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Host associations and climate influence avian haemosporidian distributions in Benin ☆

Johanna A. Harvey [a,b,*,1], Gary Voelker [a,b]

[a] Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX, USA
[b] Biodiversity Research and Teaching Collections, Texas A&M University, College Station, TX, USA

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ABSTRACT

A majority of avian haemosporidian diversity likely remains undiscovered, and each new recovery helps to further elucidate distributional patterns of diversification. We conducted the first known sampling of avian haemosporidians, *Haemoproteus*, *Leucocytozoon*, and *Plasmodium* from Benin located in tropical West Africa. We sampled 222 birds of 77 species and across distinct ecoregions with varied habitats. Haemosporidians were detected in 113 of 222 individuals, resulting in a 50.9% infection rate. By molecular analysis, we recovered a high number of novel lineages, 52.9%, and characterized the multivariate variables which influence the distributions of haemosporidian genetic lineages, including host associations and bioclimatic variables. We introduced a novel visualization method to better capture the multivariate environment of haemosporidians, and this approach resulted in the recovery of intra-generic distribution patterns of diversity, although no patterns were recovered at the genus level. Our results remain descriptive in nature, but show the promise of predictive strength with an increase in sampling localities with future work. Assessing host and bioclimatic variables at a larger geographic scale and across multiple ecoregions will help to elucidate processes regulating the distribution of haemosporidian diversity.

1. Introduction

Species are bound geographically by various factors such as oceans, mountains or islands; however, vector-borne parasites have additional constraints which include distributions of their arthropod vectors, competent host availability and other host biotic factors (e.g., age, density, sex, life history characteristics). Furthermore, arthropods that vector parasites are restricted by their own environmental constraints including temperature, precipitation and humidity, all of which combine to determine life cycle events (Gage et al., 2008). As such, the prevalence of vector-borne parasites is dependent on both vector abundance and the ecological conditions required by their vectors (van Riper III et al., 1986). Integrating bioclimatic factors as a proxy for vector distributions seems a critical consideration for studies seeking to determine the strength of association with parasites across hosts and geography.

Given the predicted effects of global climate change and associated changes in diurnal fluctuations, we expect to see the following effects on vectors and ultimately on parasite distributions: (i) expanded vector distributional ranges and associated altered parasite distributions, (ii) extension of the seasonal activity of both vectors and parasites, thus extending transmission periods, and (iii) increased introductions where vector/parasite fauna are novel (González et al., 2010; Garamszegi, 2011; Caminade et al., 2014). Range extensions and shifts will increase the introduction of parasites, and thus increase the infection of novel host species (Garamszegi, 2011; Altizer et al., 2013; Loiseau et al., 2013); the decimation of the Hawaiian avifauna remains the classic example of this effect (van Riper III et al., 1986). Increases in temperature will also directly impact parasite development, which is temperature limited (Valkiunas, 2005; LaPointe et al., 2010). For example, studies on *Plasmodium* indicate that increases in temperature will increase the rate of development and thus result in increases in prevalence and their impact on host populations (LaPointe et al., 2010). Therefore, determining current distributions and constraints of taxa is necessary to better predict future changes in distributions.

☆ Note: New nucleotide sequence data reported in this paper are available in the GenBank™ database under accession numbers MK135924-MK135931.

* Corresponding author at: Department of Ecology and Environmental Biology, University of Connecticut, Storrs, CT 06269, USA.
E-mail address: johanna.harvey@uconn.edu (J.A. Harvey).
[1] ORCID ID: orcid.org/0000-0003-4504-6777.

