectors. The avian and mammalian Plasmodium parasites have a common life cycle and similar dipteran vectors but differ in occupying nucleated versus nonnucleated erythrocytes.

Mt genes involved in the electron transport chain (ETC) might be expected to have undergone adaptive evolution associated with the transition from bird to mammal hosts. Indeed, there are known structural differences in the mitochondria from bird and mammal Plasmodium species (Rudzinska 1969 in van Dooren et al. 2006), but findings from these early studies have not been addressed in the age of genomics. In this study, we describe patterns of synonymous (S) and nonsynonymous (NS) nucleotide substitution within the phylogenetic trees of two parasite mt genes, both involved in the ETC, and one apicoplast gene. Even without understanding the molecular and biochemical mechanisms in detail, we would predict that the transition in erythrocyte environment (bird to mammal hosts) is likely to be more important to basic metabolic enzymes than transitions between vectors or patterns in the life cycle.

The reduced mt genome of haemosporidian parasites (and other Apicomplexa; Slamovits et al. 2007) contains only three genes, all of which are involved in the ETC: cytochrome b (cyt b) and cytochrome oxidases I (COI) and III (COIII). Haemosporidian mtDNA is also unusual in that multiple copies of the genome are linearly concatenated (Vaidya et al. 1989; Feagin 1994; Wilson and Williamson 1997) and mtDNA replication might involve concerted evolution (Burger et al. 2003), which could greatly reduce the observed rate of mutation (reviewed in Mather and Vaidya 2008). Moreover, unique DNA replication processes within haemosporidian parasites, involving recombination and gene conversion, could lead to rapid incorporation or rejection of mutational changes (reviewed in Preiser et al. 1996).

Haemosporidian parasite mitochondria rely on an influx of proteins manufactured in the apicoplast and nuclear genomes to carry out cellular respiration (van Dooren et al. 2006) and have lower metabolic rates than the mitochondria of vertebrate hosts (Mather and Vaidya 2008). The sole function of the haemosporidian ETC has been shown to be the regeneration of a critical enzyme during pyrimidine biosynthesis (Painter et al. 2007) and can be rendered redundant with the import of dihydroorotate dehydrogenase (van Dooren et al. 2006; Painter et al. 2007). However, ETC components, particularly cyt b, are crucial to the survival of haemosporidian parasites (e.g., Plasmodium; Painter et al. 2007) because the ETC removes byproducts of oxidative phosphorylation, which does occur in the parasite mitochondrion despite the organelle's reduced functionality (see, Mather and Vaidya 2008). Haemosporidian parasites maintain limited functionality in an

energetically expensive organelle, and the retention of only three genes might reflect a legacy of conservatism or constraint throughout the evolutionary history of these parasites.

We constructed datasets of gene sequences that spanned mammalian parasites (Plasmodium) and avian parasites in the genera Parahaemoproteus and Plasmodium (hereafter: full parasite data set 5 mammal + bird). We evaluated patterns of molecular evolution across parasite markers specifically looking for evidence of elevated adaptive evolution in the ratios of NS and S substitutions. We included parasite mt cyt b, well studied and known to be critical to parasite survival, as well as mt COI (in a separate data set) for these taxa. We also analyzed the parasite apicoplast gene, caseinolytic protease C (ClpC; Wilson et al. 1996), to provide a comparison in a different maternally inherited genome.

## Materials and Methods

### Datasets

Four parasite datasets each included 20 individual sequences, either derived from GenBank or generated in our laboratory (supplementary appendix S1, Supplementary Material online). Three of these datasets contained different genes (cyt b: ~900 bp, ~COI: 900 bp, and ClpC: ~500 bp) from the same set of parasite lineages (avian and mammalian). The fourth data set included cyt b sequences from the same haemosporidian lineages as in the first three datasets but obtained from different host individuals. Three additional datasets, with 20 individuals each, contained only mammalian Plasmodium, avian Plasmodium, or avian Parahaemoproteus (Martinsen et al. 2008; Valkiunus G, personalcommunication).

