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Diverse sampling of East African haemosporidians reveals chiropteran origin of malaria parasites in primates and rodents ☆

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ABSTRACT

Phylogenies of parasites provide hypotheses on the history of their movements between hosts, leading to important insights regarding the processes of host switching that underlie modern-day epidemics. Haemosporidian (malaria) parasites lack a well resolved phylogeny, which has impeded the study of evolutionary processes associated with host-switching in this group. Here we present a novel phylogenetic hypothesis that suggests bats served as the ancestral hosts of malaria parasites in primates and rodents. Expanding upon current taxon sampling of Afrotropical bat and bird parasites, we find strong support for all major nodes in the haemosporidian tree using both Bayesian and maximum likelihood approaches. Our analyses support a single transition of haemosporidian parasites from saurian to chiropteran hosts, and do not support a monophyletic relationship between *Plasmodium* parasites of birds and mammals. We find, for the first time, that *Hepatocystis* and *Plasmodium* parasites of mammals represent reciprocally monophyletic evolutionary lineages. These results highlight the importance of broad taxonomic sampling when analyzing phylogenetic relationships, and have important implications for our understanding of key host switching events in the history of malaria parasite evolution.

1. Introduction

Phylogenies are an important tool in the study of zoonotic epidemics, helping to determine the source of a pathogen or parasite, and enabling the prediction of future outbreaks by identifying evolutionary lineages with significant host-switching potential (Suzán et al., 2015). The utility of a phylogeny depends on adequate genetic and taxon sampling, and is limited by these two variables (Nabhan and Sarkar, 2012). Assumptions about evolutionary variables are often a necessary component of phylogenetic models, and these assumptions, too, can limit the accuracy of a phylogeny. Common problems facing phylogenetic analyses therefore are typically linked to sparse taxon sampling, gene sampling, or inaccurate evolutionary assumptions regarding outgroup assignment or rates of evolutionary change across disparate phylogenetic lineages.

Such problems have hindered phylogenetic analyses of malaria parasites (Apicomplexa: Haemosporida), which are one of the most diverse assemblages of protozoan parasites, including species that cause epidemic diseases in humans. In addition to sparse or biased taxon sampling, a lack of well-developed phylogenetic markers, and uncertainty regarding the root of the haemosporidian phylogeny, malaria systematists have also been hindered by the ambiguity of morphological features and complex life histories used to distinguish species in this group (Valkiūnas, 2005). Indeed, the evolutionary origin of the deadliest human parasite, *Plasmodium falciparum*, remains unresolved. Lateral transfer of a malaria parasite from birds to humans was once implicated in the high pathogenicity exhibited by *P. falciparum*, and was supported by early molecular studies (McCutchan et al., 1996; Waters et al., 1991). However, the discovery of a reservoir of diverse, closely-related parasites in non-human primates, and a plethora of studies in recent decades (*e.g.* Martinsen et al., 2008a; Outlaw and Ricklefs,

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2011; Perkins and Schall, 2002), have since rendered this avian origin hypothesis obsolete (Duval et al., 2010; Krief et al., 2010; Ollomo et al., 2009; Prugnolle et al., 2010).

The discovery of new malaria parasites in African primates has altered our understanding of how *P. falciparum* may have arisen in humans, and underscores the importance of thorough sampling when assessing the origin of epidemic pathogens or parasites. Major efforts to document haemosporidians in wild hosts have improved our knowledge of genetic diversity within the group (Beadell et al., 2009; Falk et al., 2011; Fecchio et al., 2013; Lacorte et al., 2013; Lutz et al., 2015; Svensson-Coelho et al., 2013), but many hosts and geographic regions remain largely unexplored. For instance, the Old World tropics contain some of the highest levels of vertebrate species richness (Davies and Buckley, 2011; Schipper et al., 2008; Jetz and Rahbek, 2001; Jetz et al., 2012), yet few broad scale surveys of Afrotropical or Asian haemosporidians have been conducted. Even fewer systematic studies in these regions have included both saurian (bird and reptile) and mammalian parasites in their phylogenetic analyses (Duval et al., 2012; Schaer et al., 2013). Parasites of Afrotropical bats are of particular interest due to their diversity and ambiguous positions in the haemosporidian tree of life. Molecular surveys of chiropteran haemosporidians have identified new species with potential to inform human malaria research (Schaer et al., 2013) and have suggested that improved sampling of chiropteran parasites may clarify the evolutionary origins of haemosporidians in other groups (Duval et al., 2007, 2012; Schaer et al., 2013; Witsenburg et al., 2012).

A major question is whether mammalian parasites form a clade, or whether chiropteran haemosporidians represent a secondary invasion of mammals by a parasite from a non-mammalian host – a hypothesis posited by several recent studies (Duval et al., 2012; Megali et al., 2011; Outlaw and Ricklefs, 2011; Witsenburg et al., 2012). Recent work employing multiple nuclear markers from both avian and mammalian parasites found support for a monophyletic relationship of all *Plasmodium* species, rendering mammalian haemosporidians a paraphyletic group. The same study found strong support for the assignment of *Leucocytozoon* as the outgroup to all other haemosporidians (Borner et al., 2016). Such discoveries have important implications for how life history traits, such as erythrocytic schizogeny, may have evolved. In light of such discoveries and new data from haemosporidians of disparate hosts and geographic regions, a re-evaluation of the phylogenetic history of malaria parasites is therefore both important and timely (Perkins, 2014; Rich and Xu, 2011).

