High diversity of West African bat malaria parasites and a tight link with rodent *Plasmodium* taxa

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As the only volant mammals, bats are captivating for their high taxonomic diversity, for their vital roles in ecosystems—particularly as pollinators and insectivores—and, more recently, for their important roles in the maintenance and transmission of zoonotic viral diseases. Genome sequences have identified evidence for a striking expansion of and positive selection in gene families associated with immunity. Bats have also been known to be hosts of malaria parasites for over a century, and as hosts, they possess perhaps the most phylogenetically diverse set of hemosporidian genera and species. To provide a molecular framework for the study of these parasites, we surveyed bats in three remote areas of the Upper Guinean forest ecosystem. We detected four distinct genera of hemosporidian parasites: *Plasmodium*, *Polychromophilus*, *Nycteria*, and *Hepatocystis*. Intriguingly, the two species of *Plasmodium* in bats fall within the clade of rodent malaria parasites, indicative of multiple host switches across mammalian orders. We show that *Nycteria* species form a very distinct phylogenetic group and that *Hepatocystis* parasites display an unusually high diversity and prevalence in epauletted fruit bats. The diversity and high prevalence of novel lineages of chiropteran hemosporidians underscore the exceptional position of bats among all other mammalian hosts of hemosporidian parasites and support hypotheses of pathogen tolerance consistent with the exceptional immunology of bats.

Haemosporida | Chiroptera | vector-borne disease | molecular phylogeny | host–pathogen coevolution

Malaria is a mosquito-borne epidemic human disease caused by protozoan parasites of the genus *Plasmodium*. Four different species known to cause human malaria have been studied intensively over several decades, and in the recent past two additional species have also been verified as human malaria parasites (1, 2). However, human-infecting *Plasmodium* species represent only a small fraction of over 550 species in the order Haemosporida that are classified into 17 extant genera (3). One hallmark of all hemosporidian parasites is the obligate host switch between a vertebrate intermediate host and an arthropod vector as a definitive host. However, across this family, a diverse array of intermediate hosts are used, including several orders of mammals, birds, squamate reptiles, turtles, and crocodilians (4). Based on solitary reports over the last century, it is thought that parasites belonging to at least seven hemosporidian genera can infect bats (Chiroptera), most of which are likely exclusive to this order (5).

Bats have an almost worldwide distribution, feature diverse life history traits, and play important ecological roles (6). Chiroptera is the second largest order of mammals after the Rodentia, with an estimated 1,232 living species and 18 families, which represent ~20% of all living mammalian species (7). Bats are also important reservoir hosts for numerous emerging and highly pathogenic viruses (8, 9). In marked contrast, the hemosporidian parasites of bats remain largely unstudied, despite the first records dating back to the late 19th century (10). The corresponding vectors for most bat parasites remain unknown. Similarly, the phylogenetic relationships for the majority of these parasites remain enigmatic.

Here, we present a unique systematic analysis of Haemosporida in a diverse species assemblage of bats. For this study, we performed surveys in three remnants of the Upper Guinean forest ecosystem, considered one of the world's biologically most diverse, but also one of the most endangered terrestrial ecosystems (Fig. 1). Our analysis highlights the overall diversity of chiropteran hemosporidian parasites and reveals distinct host–parasite associations.

Results

We captured a total of 274 bats belonging to 7 families and 31 species. Based on thorough and multiple microscopic examinations of thin blood smears, we observed hemosporidian parasites in 111 individuals, corresponding to an overall prevalence of 40% (Fig. S1). All individuals identified as infected by microscopy were then confirmed by diagnostic PCR and sequence analysis (111 of 111), establishing that there were no false-positive samples. Fifty samples scored as negative by microscopy were also tested by PCR, and all

Significance

Understanding the evolution of malaria parasites and their phylogenetic context is key to understanding this important human disease. We report an unexpected high diversity of malaria parasite genera in bats from West African forest ecosystems. Two lineages are closely related to *Plasmodium* parasites from rodents, which are common laboratory model systems, and the results are consistent with switches between these hosts over their evolutionary history. Bats are considered important reservoir hosts for many pathogens, particularly viruses, and have unusually high immunological tolerances. The abundant malaria parasite infections are consistent with this exceptional immunology and suggest that in bats the parasites repeatedly evolved life cycles away from disease-causing replication in red blood cells to less pathogenic propagation in liver tissue.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. are reported in Tables S4 and S5).