Each data set was assembled in BioEdit (Hall 1999) and aligned via the Clustal W multiple alignment feature (Thompson et al. 1994). We translated each nucleotide sequence into its amino acid sequence, verified sequence alignment, and constituent amino acid residues against a Plasmodium falciparum protein sequence (or Toxoplasma in the case of ClpC; Waller and McFadden 2005), and trimmed each data set to contain only complete codons for analysis of amino acid substitutions. For the following analyses, all datasets were analyzed in the same way.

### Tree Reconstruction and Analyses of Molecular Evolution

We reconstructed molecular phylogenies using maximum likelihood (ML) with the Hasegawa–Kishino–Yano (HKY) + I + Γ model of nucleotide substitution which allows for differing transition and tranvsersion rates, proportion of invariable sites, and rate heterogeneity across sites, in Treefinder (1,000 reconstructions; Jobb 2008).

Using PAML version 4.0 (Yang 2007), we conducted branch model analyses on each parasite sequence data set and tree (Yang et al. 2000) using M0, M1, and M2. Model 0 estimates one $d_N/d_S$ ratio across all sites under the assumption that no sites are selected over others; M1 allows $d_N/d_S$ to vary freely across all branches; and M2 allows for $d_N/d_S$ to be estimated for sets of branches, in this case a foreground (mammal parasites) and a background (bird parasite clades) rate (i.e., different $d_N/d_S$ ratios for mammal and bird parasites.

We also conducted site model analyses (N sites), models 0 (see above), 1a, 2a, 7, and 8, with the F3X4 model of amino acid substitution to account for heterogeneity in codon usage (observed codon usage) within each data set. Model 1a estimates the proportion and $d_N/d_S$ ratio of sites under negative (purifying) selection ($d_N/d_S$ between 0 and 1), assuming that the majority of sites are evolving neutrally ($d_N/ d_S = 1$). Model 2a adds to Model 1a the proportion of sites under positive (directional) selection ($d_N/d_S > 1$). Models 7 and 8 approximate $d_N/d_S$ variation over sites by a beta distribution with (Model 8) and without (Model 7) estimating the proportion and $d_N/d_S$ ratio of positively selected sites.

Finally, we conducted site model tests using the FmutSel model to evaluate selection on silent sites (Yang and Nielsen 2008). This model estimates parameters that incorporate (observed) preferred codons, which can then

Table 1. Results of Branch-Model Analyses of Parasite DataSets Using PAML Version 4.0 (Yang 2007).

| | Parasite Cyt b 1: 918 Sites | Parasite CO1: 912 Sites | Parasite ClpC: 498 Sites |
|---|---|---|---|
| Model | | | |
| M0 lnL | 24,119.88 | 24,416.63 | 22,965.36 |
| j | 1.55 | 3.03 | 1.05 |
| Parameters | $v_0$ 5 0.03 | $v_0$ 5 0.016 | $v_0$ 5 0.029 |
| Model | | | |
| M1 lnL | 24,236.36 | 24,378.38 | 22,948.88 |
| j | 1.51 | 3.12 | 1.63 |
| Model | | | |
| M2 lnL | 24,116.76 | 24,410.42 | 22,963.59 |
| j | 1.58 | 3.12 | 1.63 |
| Parameters | $v_1$ 5 0.021, $v_2$ 5 0.035 | $v_1$ 5 0.022, $v_2$ 5 0.010 | $v_1$ 5 0.038, $v_2$ 5 0.022 |

be tested against a model that incorporates only mutational biases (FmutSel0). We performed these analyses using the mammal + bird parasite datasets for each gene, designating the mammal-parasite branch as the foreground with bird parasite clades as the background.

After simultaneously running models within each of the above sets, we conducted post hoc likelihood-ratio tests to determine which model(s) produced significantly better estimates of the evolution of the sequence data (Nielsen and Yang 1998). The output of each analysis was then evaluated in several ways.

For all datasets, we calculated indices of nucleotide bias, including GC content at the third codon position. These analyses were performed in Codon W (Mobyle v. 0.96). The mean values of each index were included in analysis of variance with gene or clade as the independent factor, as appropriate (SPSS15).