In this study, we present a novel phylogenetic hypothesis for the haemosporidian tree of life, based on improved sampling of avian and mammalian hosts. Taxon sampling included the first large-scale systematic survey of neglected chiropteran parasites in the East African tropics from a range of habitats in Kenya, Malawi, Mozambique, Tanzania, and Uganda. We paired these data with comparable sampling of avian parasites, and sequenced genes from each of the three genomes present in malaria parasites (nuclear, mitochondrial, and apicoplast). Combining these new data with a broad representation of parasites from reptiles, humans, non-human primates (including novel primate parasites in the *Laverania* subgenus), and additional birds and bats, we re-evaluated major evolutionary relationships in the malaria tree of life. We explicitly tested the hypothesis that *Plasmodium* parasites from birds and mammals are monophyletic, while reconsidering the evolutionary relationships between chiropteran and non-chiropteran parasites of mammals. Support for our phylogenetic hypothesis suggests that bats, not birds or reptiles, were the ancestral hosts of extant *Plasmodium* parasites in mammals. Our results align well with previous studies, revealing that host-switching of parasites from bats to other vertebrates appears to be common throughout the haemosporidian phylogeny. This novel phylogenetic hypothesis has important implications for inferences regarding trait evolution and host shifts of haemosporidian parasites between vertebrate classes, as well as shifts between invertebrate vectors.

2. Methods

2.1. Sampling

We sampled 791 mammals, including 505 bats and 286 rodents and shrews (Supplemental Tables S1 and S2). Bats were sampled from both suborders of Chiroptera (Yinpterochiroptera and Yangochiroptera), representing 46 species from 8 families (Table 1). Sampling was conducted between 2009 and 2014, at sites in Kenya, Malawi, Mozambique, Tanzania, and Uganda (Fig. 1; Supplemental Table S3). Bats were captured using mist-nets, triple-high mist nets, harp traps, or hand nets (at roosts). In addition to bats, rodents and shrews from sites in Malawi, Mozambique, and Uganda were collected using a combination of Sherman, pitfall, and snap traps. Bird sampling was conducted concurrently at all sites, except for those in Kenya and Tanzania, according to previously described methods (Lutz et al., 2015), and included 1745 individuals representing 20 avian orders and 112 families (>400 species) (bird data available via the Field Museum of Natural History Bird Collection Database, fm1.fieldmuseum.org/birds/). Blood was stored on Whatman Classic FTA cards, and thin blood films were prepared when possible. All sampling was conducted in accordance with the Field Museum of Natural History IACUC, and voucher specimens of both mammals and birds are accessioned at the Field Museum of Natural History.

2.2. Identification and sequencing of haemosporidian parasites

Genomic DNA was extracted from whole blood that was stored on Whatman FTA Classic Cards, using the dried blood spot protocol of Qiagen Blood and Tissue Mini Kits (Qiagen, Valencia, CA). A polymerase chain reaction (PCR) protocol targeting a standard 478 bp barcoding region of the haemosporidian mitochondrial cytochrome *b* (Cytb) gene (Bensch et al., 2009a) was applied to all DNA samples in triplicate (*sensu* Lutz et al., 2015). Thin blood films prepared from fresh blood were fixed in the field with 100% methanol and subsequently stained with a 10% Giemsa solution in the lab. Blood smears from individuals detected to be positive for haemosporidians by PCR were screened under 1000× magnification for 100 fields for visual confirmation of infection and morphological identification of parasites when possible (Fig. 2). Blood films from rodents, shrews, and bats that screened negative by PCR were also examined to verify absence of haemosporidian parasites when possible (blood films were not available for all individuals).

Following detection of parasites, PCR products from barcoding screens were purified and sequenced on an ABI 3730 Automated DNA Sequencer (Applied Biosystems, Foster City, California). We then grouped sequences into unique lineages using the FaBox haplotype collapser (Villesen, 2007), with unique lineages being defined as having one or more SNPs at the Cytb locus. Due to the vast number of unique parasite lineages identified in birds we sampled, data from all countries were included in preliminary phylogenetic analyses to provide phylogenetic context for the selection of a subset of lineages, and ultimately parasites from Malawi and Mozambique were included in this study (Supplemental Table S2). For additional sequencing and phylogenetic analyses, we selected a subset of these collapsed parasite lineages (which included representatives from *Plasmodium*, *Haemoproteus*, *Parahaemoproteus*, *Leucocytozoon*, *Polychromophilus*, *Nycteria*, and *Hepatocystis*) such that broad and even coverage of genetic diver-

Table 1
Taxonomic sampling of bats from East Africa, with number of individuals sampled (n) and infected (n_i). Hosts from which haemosporidian parasites were molecularly identified are indicated in bold.