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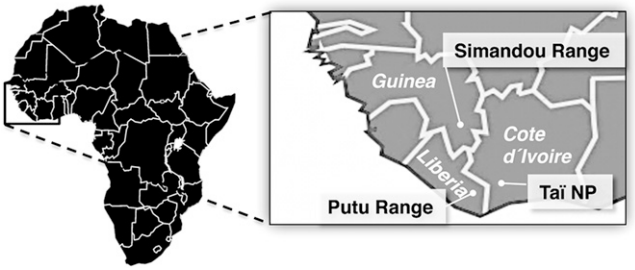

Fig. 1. Bat sampling areas in West Africa. Bats were captured during the dry season between November and December 2006 in Taï National Park, Côte d'Ivoire, in December 2008 in the Forêt Classée de Pic de Fon in the Simandou range of Guinea, and between November and December 2010 in the Putu range in southeastern Liberia.

proved negative, indicating the false-negative rate is low. Collectively, infected bats belonged to 13 different host species from five families: *Pteropodidae*, *Vespertilionidae*, *Miniopteridae*, *Hipposideridae*, and *Rhinolophidae* (Table 1).

To classify the parasites, we used morphological characteristics (*SI Text*), a multigene phylogenetic approach from all three parasite genomes, consulted previous records, and considered the corresponding hosts and ecological localities. Robust phylogenetic analyses using both maximum-likelihood and Bayesian algorithms, including multiple previously published sequences from diverse hemosporid genera from other bat hosts, as well as rodents, primates, birds, and lizards, showed clear separation of the West African parasites into four well-supported, distinct genera: *Plasmodium*, *Polychromophilus*, *Nycteria*, and *Hepatocystis* (Fig. 2*A* and Fig. S2). We did not observe any individual bat host to be infected with more than one parasite genus or morphologically identified species, nor did the molecular data suggest that there were mixed-species or mixed-genus infections present in these bats.

Plasmodium species were restricted to the fruit bat species *Myonycteris angolensis* (Pteropodidae; formerly *Lissonycteris angolensis*) and the insectivorous *Hipposideros cyclops* (Hipposideridae) and showed high prevalences in these species (Fig. 2 and Table 1). *M. angolensis* harbored parasites classified as *Plasmodium voltaicum* based on morphological features (Fig. 3*A* and Fig. S3*A*), a finding that is consistent with previous records of this parasite species in this host (11). Individuals of *H. cyclops* were infected with parasites identified as *Plasmodium cyclopsi* (Fig. 3*A* and Fig. S3*B*), a species that has been reported from the same host species in Gabon (12). Unexpectedly, in the phylogenetic analysis, both of these *Plasmodium* species fell within the clade of rodent malaria parasites and showed patterns of multiple switches between chiropteran and rodent hosts (Fig. 2 and Fig. S4). Sequencing of nine additional nuclear-encoded genes from these chiropteran *Plasmodium* samples yielded slightly different within-clade relationships, but consistently showed a close relationship with *Plasmodium yoelii* and *Plasmodium berghei* (Fig. 2*B* and Fig. S4). This tight link was entirely maintained when we included sequences from all known rodent *Plasmodium* strains in our analysis (Fig. S4). These rodent malaria species are widely used model parasites to study *Plasmodium* biology, disease progression, and novel malaria intervention strategies (13). We conclude that the surprising and tight relationship of bat *Plasmodium* species with those infecting rodents suggests multiple host switches between the two host orders.