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Haemosporidians are protozoan blood parasites infecting vertebrate taxa including reptiles, mammals and birds, with transmission occurring via bloodsucking dipterans. Avian haemosporidians range nearly worldwide and consist of the genera *Haemoproteus* (including *Parahaemoproteus*), *Leucocytozoon*, and *Plasmodium*. Each of these genera is vectored by a different suite of dipterans and these dipterans have varying ecological constraints to include moisture (ranging from humidity, moisture content of soil, precipitation, pooling of water, and running streams) and temperature regimes, which increases the complexity for understanding and modeling haemosporidian distributional limits. It is known that *Haemoproteus*/*Parahaemoproteus* are vectored by hippoboscid flies (Hippoboscidae) and biting midges (Ceratopogonidae). Biting midges exhibit highly varied habitat preferences with most biting midges inhabiting damp sand or soil, particularly along rivers and marshes, while they can also be found breeding in detritus, pools and streams (Meillon and de Wirth, 1991; Mellor et al., 2000; Meiswinkel et al., 2004). Louse flies (Hippoboscidae), are highly restricted to hosts and their larvae are laid in or near bird nests; given that louse flies do not lay eggs and instead deposit larvae directly, their moisture constraints are limited (Lamerton, 1965). *Plasmodium* is vectored by mosquitos (Culicidae) which vary in breeding site preference across species and have high moisture requirements for breeding and emergence, yet are preferentially found in swamp and upland forest versus open habitats (Njabo et al., 2009). *Leucocytozoon* is vectored by black flies (Simuliidae) and requires some degree of flowing water for egg laying, and is thereby primarily found near rivers and other bodies of water (Sutcliffe, 1986).

Although there is a basic knowledge of which insect vectors transmit haemosporidian genera, the specific vector species for most haemosporidian species remain unknown, and overall there are few studies linking vectors to both the vertebrate hosts and the haemosporidians (Malmqvist et al., 2004; Martínez-De La Puente et al., 2011; Santiago-Alarcon et al., 2012). Additionally, there appears to be a great deal of undiscovered Haemosporidia diversity, as new clades are being described with regularity (Bertram et al., 2017). Further, many geographic regions and host taxa have not been sampled for haemosporidians, thereby impacting the ability to map parasite associations and distributions, which in turn impacts the ability to predict range shifts under climate change scenarios. Therefore, although avian haemosporidians have become a model parasite system, much remains to be learned before we can reliably use this system to track climate change impacts on communities.

Several studies have attempted to assess the importance of environmental variables in predicting the distribution and prevalence of haemosporidian parasites, although many of these studies have been on single or a limited number of host species and their associated haemosporidians (Loiseau et al., 2012b, 2013). Researchers examining the distribution of haemosporidians across avian communities along two mountain ranges found that indeed each parasite genus demonstrated some environmental preferences, including preference across elevational gradients (Illera et al., 2017). While examining a single widespread tropical forest species, researchers found that the maximum temperature of the warmest month was the strongest predictor of *Plasmodium* prevalence (Sehgal et al., 2011). These studies indicate the need for assessment within and across avian haemosporidian communities.

Climate change is already having an effect; mortality due to chronic malaria was recently documented in a Common Loon (*Gavia immer*) individual in New Hampshire, USA. This is a previously monitored and believed to be uninfected Nearctic breeding species, which indicates a possible shift in the *Plasmodium* range or a shift in host species susceptibility, as suggested by the high level of virulence detected histologically (Martinsen et al., 2017). Indeed, the northern latitudinal limit of *Plasmodium* has expanded and is associated with temperature changes; further latitudinal expansions poleward are predicted, as are elevational expansions in montane areas (Loiseau et al., 2012a, 2013). An unknown portion of haemosporidian diversity remains unsampled across avian diversity and ecoregions. This under-sampling along with a lack of understanding of vector species associations present challenges in characterizing the dimensionality of the relationship of parasites and the associations with host and bioclimatic data variables.