### Relative Timing of Recent Common Ancestry

We evaluated the relative time to the most recent common ancestor (TMRCA) within each data set using the program BEAST (Drummond and Rambaut 2007). Each data set incorporated the HKY + I + Γ model of nucleotide substitution (as above). Our analyses allowed mutation rate heterogeneity among branches of the phylogeny, producing relative root ‘‘age’’ (root height) estimates in which any bias due to disproportionately long branches was reduced (relaxed clock: uncorrelated lognormal). Priors were optimized by the program using the Yule tree option. Unlike coalescent approaches in which only some lineages are assumed to leave descendants, the Yule tree option assumes that such lineages have already been pruned (Drummond and Rambaut 2007).

## RESULTS

### Models of Codon Evolution

Among branch models across parasite datasets, M1 provided a significantly better fit ($P < 0.01$; table 1) than either M0 or M2 indicating substantial heterogeneity in $d_N/d_S$ throughout each gene tree. In the mt genes, cyt b ($P = 0.01$) and COI ($P < 0.001$), M2 (different $d_N/d_S$ in bird vs. mammal parasites) provided a significantly better fit than M0, but not in the apicoplast gene, ClpC ($P = 0.06$).

In site-based models, M7 provided the best fit to the data, which suggests that although substantial heterogeneity in codon substitution rates exist within each data set, the data are adequately fit by a model in which most sites are dominated by negative or purifying selection ($\omega < 1$; supplementary appendix S2, Supplementary Material online), that is a lack of positive selection. More NS substitutions occur at cyt *b* binding sites in the internode between bird and mammal parasites than at

any other internal or terminal branch (Conway et al. 2000; supplementary appendix S3, Supplementary Material online).

The results of models incorporating selection on silent sites (mammal vs. bird parasites) differed between genes. For both cyt b and ClpC, the FmutSel model provided a significantly better fit than the FmutSel0 model (df = 41, cyt b: $P = 0.005$; ClpC: $P \ll 0.001$), suggesting that codon usage differs between bird and mammal parasites. For CO1, however, the fits obtained by the FMutSel and FmutSel0 models did not differ significantly.

## Gene Trees

Gene trees for the mammal + bird parasite data set, with branch lengths proportional to the number of nucleotide substitutions per codon, produced strikingly different patterns for the three genes (cyt b, COI, and ClpC; fig. 1). The total number of substitutions does not differ between genes, as shown by their similar values of sequence divergence (table 2). However, in the cyt b gene tree, the internal branch separating parasites of birds and mammals is long compared with terminal branches and other internal branches and includes a greater number of active site substitutions than other branches; nucleotide substitutions are also concentrated on the branch leading to P. falciparum (fig. 2, supplementary appendix S3, Supplementary Material online). Substitutions per codon position are more evenly distributed between internal and terminal branches within COI (fig. 1b), whereas change occurs primarily along terminal branches in ClpC, with no separation between bird and mammal-parasite clades (fig. 1c). An analysis conducted using a nuclear marker (diacylglycerol Oacyltransferase) also showed no separation between bird and mammal parasites (results not shown but data and results available on request). Cyt b and CO1 gene trees based solely on S substitutions are qualitatively similar, in that the branch between bird and mammal parasites in cyt b is not disproportionately long (fig. 3). These results suggest that the different appearance of the haplotype networks does not reflect a difference between internal and terminal branches so much as a difference in the divergence between bird and mammal-parasite sequences.

## Codon-Usage Bias

Haemosporidian genomes are AT rich, and the GC content of third position sites is low across parasite genes: cyt b, 0.067; CO1, 0.052; and ClpC, 0.020. GC content of third position sites in ClpC differs significantly from the two mt genes ($P < 0.0001$), but the mt genes differ only marginally from one another ($P = 0.025$). In analyses with expanded datasets (by clade), mammal and bird Plasmodium clades do not differ in GC content, but both differ significantly from bird Parahaemoproteus in GC content ($P < 0.0001$).

## Genetic Diversity and Relative Divergence Times

For mammal + bird parasite lineages across the three genes, uncorrected sequence divergence values were almost identical (0.170–0.176, table 2). Thus, average rates of nucleotide substitution have been the same in these genes over the diversification of contemporary haemosporidian parasites, in spite of significant heterogeneity in the distribution of evolutionary change between parasites of birds versus mammals.