Several infections in insectivorous bats were morphologically identified as belonging to the genus *Polychromophilus* (Fig. 3*B* and Fig. S3*C*). The samples from *Miniopterus villiersi* possessed slightly coarser-grained pigment, a characteristic attributed to *Polychromophilus melanipherus* (10) and are part of the same clade as *P. melanipherus* samples from *Miniopterus schreibersii* (Fig. 2*A* and Fig. S2). Similarly, the parasites of *Pipistrellus* aff. *grandidieri* and *Neoromicia capensis* are very similar but do not group closely with *P. murinus* samples from European vespertilionid hosts (14), and thus may represent a distinct species. All samples showed very low parasitemias (Table S1), consisting only of mature gametocytes, most likely because *Polychromophilus* infections were chronic in the captured hosts. Taken together, our findings support the notion that *Polychromophilus* is restricted to insectivorous bats, but has a wide cross-equatorial geographic range.

Nycteria parasites were found in two horseshoe bats, *Rhinolophus alcyone* and *Rhinolophus landeri* (Fig. 3*C* and Fig. S3 *D* and *E*). Only sexual stages were found in the blood, consistent with the characteristics of this genus (5). Our analyses place *Nycteria* as a very distinct sister clade to the mammalian *Plasmodium*/*Hepatocystis* clade (Fig. 2*A*), suggesting that the origin

Table 1. Investigated bat species

Bat suborder	Bat family	Species*	Parasite genus
Yinpterochiroptera	Pteropodidae	***Myonycteris angolensis***† (3/3)	*Plasmodium*
		Epomophorus gambianus (1/1), ***Epomops buettikoferi*** (7/25), ***Hypsignathus monstrosus*** (5/10), ***Micropteropus pusillus*** (68/72), ***Myonycteris leptodon***‡ (11/63), ***Nanonycteris veldkampii*** (6/8)	*Hepatocystis*
		Eidolon helvum (0/2), *Megaloglossus azagnyi*§ (0/12), *Rousettus aegyptiacus* (0/6), *Scotonycteris ophiodon* (0/5), *Scotonycteris zenkeri* (0/5)	
	Rhinolophidae	***Rhinolophus alcyone*** (1/1), ***Rhinolophus landeri*** (1/2)	*Nycteria*
		Rhinolophus guineensis (0/5)	
	Hipposideridae	***Hipposideros cyclops*** (3/4)	*Plasmodium*
		Hipposideros jonesi (0/2), Hipposideros cf ruber (0/10)	
Yangochiroptera	Miniopteridae	***Miniopterus villiersi*** (3/12)	*Polychromophilus*
	Vespertilionidae	***Neoromicia capensis*** (1/3), ***Pipistrellus* aff. *grandidieri***¶ (1/3)	*Polychromophilus*
		Neoromicia guineensis (0/1), *Pipistrellus bellieri* (0/3), *Pipistrellus nanulus* (0/2)	
	Molossidae	*Chaerephon nigeriae* (0/1), *Mops condylurus* (0/1), *Mops leonis* (0/2), *Mops thersites* (0/7), *Otomops martiensseni* (0/1)	
	Nycteridae	*Nycteris arge* (0/1), *Nycteris hispida* (0/1)	

*Infected species are highlighted in bold and numbers of infected per captured individuals are shown in brackets.
Bat species synonyms: †*Rousettus smithi*/ *Lissonycteris angolensis*, ‡*Myonycteris torquata*, §*Megaloglossus woermanni*.
¶The West African *Pipistrellus* aff. *grandidieri* might represent a yet undescribed species.