Here we examine haemosporidian distributions in the western African country of Benin; previously a subset of this data was assessed for detections across source materials (Harvey and Voelker, 2017). Our sampling is ideal due to the small geographic scale, yet ecologically diverse, sites. Benin is located within the Sudanian bioregion and across two contrasting ecoregions: west Sudanian savanna and Guinean forest-savanna (Linder et al., 2012; Dinerstein et al., 2017). The localities sampled have generally tropical savanna climates (Peel et al., 2007), with mean temperatures above 18 °C and a marked dry season, but they are differentiated by length and timing of wet seasons which correspond to Northern (arid) and Southern (generally moist) localities (Fig. 1). Both of the west Sudanian savanna ecoregion localities, Chutes de Koudou and Point Triplo, are characterized by a long dry season with a pronounced single wet season, which occurs from June to September (http://worldclim.org; Hijmans et al., 2005). All of the Guinean forest-savanna localities, Dogo Forest, Lama Forest, Lake Toho, and Abomey-Calavi, have two wet seasons. Our sampling (Fig. 1) thereby provides a latitudinal gradient framework from the northern west Sudanian savanna localities to the southern Guinean forest savanna localities. Here, we examine avian haemosporidians and assess how their host relationships and environments are shaping the resulting diversity and distributions in Benin. We seek to elucidate: (i) host-parasite associations across a climatic gradient, (ii) the association of haemosporidian distributional patterns with bioclimatic variables, and (iii) how the associations of (i) and (ii) inform the observed distributional patterns. We associate these objectives across scales, addressing the bioregion, the ecoregions and the localities sampled.

2. Materials and methods

2.1. Sampling localities and avian sampling

Birds were sampled between May and June of 2010, as described in Harvey and Voelker (2017). Avian sampling included the 199 individual birds previously sampled (from blood and pectoral muscle source materials: Genbank accession numbers MG018625-MG018709) and the addition of 23 individuals for which blood ($n = 13$) or pectoral muscle ($n = 10$) were sampled. All 222 individual birds were addressed for haemosporidian detection, including all detections recovered from both source materials.

Briefly, birds were sampled at six localities across Benin. Sampling at the northern localities, Point Triplo ($n = 34$) and Chutes de Koudou ($n = 34$), occurred between 21 May–28 May, 2010. All southern localities (Dogo Forest ($n = 32$), Lama Forest ($n = 29$), Lake Toho ($n = 20$) and Abomey-Calavi ($n = 3$)) were sampled from 1 June to 10 June, 2010. Given the similar habitat and bioclimatic variables of Lake Toho and Abomey-Calavi, and that only three samples were collected in Abomey-Calavi, these samples were grouped together. Of the 222 individual birds sampled, 174 were determined to be adults and 23 were determined to be juveniles via skull ossification data. The remaining 22 individuals were lacking ossification data.

All voucher specimens collected are accessioned in the Biodiversity Research and Teaching Collections, at Texas A&M Univer-