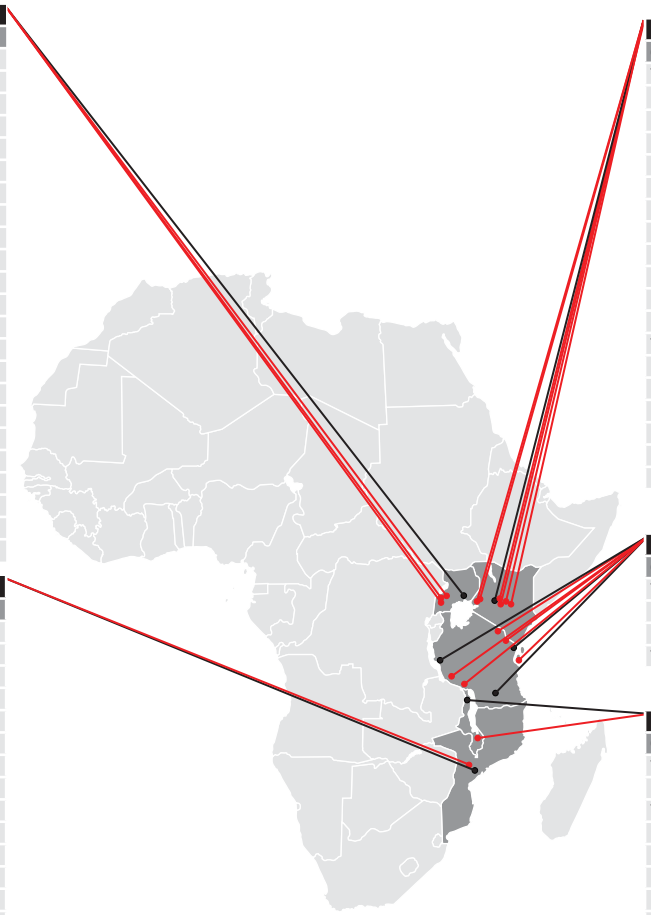
Host suborder	Host family	Host species	n_i	n	Parasite
Yangochiroptera	Emballonuridae	*Coleura afra*	0	7	
	Emballonuridae	*Taphozous mauritianus*	0	1	
	Miniopteridae	***Miniopterus africanus***	**4**	**20**	***Polychromophilus melanipherus***
	Miniopteridae	***Miniopterus cf. fraterculus***	**2**	**6**	***Polychromophilus melanipherus***
	Miniopteridae	***Miniopterus minor***	**5**	**13**	***Polychromophilus melanipherus***
	Miniopteridae	***Miniopterus natalensis***	**20**	**42**	***Polychromophilus melanipherus***
	Miniopteridae	***Miniopterus rufus***	**22**	**31**	***Polychromophilus melanipherus***
	Miniopteridae	***Miniopterus* sp.**	**2**	**2**	***Polychromophilus melanipherus***
	Molossidae	*Chaerephon pumilus*	0	2	
	Molossidae	*Tadarida cf. lobata*	0	1	
	Nycteridae	*Nycteris arge*	0	1	
	Nycteridae	*Nycteris aurita*	0	1	
	Nycteridae	*Nycteris macrotis*	0	1	
	Nycteridae	*Nycteris* sp.	0	2	
	Nycteridae	*Nycteris thebaica*	0	7	
	Vespertilionidae	*Glauconycteris humeralis*	0	1	
	Vespertilionidae	*Laephotis wintoni*	0	1	
	Vespertilionidae	*Myotis bocagii*	0	1	
	Vespertilionidae	***Myotis tricolor***	**1**	**3**	***Polychromophilus* sp.**
	Vespertilionidae	*Neoromicia capensis*	0	16	
	Vespertilionidae	*Neoromicia nana*	0	16	
	Vespertilionidae	*Neoromicia* sp.	0	6	
	Vespertilionidae	*Neoromicia tenuipinnis*	0	8	
	Vespertilionidae	*Pipistrellus hesperidus fuscatus*	0	11	
	Vespertilionidae	*Pipistrellus rueppellii*	0	2	
	Vespertilionidae	*Pipistrellus* sp.	0	1	
	Vespertilionidae	*Scotophilus dinganii*	0	1	
	Vespertilionidae	*Scotophilus* cf. *leucogaster*	0	1	
Yinpterochiroptera	Hipposideridae	*Hipposideros beatus*	0	3	
	Hipposideridae	*Hipposideros caffer*	0	38	
	Hipposideridae	***Hipposideros cyclops***	**3**	**3**	***Nycteria* sp.**
	Hipposideridae	*Hipposideros ruber*	0	43	
	Hipposideridae	*Hipposideros* sp.	0	1	
	Megadermatidae	*Cardioderma cor*	0	2	
	Megadermatidae	*Lavia frons*	0	11	
	Pteropodidae	*Epomophorus labiatus*	0	6	
	Pteropodidae	*Epomophorus wahlbergi*	0	8	
	Pteropodidae	***Epomops franqueti***	**35**	**52**	***Hepatocystis* sp.**
	Pteropodidae	*Micropteropus pusillus*	0	1	
	Pteropodidae	*Myonycteris angolensis*	0	15	
	Pteropodidae	***Myonycteris torquata***	**4**	**7**	***Hepatocystis* sp.**
	Pteropodidae	*Rousettus aegyptiacus*	0	3	
	Rhinolophidae	*Rhinolophus clivosus*	0	9	
	Rhinolophidae	*Rhinolophus deckeni*	0	2	
	Rhinolophidae	*Rhinolophus eloquens*	0	39	
	Rhinolophidae	***Rhinolophus fumigatus***	**2**	**5**	***Nycteria* sp.**
	Rhinolophidae	***Rhinolophus hildebrandti***	**8**	**14**	***Nycteria* sp.**
	Rhinolophidae	*Rhinolophus landeri*	0	36	
	Rhinolophidae	*Rhinolophus simulator*	0	1	
	Rhinolophidae	*Rhinolophus* sp.	0	1	
Total			108	505	

sity was obtained. For these parasite lineages, we sequenced additional loci from each of the three genomes present in Haemosporida, using the methods of (Martinsen et al., 2008b): mitochondrial cytochrome oxidase I (998 bp; trimmed to 886 for phylogenetic analyses), nuclear adenylosuccinate lyase (206 bp), and the apicoplast Caseinolytic protease C (531 bp). All sequences are accessioned on GenBank (KT750341–KT750753) (Supplemental Table S4), and all avian Cytb sequences are also available via the MalAvi database (Bensch et al., 2009b) (http://mbio-serv2.mbioekol.lu.se/Malavi/; accessed 1 Feb 2015).