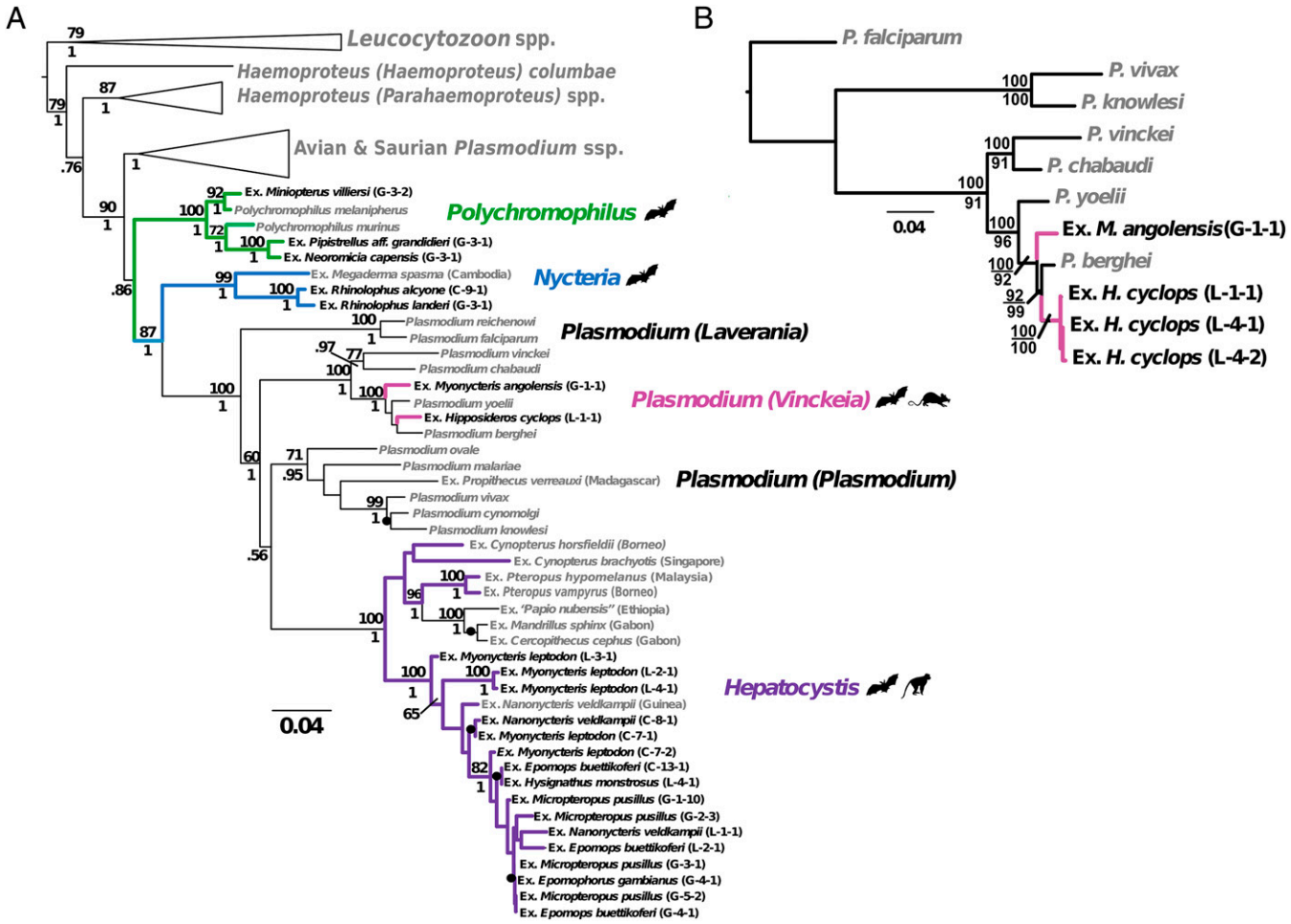

Fig. 2. Haemosporida from West African bats separate into four distinct genera. (*A*) Three-genome phylogeny of the hemosporidian parasites of the bats of this study, including published sequences for context, obtained by ML. Phylogeny was constructed with partitioned analysis of mitochondrial (*cytb*, *cox1*), apicoplast (*clpc*), and nuclear (*ef2a*) genes, rooted with *Leucocytozoon* taxa. ML bootstrap values (100 replicates) and Bayesian posterior probabilities are indicated above and below nodes, respectively. Recent divergences with high nodal support are indicated by black dots. Genera of bat malaria parasites are labeled to the right. Hemosporidian parasites from this study are highlighted in bold type. (*B*) ML phylogeny of bat and rodent *Plasmodium* species obtained via analysis of four genes as in *A*, plus nine additional nuclear genes (actin-1, actin-2, adenylosuccinate lyase, cysteine proteinase, dihydrofolate reductase/thymidylate synthase, histone H2A, inosine monophosphate-dehydrogenase, ookinete surface protein P25, and polyubiquitin), with three primate taxa included as outgroups.

of the mammalian hemosporidians may have been in African bats. A parasite found in *Megaderma spasma* from Cambodia (15), included in our phylogenetic dataset, grouped with the *Nycteria* sequences. Collectively, this first molecular analysis of *Nycteria* parasites underscores the evolutionary distance of these Haemosporida and implies still unrecognized biological peculiarities in this parasite genus.