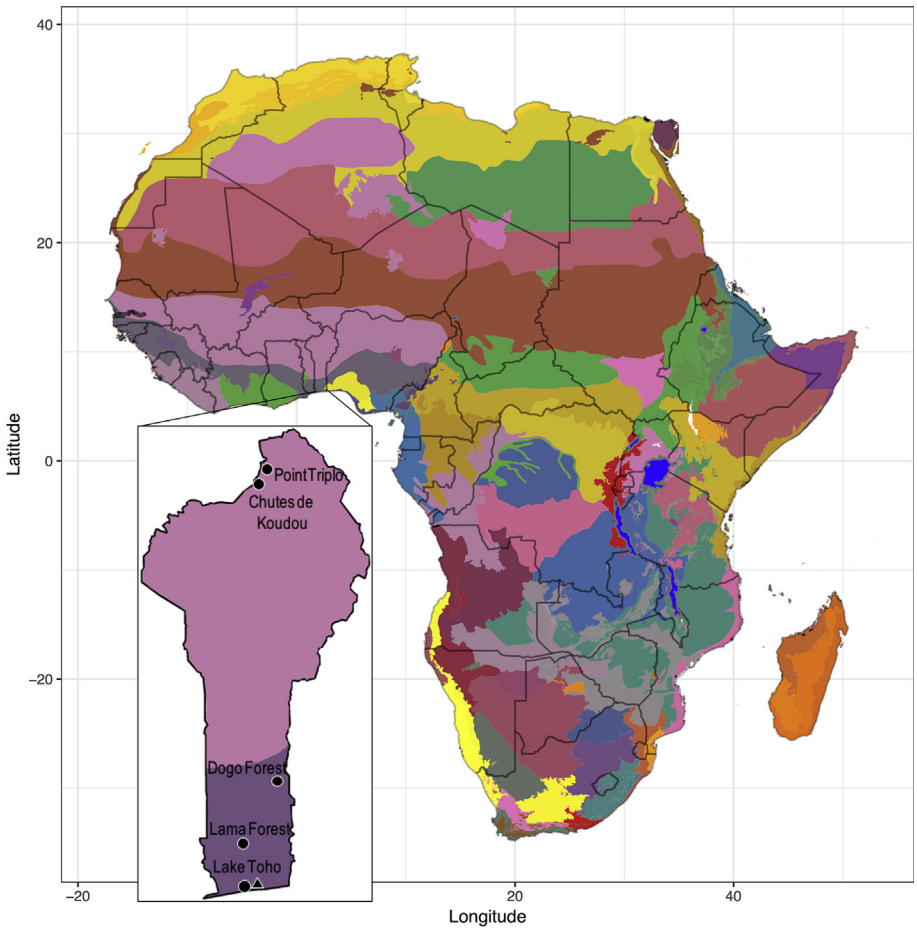

Fig. 1. Map demonstrating diversity of ecoregions across the African continent (n = 110). Inset map shows Benin's ecoregions (n = 2) as West Sudanian savanna denoted in lilac (north), and Guinean forest savanna in purple (south). Sampling localities are denoted with black circles. Abomey Calavi (n = 3) is denoted by a black triangle and these samples were grouped with Lake Toho sampling for analysis. Ecoregions map adapted from Dinerstein et al. (2017). (For full colour resolution, please see the online version.)

sity, USA. All specimens were collected under protocols approved by the Institutional Animal Care and Use Committee at Texas A&M University.

2.2. Molecular assessment of avian haemosporidians

We followed molecular protocols previously described in Harvey and Voelker (2017) and targeted a fragment of the mitochondrial DNA (mtDNA) cytochrome b (Cyt *b*) gene using multiple primer pairs previously published in Drovetski et al. (2014). This fragment encompassed the entire 479 bp of the standard gene region collected in the MalAvi avian haemosporidian database (Bensch et al., 2009). Collectively, the primers from Drovetski et al. (2014) amplify all three genera of avian haemosporidians: *Haemoproteus* (to include subgenera *Parahaemoproteus*), *Leucocytozoon*, and *Plasmodium*.

The quality of sequences was verified base by base and aligned by eye using Geneious 6.1.8 (http://www.geneious.com, Kearse et al., 2012). Multiple infections were determined by the presence of multiple peaks on both chromatograms at one or more base positions (Harvey and Voelker, 2017). Due to the high probability that sequences with less than three DNA positions displaying multiple peaks were sequencing errors and not true multiple infections (Szymanski and Lovette, 2005), we treated these as single infections. After being verified with criteria for peak similarity, confidence score and visual assessment, we processed double infection data (n = 5) with assigned International Union of Pure and Applied Chemistry] (IUPAC) nomenclature ambiguity codes. We then reconstructed single infection haplotypes (Browning and Browning, 2011) using Phase 2.1 (Stephens et al., 2001) as implemented in DnaSP 25.10.1 (Librado and Rozas, 2009) together with all similar existing sequences (≥97% BLAST match) from Benin and MalAvi data.