2.3. Phylogenetic analysis

Phylogenetic analyses were performed on a concatenated DNA sequence alignment of four genes (Cytb, CoI, Asl, ClpC; 2536 bp) via the CIPRES Science Gateway Web Portal V3.3 (Miller et al., 2010) and the Computational Biology Service Unit at Cornell University (using a large memory machine with 512 GB RAM and 64 cores). Our alignment consisted of chiropteran and avian parasite sequences from this study, combined with data available from GenBank representing additional parasites of bats, birds, primates, and squamate reptiles for a total of 170 unique parasite lineages (Supplemental Table S4). Sequences were aligned using the MUSCLE plugin for Geneious v7.1.7 (Biomatters Ltd.), and the best partitioning scheme and model of evolution were determined using PartitionFinder (Lanfear et al., 2012). We compared four partitioning schemes (no partition, partitioned by gene, partitioned by codon, and mtDNA combined versus nuDNA and apDNA) and found no well-supported differences in the resulting maximum likelihood tree topologies. A concatenated unpartitioned dataset and GTR + I + G model of evolution were used for subsequent phylogenetic analyses.

Bayesian inference (BI) analyses were implemented using BEAST v1.8.0 and its associated utilities (Drummond et al., 2012). We assigned *Leucocytozoon* as the outgroup, based on the conclusions of Borner et al. (2016) regarding proper root assign-



UGANDA

Order / Suborder	Genus	n_i / n
Yangochiroptera	Nycteris	0 / 3
	Pipistrellus	0 / 1
Yinpterochiroptera	Epomops	35 / 52
	Hipposideros	3 / 35
	Myonycteris	4 / 12
	Rousettus	0 / 3
Rodentia	Colomys	0 / 2
	Dendromus	0 / 1
	Funisciurus	0 / 5
	Grammomys	0 / 1
	Hybomys	0 / 10
	Hylomyscus	0 / 31
	Lemniscomys	0 / 9
	Lophuromys	0 / 14
	Malacomys	0 / 23
	Mus	0 / 9
	Otomys	0 / 1
	Praomys	0 / 40
	Rattus	0 / 8
Soricomorpha	Crocidura	0 / 32
	Scutisorex	0 / 3
	Suncus	0 / 4
	Sylvisorex	0 / 9

MOZAMBIQUE

Order / Suborder	Genus	n_i / n
Yangochiroptera	Miniopterus	1 / 1
	Neoromicia	0 / 2
	Nycteris	0 / 1
Yinpterochiroptera	Epomophorus	0 / 2
	Myonycteris	0 / 8
	Rhinolophus	8 / 14
Rodentia	Acomys	0 / 2
	Aethomys	0 / 1
	Cricetomys	0 / 1
	Cryptomys	0 / 2
	Dendromus	0 / 4
	Grammomys	0 / 6
	Graphiurus	0 / 1
	Mastomys	0 / 2
	Mus	0 / 2
	Rhabdomys	0 / 10
	Saccostomus	0 / 1
Soricomorpha	Crocidura	0 / 6
	Myosorex	0 / 6

KENYA

Order / Suborder	Genus	n_i / n
Yangochiroptera	Chaerophon	0 / 2
	Coleura	0 / 7
	Glauconycteris	0 / 1
	Laephotis	0 / 1
	Miniopterus	49 / 100
	Myotis	1 / 4
	Neoromicia	0 / 29
	Nycteris	0 / 6
	Pipistrellus	0 / 2
	Scotophilus	0 / 2
	Tadarida	0 / 1
	Taphozous	0 / 1
Yinpterochiroptera	Cardioderma	0 / 2
	Epomophorus	0 / 12
	Hipposideros	0 / 53
	Lavia	0 / 11
	Micropteropus	0 / 1
	Myonycteris	0 / 2
	Rhinolophus	0 / 83

TANZANIA

Order / Suborder	Genus	n_i / n
Yangochiroptera	Miniopterus	5 / 13
	Neoromicia	0 / 3
	Pipistrellus	0 / 11
Yinpterochiroptera	Rhinolophus	0 / 5

MALAWI

Order / Suborder	Genus	n_i / n
Yangochiroptera	Neoromicia	0 / 12
	Nycteris	0 / 2
Yinpterochiroptera	Rhinolophus	2 / 5
Rodentia	Grammomys	0 / 1
	Hylomyscus	0 / 1
	Lophuromys	0 / 8
	Mus	0 / 1
	Otomys	0 / 4
	Praomys	0 / 8
	Rhabdomys	0 / 3
Soricomorpha	Crocidura	0 / 10
	Myosorex	0 / 1
	Suncus	0 / 3

Fig. 1. Map of sampling localities, and mammal genera sampled by country. Red text and red lines indicate genera and localities from which haemosporidian parasites were recovered.

ment for Haemosporida. Following preliminary runs to optimize chain length and ensure proper mixing and convergence, we conducted four independent runs consisting of 150,000,000 generations each using an uncorrelated relaxed lognormal clock and a Yule speciation prior. Appropriate mixing and convergence of runs, and effective sample size (ESS > 1000 in all cases, ESS > 2000 in most), were assessed in the program Tracer v1.6 (Rambaut et al., 2014). After removal of burn-in (10%), runs were combined using the ancillary BEAST program, LogCombiner v1.8.0, and a maximum clade credibility tree was produced using the program TreeAnnotator v1.8.0 (Supplemental Fig. S1).