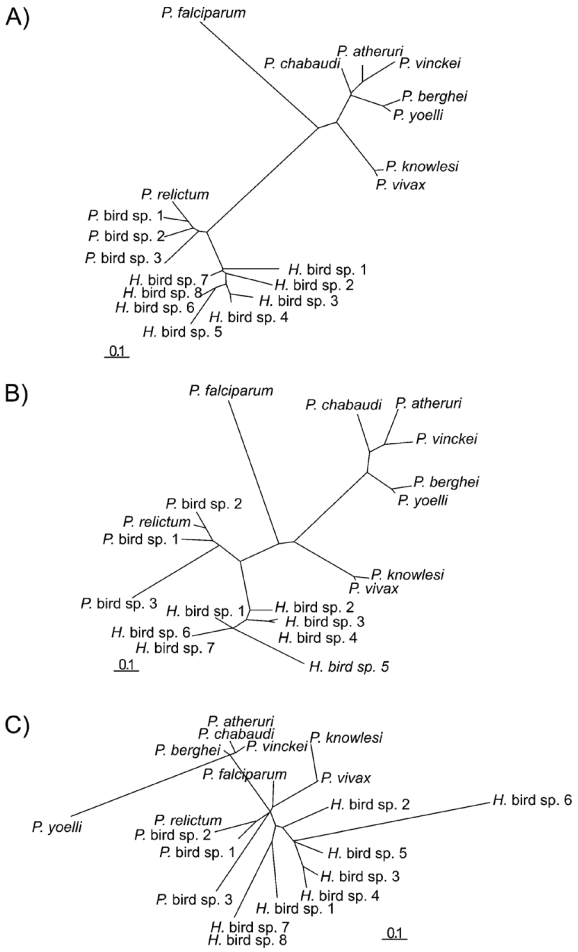


FIG. 1. Parasite gene trees reconstructed with ML (HKY + I + Γ). Branch lengths are proportional to the number of NS and S substitutions per codon using the nearly neutral model of codon substitution. (a) Parasite cyt b, (b) parasite CO1, and (c) parasite ClpC. Note that avian Parahaemoproteus lineages are referred to as H. species, reflecting their original taxonomic designation, Haemoproteus, to distinguish them from avian Plasmodium.

## Discussion

Parasite gene sequences have diverged substantially with respect to resulting amino acid substitutions in association

Table 2. Summary of Site-Model Results by Data Set.

| Parameters | Divergence | Model 1a | TMRCA |
|---|---|---|---|
| All parasite cyt b | 0.02–0.18 | v< 1, 0.98; v5 1, 0.02 | 0.172 |
| All parasite CO1 | 0.03–0.17 | v< 1, 0.98; v5 1, 0.02 | 0.170 |
| All parasite ClpC | 0.00–0.17 | v< 1, 0.90; v5 1, 0.10 | 0.176 |
| Avian Parahaemoproteus cyt b | 0.00–0.09 | v< 1, 0.99; v5 1, 0.01 | 0.083 |
| Avian Plasmodium cyt b | 0.00–0.08 | v< 1, 0.99; v5 1, 0.01 | 0.084 |
| Mammal Plasmodium cyt b | 0.00–0.13 | v< 1, 0.98; v5 1, 0.02 | 0.156 |

Divergence: the range of uncorrected sequence divergence values within parasite lineages. Model 1a parameters: proportions of sites under negative selection (x, 1) and neutral evolution (x5 1). TMRCA: estimates of clade age based on a model accounting for heterogeneity in branch lengths.

with the bird–mammal split, but only in cyt b, and not in COI and ClpC. None of the genes shows a similar level of adaptive diversification associated with the vector and lifehistory differences between Parahaemoproteus and Plasmodium parasites. The unusual nature of parasite cyt b becomes clearer by examining the cyt b gene tree relative to those of parasite COI and ClpC (fig. 1), which, remarkably, have similar levels of uncorrected pairwise genetic divergence (table 2). When NS substitutions are removed from the cyt b data set (by nucleotide rather than codon), internal and terminal branch lengths become quite similar (fig. 3a), as in the case of a COI gene tree based on both substitution types (fig. 1b) or only S substitutions (fig. 3b). Like cyt b, COI is important in cellular respiration yet does not exhibit nearly the degree of terminal versus internal branch segregation of substitution types (fig. 1a and b). This may be indicative of selection on cyt b in the transition between bird and mammal hosts and/or the relative absence of a similar level of amino acid substitution in COI may be suggestive of further reduction and/or redundancy in the parasite ETC, that is, COI is becoming obsolete.