The vast majority of parasites we observed in our sampling were *Hepatocystis* species. All were found in fruit bats (Pteropodidae) with a high overall prevalence of 58% (Fig. 3*D* and Table 1). Apparently, *Hepatocystis* infections are almost universal in several West African pteropodid bats, indicative of continuous and highly efficient transmission cycles. The *Hepatocystis* group of the West African samples might comprise different species and phylogenetically clearly places as a sister group to another *Hepatocystis* clade, which contains parasites in both African primate (baboon and mandrill) and Asian pteropodid fruit bat hosts (Fig. 2*A*). As with previous reports (16, 17), *Hepatocystis* appears to be a derived clade from the mammalian *Plasmodium* parasites. The remarkable phylogenetic diversity of *Hepatocystis* correlates with the diverse array of vertebrate hosts. Based on our data, we hypothesize that bats were also likely the first hosts of *Hepatocystis*, although our phylogeny also supports one invasion into African primates. However, this clade was more closely related to Asian bat *Hepatocystis* parasites than the West African bat *Hepatocystis* (Fig. 2*A*).

Discussion

The close phylogenetic relationship of the chiropteran *Plasmodium* species with those of rodents offers a fresh look at the evolution of rodent malaria, which is used as an experimental model for human disease (13) and evolutionary biology (18). All four known murine *Plasmodium* species, *Plasmodium chabaudi*, *P. yoelii*, *P. berghei* and *Plasmodium vinckei* naturally infect African thicket rats (Muridae: *Grammomys*), which are arboreal. The *Plasmodium*-infected bat species, *H. cyclops*, forms small colonies in cavities in large hollow trees, and *M. angolensis* usually roosts in caves, but is also occasionally found in hollow trees (19, 20). These distinct roosting behaviors might attract the same dipteran vectors as those seeking tree-dwelling rodents, and provides a plausible explanation for the phylogenetic relationship of their blood parasites. An additional link might be scaly-tailed flying squirrels (Anomaluridae) (19), which sometimes share their roosts with *H. cyclops* and have been found