Maximum likelihood (ML) analyses were conducted in RAxML via the Cipres Science Gateway Web Portal (Miller et al., 2010), using a GTR + I + G model and 1000 bootstrap pseudoreplicates, with *Leucocytozoon* assigned as the outgroup. We also performed an ML analysis of the same data set, but constraining avian and mammalian *Plasmodium* parasites to be monophyletic. The resulting topologies were compared by performing approximately unbiased (AU), Kishino–Hasegawa (KH), and weighted Shimodaira–Hasegawa (wSH) tests in the programs PAUP* (Swofford, 2003) and CONSEL (Shimodaira and Hasegawa, 2001) using 1000 bootstrap pseudoreplicates and resampling of estimated log-likelihood approximation (RELL) (Hasegawa and Kishino, 1994; Kishino et al., 1990) (Table 2).

3. Results and discussion

Our phylogenetic analysis of 170 haemosporidian parasite lineages from bats, birds, primates, rodents, and squamate reptiles supports the hypothesis that *Plasmodium* parasites of mammals evolved from parasites of chiropteran hosts (Fig. 3). In contrast to most recent phylogenetic hypotheses (Borner et al., 2016; Martinsen et al., 2008b; Outlaw and Ricklefs, 2011; Schaer et al., 2013, 2015), our analyses strongly support the reciprocal monophyly of *Hepatocystis* and mammalian *Plasmodium* species (Fig. 4). In further contrast to the recent phylogenetic study of Borner et al. (2016), topology tests did not support the monophyly of avian and mammalian *Plasmodium* parasites (Table 2), rendering the genus paraphyletic. Our analyses utilized genetic information from the broadest taxonomic sampling of Haemosporida currently available, and identified well-supported relationships that differ from analyses relying on large data sets of orthologous genes derived from a fewer number of haemosporidian taxa (*e.g.* Borner et al., 2016; Dávalos and Perkins, 2008). The effect of increasing genes versus taxa in phylogenetic reconstruction remains a controversial and important topic (Nabhan and Sarkar, 2012), and a combination of both approaches will likely be required before arriving at our best estimate of the true evolutionary history of Haemosporida.

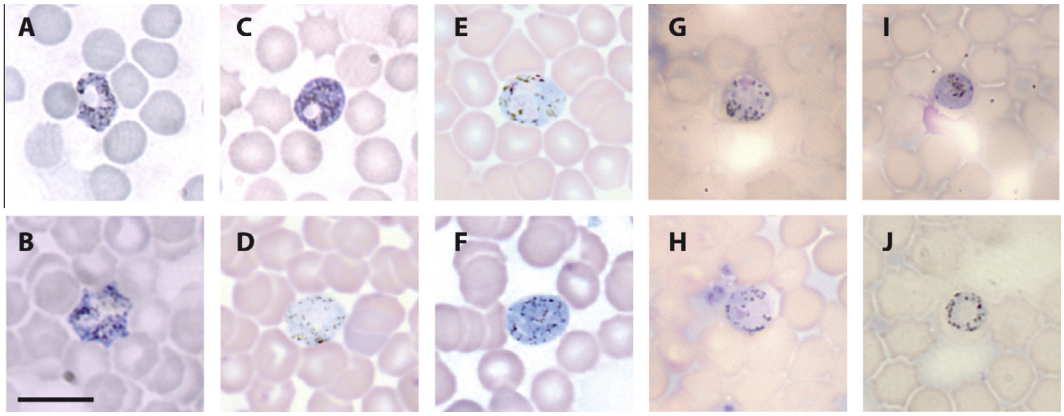


Fig. 2. Micrographs of blood stage gametocytes of bat haemosporidians; scale bar = 10 μm. (A–C) *Hepatocystis* cf. *epomophori* macrogametocytes ex. *Epomops franquetti*; (D–E) *Polychromophilus melanipherus* microgametocytes and (F) macrogametocyte ex. *Miniopterus natalensis*; (G–H) *Nycteria* microgametocytes, (I) macrogametocyte, and (J) gametocyte ex. *Hipposideros cyclops*.

The topology of our tree, rooted with *Leucocytozoon*, provides modest support for a single invasion of haemosporidian parasites from sauropsids into mammals (BI = 0.95, ML = 53), with subsequent diversification among lineages of bat parasites and multiple invasions into other mammals. This emerging pattern of chiropteran parasites transitioning into non-chiropteran hosts, including primates and rodents, is observed both at deep and shallow levels in the phylogeny (Fig. 4). Specifically, we observe a transition of *Hepatocystis* parasites from Asian pteropodids (megabats) into Asian and African primates, and a similar transition of *Plasmodium* in African pteropodids into African rodents. The large genetic and geographic diversity of *Hepatocystis* lineages found in pteropodids and their paraphyletic relationship to *Hepatocystis* of primates suggest these bats may be an important source of *Hepatocystis* parasites with the potential to shift into other mammal (*e.g.* primate) groups. However, concentrated sampling of pteropodids in both East Africa (this study) and West Africa (Schaer et al., 2013) may be biasing the diversity we observe. Further sampling of Old World primates and Asian bats will likely improve the resolution of evolutionary host switches between bats and primates. On a larger phylogenetic scale, the sister relationship of *Polychromophilus* and *Nycteria* parasites (which exclusively infect bats) to the clade containing *Plasmodium* and *Hepatocystis* suggest that the ancestor of primate-infecting *Plasmodium* species was likely a chiropteran parasite.

