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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-