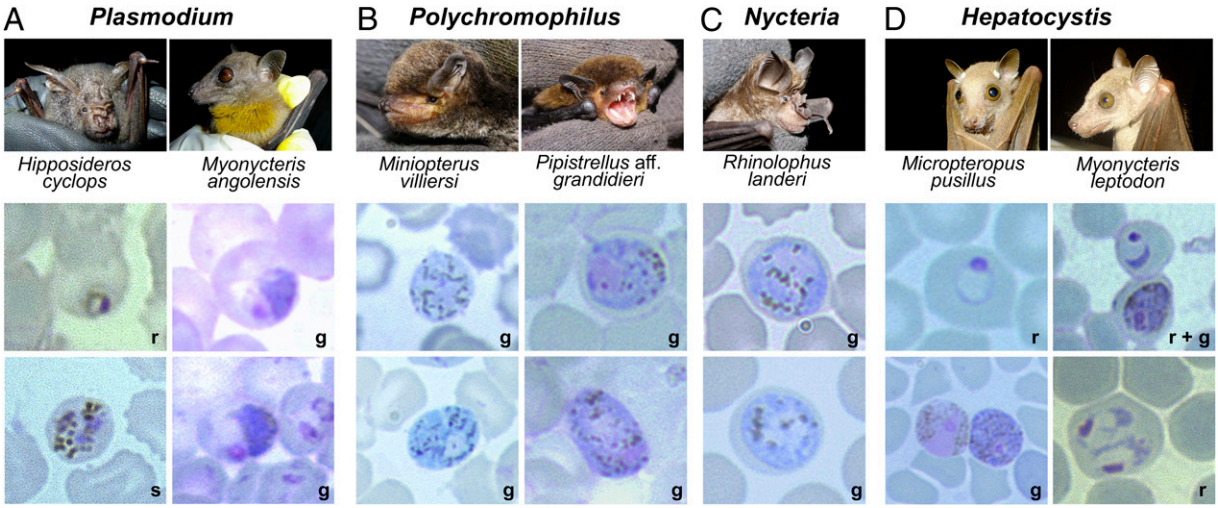

Fig. 3. Hemosporidian parasites and their host species. Shown are captured bats and representative micrographs of Giemsa-stained thin blood films of their respective hemosporidian parasite blood stages (r, ring stage; s, schizont; g, gametocyte). (*A*) *P. cyclopsi* and *P. voltaicum* blood stages isolated from *H. cyclops* and *M. angolensis,* respectively. (*B*) *Polychromophilus* gametocytes isolated from miniopterid and vespertilionid bats. (*C*) *Nycteria* gametocytes isolated from two rhinolophid bats. (*D*) *Hepatocystis* blood stages isolated from six pteropodid bats. Shown are two (*Micropteropus pusillus*, *Myonycteris leptodon*) of six host species and different blood stages of their parasites. Micrographs were taken at 1,000× magnification.

infected with *Plasmodium anomaluri* (12), a species that morphologically appears to be the closest to *P. cyclopsi* (12). Based on our data, we propose that *P. cyclopsi* and *P. voltaicum* should be readily transferrable from naturally infected bats to laboratory mice and mosquito colonies. Adaptation of these *Plasmodium* species to experimental murine models could offer unprecedented insights into phenotypic and genetic evolution of malarial parasites. The first identification of rodent *Plasmodium* species that can eventually be transmitted to laboratory rodents was by Vincke and Lips, who found sporozoites of what is now known as *P. berghei* in the salivary glands of infected *Anopheles dureni millecampsi* (21). This landmark discovery marked the beginning of the widespread use of murine malaria parasites as in vivo models for biomedical malaria research. These model systems became invaluable tools for large-scale drug testing, experimental vaccine development, and molecular and cellular studies of malaria-associated pathology and host immune responses (22, 23). The importance of these murine hosts is such that the complete genome of one of them, *P. yoelii*, was sequenced in parallel with that of *P. falciparum* (13). However, many currently used strains of rodent malaria used in experimental studies derive from isolates that have been adapted to inbred laboratory hosts for over three decades, thus representing a somewhat unnatural parasite–host system.

Recent studies (24) addressed the exceptional ability of bats to endure numerous viruses, some of which are highly pathogenic for humans. Tolerating high prevalences of hemosporidian parasites along with high parasitemias, as shown for *Hepatocystis* infections in this study, might be another example of the well-adapted immune responses of bats. It is possible that even the change in the parasites' life cycle away from erythrocytic schizogony in three of the bat-infecting genera (*Hepatocystis*, *Nycteria*, and *Polychromophilus*) could have been an evolutionary "compromise" of using these metabolically active hosts and driven by the immune responses of the bat hosts. Similarly, the *Plasmodium* species infecting bats could offer a unique comparative system with the rodent malaria parasites to investigate the response of these parasites to the very different immunological environment of chiropterans.

Our phylogenetic analysis also challenges several traditional and recently proposed ideas in hemosporidian relationships. This analysis, rooted with *Leucocytozoon* species, places the avian and squamate *Plasmodium* species as polyphyletic to the mammalian *Plasmodium* species, and further suggests that bats were likely the first mammalian hosts of *Plasmodium* before the parasites began using rodents and primates, including humans, as vertebrate hosts. The topology of the phylogeny (Fig. 2 and Fig. S2) also suggests that once *Plasmodium* parasites invaded mammals, they never switched back into nonmammalian hosts. We note that this interpretation contrasts with suggestions that across-clade switching might have occurred multiple times (15), or that *Polychromophilus* parasites are more closely related to the parasites of birds and squamate reptiles (25). To eventually resolve which of these two scenarios is more likely, additional taxa, perhaps including lineages external to Haemosporida but more closely related than other sequenced Apicomplexa, will likely be required (26).