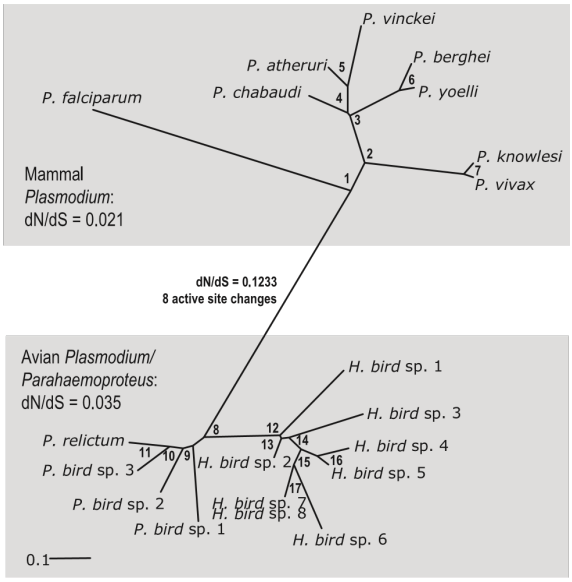


FIG. 2. Parasite cyt b gene tree (as in fig. 1a). $d_N/d_S$ is indicated on the branch between bird and mammal parasites, along with the number of NS substitutions that occur at active sites. Nodes are numbered to correspond with supplementary appendix S3, Supplementary Material online, in which NS substitutions are detailed for each internal and terminal branch. Note that avian Parahaemoproteus lineages are referred to as H. species, reflecting their original taxonomic designation, Haemoproteus, to distinguish them from avian Plasmodium.

Future studies will need to include more genes, although if COIII, the one mt gene not included here, likewise shows a low $d_N/d_S$ ratio between bird and mammal parasites, that would provide more evidence that cyt b is unique. In fact, all phylogenetic analyses based on cyt b to date that include both bird and mammal-parasite lineages show that mammal parasites are derived on a very long branch.

The separation between bird and mammal parasites cannot be explained by codon usage: third position GC content. This index does not differ between mt genes across all parasites; both mammal and bird Plasmodium lineages are essentially identical in GC content, and both differ from bird Parahaemoproteus, suggesting retention of genetic similarity due to shared evolutionary history. However, patterns of selection on silent sites are in fact different between bird (both genera) and mammal parasites in cyt b and ClpC.

Our results are all the more striking because the parasite lineages chosen within the three clades (avian Parahaemoproteus, avian Plasmodium, and mammal Plasmodium) represent virtually the extent of known diversity within each clade. According to multigene phylogenetic hypotheses, avian Plasmodium is a sister taxon to mammal Plasmodium rather than to avian Parahaemoproteus (Martinsen et al. 2008, Outlaw DC and Ricklefs RE, unpublished data). Clearly in the case of cyt b, selection