We find strong support from both BI and ML analyses for a sister group relationship between *Hepatocystis* and *Plasmodium* (BI = 1, ML = 100), as well as the respective monophyly of each group (*Hepatocystis*: BI = 1, ML = 100; *Plasmodium*: BI = 1, ML = 90), which is contrary to previous phylogenetic hypotheses (Duval et al., 2012; Martinsen et al., 2008b; Schaer et al., 2013). This novel relationship may be attributable to our inclusion of recently discovered primate parasites in the subgenus *Laverania* (*P. billcollinsi*, *P. billbrayi*), which also contains the deadly human malaria parasite *P. falciparum* and other closely related parasites of great apes. Our phylogeny suggests that the defining feature of true "malaria" parasites – erythrocytic schizogony, or the asexual reproduction in red blood cells of hosts – may have arisen twice in the haemosporidian tree of life (once in the saurian *Plasmodium* lineage, and once in the mammalian *Plasmodium* lineage), possibly in association with the dependence on mosquitoes as definitive hosts (Fig. 3). Our placement of *Hepatocystis* as sister to mammalian *Plasmodium* eliminates the requisite assumption that erythrocytic schizogony was a trait lost in the *Hepatocystis* lineage (Borner et al., 2016; Martinsen et al., 2008b; Outlaw and Ricklefs, 2011; Schaer et al., 2013), and instead suggests that this trait has convergently evolved. The correlation between erythrocytic schizogony and dependence on mosquitoes for transmission highlights an intriguing area of study in the coevolution of haemosporidians and their definitive hosts (dipteran insects, which also serve as vectors). The evolutionary relationships between dipteran vectors and haemosporidian parasites have been poorly studied, but are presumed to be influenced by strong selective forces on both parasites and insect hosts (Valkiūnas, 2005). Indeed, recent experiments demonstrated that the ingestion of avian *Haemoproteus* parasites (*H. balmorali*, *H. tartakovskyi*, and *H. lanii*) can be fatal to culicid mosquitoes, which are not the natural hosts of these parasite species (Valkiunas et al., 2014). Such significant survival effects of, in this case, geographically widespread parasites on a similarly widespread vector, suggest that parasite life-history traits are tightly linked to their insect hosts, and underscore the need for additional studies in this area.

We found only 24% of bat species (21% of individuals) sampled to be infected by haemosporidian parasites, with those infected belonging to only six of the 21 genera sampled (Table 1). Notably, no other mammals (rodents or shrews) were found to be infected. Although the primers we used for molecular detection of parasites are able to amplify parasites from many diverse haemosporidian groups (*e.g. Haemoproteus*, *Hepatocystis*, *Leucocytozoon*, *Nycteria*, *Plasmodium*, *Polychromophilus*), it is possible that some parasites of rodents and shrews may have diverged sufficiently at the Cytb locus such that we were unable to molecularly detect them. Microscopic analysis of a subset of rodent and shrew blood films (~100 and 50 randomly selected blood films of rodents and shrews, respectively), however, did not reveal any haemosporidian parasites. Microscopic analysis of PCR-negative blood films from

Table 2
Maximum likelihood (ML) topology tests, comparing likelihood of unconstrained topology, and a constraint forcing the monophyly of all *Plasmodium* lineages.

Maximum likelihood Topological constraints	ln*L*	Δln*L*	KH test *p*-value	AU test *p*-value	SH test *p*-value	wSH test *p*-value
Unconstrained	−51,783.01	(best)	–	–	–	–
Plasmodium monophyletic	−52,148.49	365.49	<0.0001	<0.0001	<0.0001	<0.0001

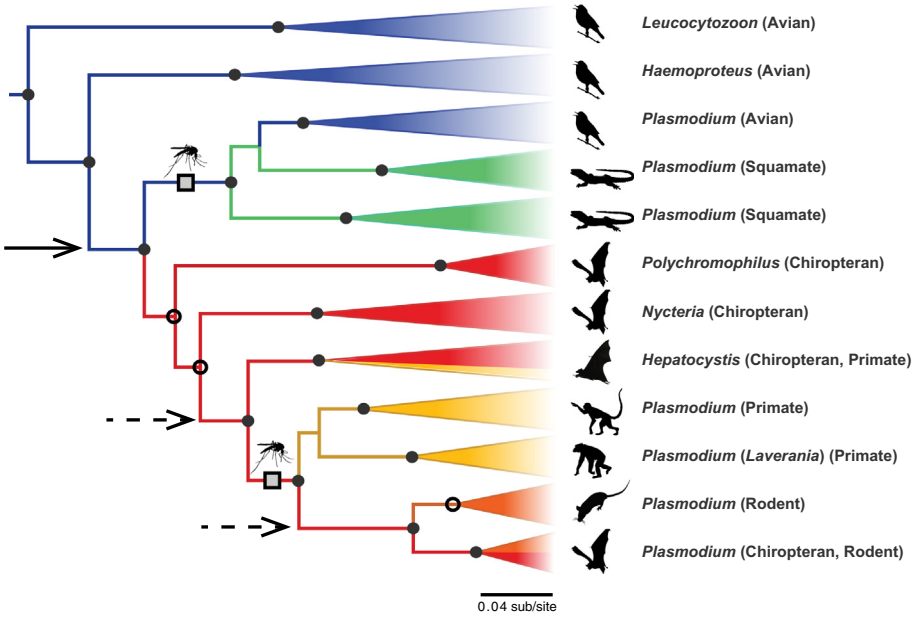


Fig. 3. BEAST maximum clade credibility tree, based on four runs of 150 million generations with sampling every 5000th tree (10% burn-in, 108,000 trees sampled, ESS > 1000). Closed circle indicates >99% posterior probability; open circle indicates >95% posterior probability. Colored triangles correspond to collapsed nodes of major haemosporidian parasite lineages from vertebrate hosts. Bold arrow indicates hypothesized transition from avian to mammalian hosts, and dotted arrows indicate transitions between chiropteran and primate or rodent hosts. Gray squares indicate lineages that exhibit erythrocytic schizogeny, which also depend on mosquitoes as definitive hosts.