The sampling of our study presents only a snapshot of the diversity of bats and thus of potential host species. Although the pace of discovery of novel mammalian parasite lineages slowed over the past few decades (3), more recently there has been a resurgence of investigating wild hosts, particularly primates in both the New and Old Worlds, using molecular screening techniques (e.g., refs. 27–30). These studies have broadened our conceptions of the diversity of hemosporidian lineages in these hosts and, in some cases, will challenge our concepts of the origin of the human-infecting species and their natural epidemiology (31, 32). These surveys, like our study, illustrate that minimally invasive sampling in biodiversity hotspots is likely to reveal numerous previously unrecognized parasitic infections in most mammalian host taxa. Renewed investigation of hemosporidian parasites in natural hosts is critical to eventually gain a better understanding of host–pathogen coevolution.

The exceptional phylogenetic and ecological diversity of bats have certainly contributed to the diversity of malaria parasites in these hosts. Bats have a deep evolutionary history, with an inferred origin of 66 Mya, and it has been proposed that the biogeographic center of origin of the order was in Africa, but with numerous dispersal events (33). The diverse array of hemosporidian genera currently present in African bats may thus be a reflection of the long and complex history of their hosts. The taxa sampled in this study include members of both chiropteran

suborders (Table 1). The arthropod vectors, where sexual recombination occurs, should also be included in future considerations, because they likely play a major role in parasite diversification (34). In conclusion, our findings reveal an intriguing correlation between exceptional diversity and phylogenetic relationships of arthropod-borne, obligate intracellular blood parasites and the unique ecological niche and species range that distinguish bats from all other mammals as potential hosts.

Materials and Methods

Bats were captured in Côte d'Ivoire, Guinea, and Liberia (Fig. 1, Fig. S1, and Table S2). For molecular analysis, blood and wing punches were sampled. For morphological parasite identification thin blood smears were prepared for Romanowsky–Giemsa staining and microscopic analysis. Confirmatory diagnosis at the genus and, where possible, species level was defined as combination of microscopy, PCR, and sequencing. Parasitemias were recorded for each infected bat (Table S1) by direct counting of parasite cells in 40 microscopic fields (see *SI Materials and Methods* for full details), except in eight cases, where the quality of the thin blood smear was too poor to obtain an accurate count. Four signature genes representing the three parasite genomes (mitochondrial: cytochrome *b*, cytochrome oxidase I; plastid: caseinolytic protease C; nuclear: elongation factor 2A) were selected for the phylogenetic analyses. For just the *Plasmodium* isolates, nine additional nuclear genes were amplified and sequenced (actin-1, actin-2, adenylosuccinate lyase, cysteine proteinase, dihydrofolate reductase/thymidylate synthase, histone H2A, inosine monophosphate-dehydrogenase, ookinete surface protein P25, and polyubiquitin). Primers are listed in Table S3; GenBank accession numbers are listed in Tables S4 and S5. After Sanger sequencing and assembly, alignments were done using MUSCLE (35). We evaluated the phylogenetic relationships using both maximum-likelihood (ML) and Bayesian inference methods. Data were divided into four partitions according to genes. For ML analysis, we used raxmlGUI (36). Nodal support was evaluated using 100 rapid bootstrap pseudoreplicates (37). Bayesian inference was conducted in MrBayes v3.2.0 (38), with two runs of four chains (three heated, one cold, temperature = 0.20) each for 10 million generations. The GTR + I + Γ type model was used for each independent partition. Reversible rate matrices, partition-specific rate multipliers and stationary state frequencies had a Dirichlet prior. The α and proportion of invariant sites had uniform priors. A prior of all topologies equally likely was used for τ and the prior on branch lengths was set as unconstrained exponential (parameter 10). Convergence was tested in the program AWTY (39).

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