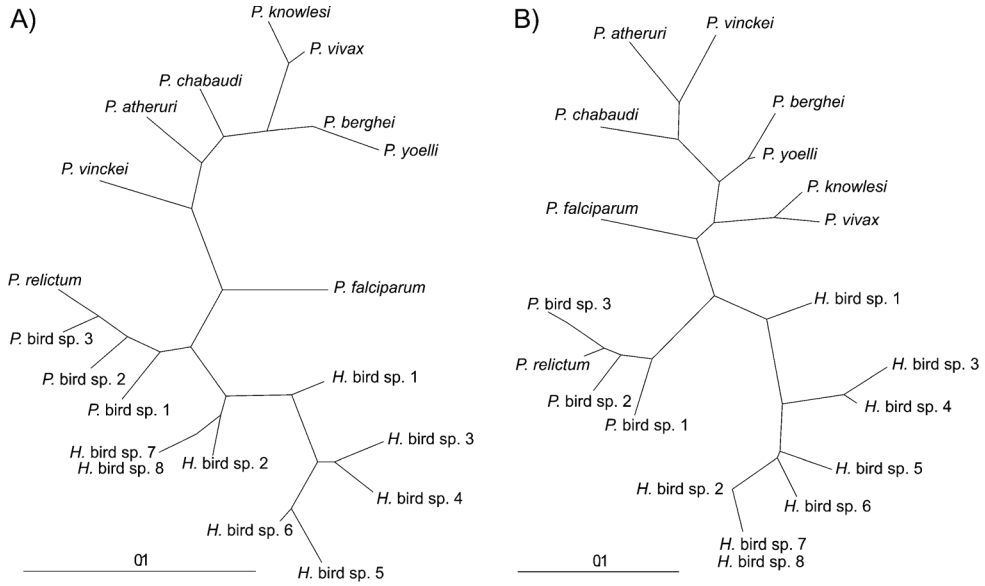


FIG. 3. Cyt b (a) and COI (b) gene trees based on S substitutions only. Gene trees were reconstructed with ML (HKY + I + Γ). Branch lengths are proportional to the number of S substitutions per nucleotide site. Note that avian Parahaemoproteus lineages are referred to as H. species, reflecting their original taxonomic designation, Haemoproteus, to distinguish them from avian Plasmodium.

(on mammal parasites), constraint (on bird Plasmodium and Parahaemoproteus), or some other mechanism has led to separation between bird and mammal parasites (figs. 1 and 2).

Haemosporidian mt function has been examined only in mammalian Plasmodium, and therefore, we cannot speculate on potential functional differences between bird and mammal-parasite cyt b proteins. Two factors might be relevant, however. The first is the fact that mammal erythrocytes are nonnucleated, whereas bird erythrocytes (and those of lizards, which have parasites closely related to avian parasites) are nucleated. Indeed, in preliminary codon analyses for this study in which lizard parasite cyt b data were included, lizard and bird parasites exhibited similar substitution patterns, and both differed markedly from those in mammal parasites (results not shown). The second is that differences in insect vectors between the groups, albeit based on limited data for birds and nonanthropoid mammals, do not match the separation of bird and mammal-parasite cyt b sequences. A consensus from many studies suggests that vectors of bird and mammal Plasmodium (culicine mosquitoes) are more similar to each other than they are to the vectors of Parahaemoproteus (ceratopogonid Cuclicoides midges). Thus, cyt b amino acid evolution in parasites appears to be associated with vertebrate hosts rather than with vectors.

Why the differences between parasitizing bird/reptile versus mammal hosts would produce so much evolutionary change in parasite cyt b is not a question we can answer with current data, but we can speculate that mammal Plasmodium embarked on a nonreversible trajectory when it invaded the unique mammalian erythrocytes (Hagner et al. 2007). Mammal haemosporidian parasites appear to be derived from those of birds and reptiles (Martinsen et al. 2008). Not coincidentally perhaps, several structural differences distinguish the mitochondria of avian and mammalian Plasmodium parasites (see van Dooren et al. 2006). For example, in the vertebrate host, the mitochondria of avian Plasmodium have more cristae than those of mammalian Plasmodium (Rudzinska 1969, in van Dooren et al. 2006). Cristae increase the inner-membrane reactive surface area of mitochondria and are permeated with enzymes such as the cytochromes; reduced cristae imply a reduction in cytochrome activity in mammal parasites. The currently accepted relationships between these three clades—avian and mammal Plasmodium are sister clades (Martinsen et al. 2008)—adds strength to the argument that mammalian Plasmodium has adapted to mammal erythrocytes, especially if we assume that haemosporidian parasites originated in hosts with nucleated erythrocytes (e.g., lizards or birds).

## Supplementary Material

Supplementary appendixes 1–3 are available at Molecular Biology and Evolution online (http://www.mbe. oxfordjournals.org/).

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