bats also did not reveal haemosporidian parasites, whereas microscopy of PCR-positive samples confirmed the presence of morphologically-identifiable haemosporidians in all cases sampled (Fig. 2). We emphasize that a thorough examination of all blood films – not a subset – collected for this study is needed to definitively conclude that no rodents or shrews were infected by haemosporidian parasites. Parasite diversity in the bats we sampled included 50 unique lineages (unique lineages being defined, conservatively, as having a single nucleotide polymorphism at the Cytb locus) (Supplemental Table S2). In stark contrast, a recent study using identical parasite detection methods and comparably broad taxonomic sampling found ~93% of bird species (80% of individuals) sampled in Malawi to be infected with 248 unique haemosporidian parasite lineages (Lutz et al., 2015). In general, broad taxonomic surveys of Afrotropical bats, both in our study and in others (Duval et al., 2012; Schaer et al., 2013, 2015), indicate that only a small proportion of potential chiropteran host species harbor haemosporidian parasites, whereas the majority of potential avian host species in any given region are likely to be associated with one or more parasite lineages (Beadell et al., 2009; Belo et al., 2011; Ishtiaq et al., 2007; Lutz et al., 2015; Okanga et al., 2014; Ribeiro et al., 2005; Svensson-Coelho et al., 2013).

The difference in haemosporidian prevalence among species of birds and species of bats may be attributable to several factors, including ecological barriers to host-vector interactions that limit or accelerate opportunities for parasite transmission, and immunological responses in hosts that render them either viable or dead-end hosts for parasites. Coevolutionary hypotheses have been tested for several major lineages of haemosporidians, including mammalian and avian *Plasmodium* (Beadell et al., 2009; Jenkins et al., 2012; Silva et al., 2011, 2015). *Plasmodium* parasites of mammals exhibit high host specificity at the genus level (Garnham, 1966), relative to avian parasites, which are strikingly labile with respect to host specificity (Valkiūnas, 2005). Whereas comparative genomic studies of mammalian *Plasmodium* species reveal deep temporal connections between hosts and parasites (Silva et al., 2011, 2015), analyses of avian haemosporidians rarely find such relationships (Beadell et al., 2009; Hellgren et al., 2009), but rather, indicate tight links between ecology or host life history traits and parasite associations (Fecchio et al., 2013; Jenkins et al., 2012; Lacorte et al., 2013). Whether ecology and life history influence the relationships between bats and their haemosporidian parasites remains to be seen. Host-parasite specificity of *Nycteria* (Schaer et al., 2015) and *Polychromophilus* (Duval et al., 2012) parasites does appear to be high relative to avian host-parasite associations, but sampling of potential hosts for these two parasite genera has also been relatively low (as it has been with many other mammal groups). Few studies have examined feeding preferences of dipteran vectors of haemosporidians in wild bats (Witsenburg et al., 2015), and it is unknown how they might compare to those of avian hosts. Indeed, the dipteran vectors for most chiropteran parasites are unknown. The life history traits of chiropteran haemosporidians, however, suggest that host immunology represents an important factor in chiropteran parasite evolution. For example, *Hepatocystis* parasites exhibit prolonged developmental stages in the liver, with only brief periods of gametocyte circulation in the blood (Garnham, 1966), which has been suggested to be an adaptation to the unique immunological environment and metabolic demands of chiropteran hosts (Schaer et al., 2013).

The suggestion that bats harbor exceptional prevalence and diversity of parasites and pathogens relative to other mammalian hosts (Luis et al., 2013; Schaer et al., 2013) may hold true as knowledge of parasite diversity among mammals increases. However, evidence from recent Afrotropical surveys suggests comparably high parasite diversity in other mammalian groups, such as primates (Duval et al., 2010; Krief et al., 2010; Liu et al., 2010; Ollomo et al., 2009; Prugnolle et al., 2010). Perhaps more interesting than the prevalence of chiropteran haemosporidians (21% in this study), which is low compared to avian parasite prevalence (~80% in the same region; Lutz et al., 2015), is the range of phylogenetic positions that they occupy in the haemosporidian tree of life. *Polychromophilus* and *Nycteria* parasites are the closest known mammal-infecting relatives of avian haemosporidians, the chiropteran *Plasmodium* parasites *P. voltaicum* and *P. cyclopsi* are

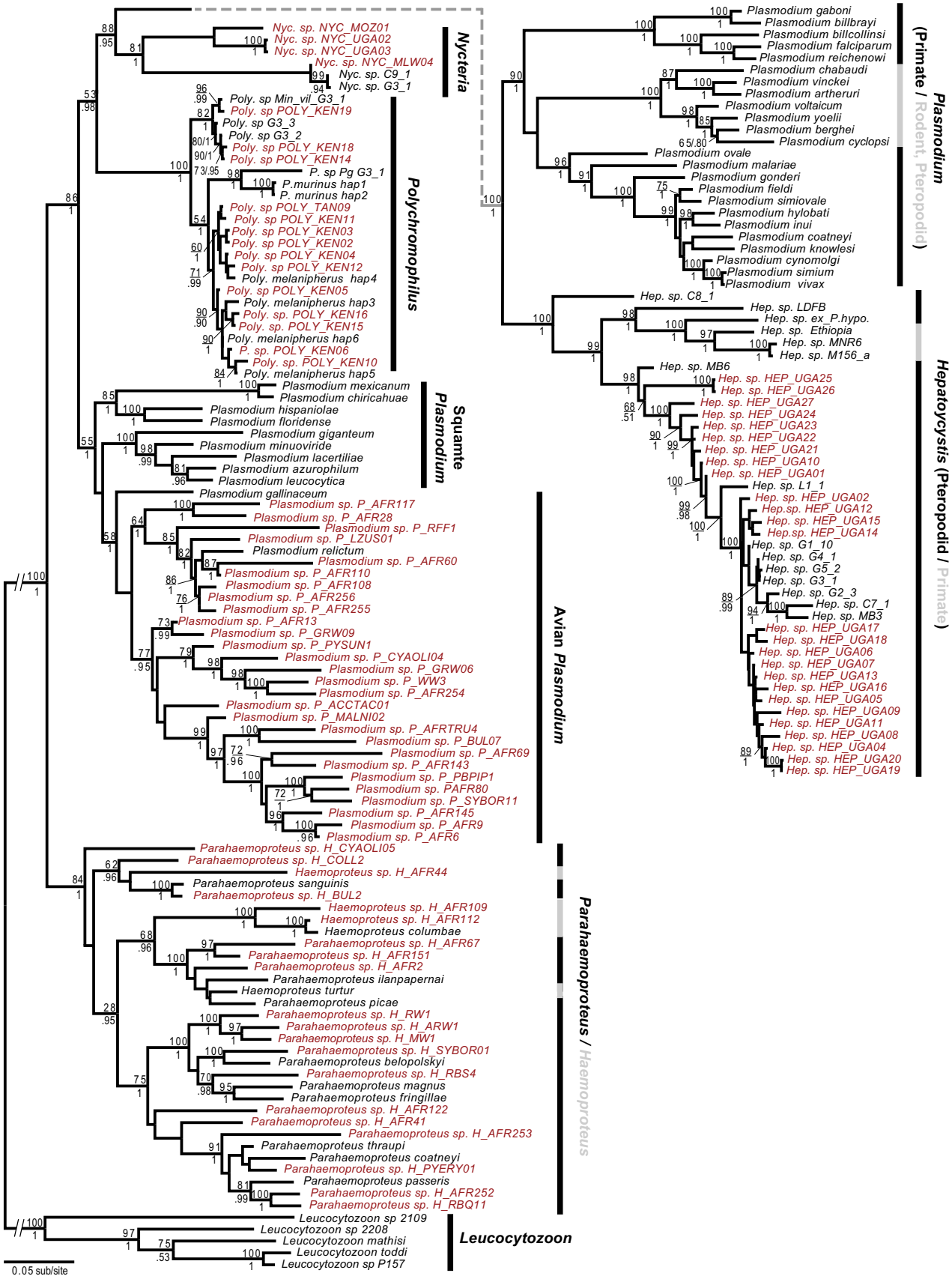


Fig. 4. Maximum likelihood (ML) phylogeny, rooted with *Leucocytozoon*. Support values indicated at nodes include ML support based on 1000 bootstrap pseudoreplicates, and Bayesian inference (BI) posterior probabilities from maximum clade credibility tree summarized from four independent mcmc chains of 150mil generations (above and below nodes, respectively; missing values indicate low BI/ML support). Terminal taxa labeled in red represent parasite lineages sequenced in this study.

sister to rodent *Plasmodium* species, and chiropteran *Hepatocystis* parasites are sister to primate-infecting *Hepatocystis* species. As noted in previous studies of pathogen spillover from bats (Calisher et al., 2006; Dobson, 2005), the exceptional nature of chiropteran pathogens lies not in their diversity, but rather, in their pronounced pathogenicity once they transition to novel hosts – a feature possibly linked to the evolution of flight in bats (O'Shea et al., 2014; Zhang et al., 2013). Such evolutionary forces acting on bats and their pathogens may very well be driving the apparent phylogenetic mobility of chiropteran haemosporidians.

4. Conclusion

The study of haemosporidian diversity and evolution has benefited from the steady efforts of biologists seeking to explore new host-parasite associations throughout the world. The importance of taxon sampling when assessing evolutionary relationships cannot be understated, and in this study, we present a novel hypothesis for haemosporidian parasites based on improved sampling of both saurian and mammalian hosts from the Afrotropics. In the future, sampling of haemosporidians from other additional geographic locations and host taxa (*e.g.* Asian mammals, New World bats, ungulates, *etc.*) should continue to improve our understanding of the evolution of parasites in this extraordinary group. Our phylogenetic analyses produced strong support for relationships at major nodes within the haemosporidian tree, and did not support the monophyly of *Plasmodium* parasites from birds and mammals. The consistent and strongly supported placement of *Polychromophilus* and *Nycteria* parasites as sister to mammalian *Plasmodium* and *Hepatocystis* suggest that malaria parasites in primates and rodents are derived from an ancestor infecting chiropteran hosts. The transition of parasites from chiropteran to non-chiropteran hosts appears to be a pattern throughout the evolutionary history of mammalian haemosporidian parasites, and may be linked to ecological or immunological factors that are unique to bats.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.03.004.

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