All data were identified to genus by use of the MalAvi BLAST (Version 2.2.8, Bensch et al., 2009) and National Center for Biotechnology Information (NCBI) BLAST (Altschul et al., 1990) functions. The criteria of 1 bp was followed to differentiate genetic lineages (i.e. unique haplotype) (Bensch et al., 2000, 2004). Sequences were assigned the MalAvi lineage name (if identified as a complete match) or identified as novel detections and given a unique lineage name (GenBank accession numbers MK135924-MK135931).

2.3. Delimitation of parasite clades of interest

To assess diversity of haemosporidians we first needed to determine the biological units to be addressed. The delineations for haemosporidian evolutionary units, species and lineages are poorly understood. Lineage formations have been found to develop primarily through host switching and allopatric speciation (Ricklefs and Fallon, 2002; Ricklefs et al., 2014). Species descriptions have been primarily based on morphological descriptions of circulating red blood cell life stages (meronts and gametocytes) using microscopy of blood smears, while some species description are across all life stages (this is methodologically more challenging and there-

fore rare) (Valkiunas, 2005). Currently there are 220 morphologically described haemosporidian species (MalAvi 2.3.3). Molecular determinations, using the single nucleotide substitutions within the 479 bp Cyt b region, have yielded 2,876 molecular lineages thus far (MalAvi 2.3.3). The single base pair delimitation for genetic lineages has been accepted as best practice (Bensch et al., 2000, 2004), although species may consist of several lineages which are simply variants, and is supported by the fact that haemosporidian lineages have not shown evidence of recombination events (Joy, 2003; Bensch et al., 2004, 2009).

Delimitations of species are more complex and not standardized across haemosporidian taxa. For example, between the human *Plasmodium falcip*arum and the chimpanzee *Plasmodium reichenowi* sequence divergence is 3.3%, and within *Plasmodium falciparum* ($N = 96$) reaches 0.2% across all of Cyt *b*, consisting of six haplotypes with single base pair substitutions (Joy, 2003). When examining the same *P. falciparum* data set restricted down to the 505 bp assessed in this study, pairwise sequence divergence reaches 0.4% consisting of four haplotypes with single base pair substitutions (Joy, 2003). Avian *Plasmodium relictum* from the Hawaiian Islands was previously believed to show no sequence divergence, while globally distributed *P. relictum* lineages demonstrate a 7.6% sequence divergence (Beadell et al., 2006). A study by Jarvi et al. (2013) demonstrated 23 variant haplotypes recovered from Hawaiian *P. relictum* using deep sequencing. However, these variants had low coverage (ranging from one to nine) and an average read depth of 4.6x. A number of these variants included non-synonymous substitutions or resulted in stop codons, suggesting that these variants may be a result of sequencing error or random mutations which are not biologically meaningful, thus supporting the genetic conservatism previously detected in Hawaiian *P. relictum*. Hellgren et al. (2015) recovered higher allelic diversity with a more quickly evolving independent marker within differentiated lineages of *P. relictum* which were supported by occurrence across distinct distributional regions. This variability in the divergence rates across groups supports the argument to range criteria (1–5%) for species delimitations, while multiple markers may be needed for confirmation, and use other characteristics as further support including morphology (where available), host association, and locality (Outlaw and Ricklefs, 2014).

Here we are not attempting to determine species as morphological data is missing. Instead we address phylogenetic clades of haemosporidians to determine patterns of association across hosts and climate. An important caveat is that host competence was not confirmed due to a lack of blood smears, thereby not allowing us to confirm the presence of gametocytes, the transmissive and reproductive life stage of parasites. Clades are selected with the criteria of being reciprocally monophyletic and having a within group pair wise sequence divergence of less than 5.5%, although most are more conservative. Pairwise sequence divergence was measured using MEGA version 7.0.14 (Kumar et al., 2016).

2.4. Bioclimatic data

We used the WorldClim 2 database and the ENVIREM data set, and their bioclimatic and topographic variables, as these have been determined to be biologically important for species distributions (Fick and Hijmans, 2017; Title and Bemmels, 2017). WorldClim 2 data are based on interpolated minimum, maximum and monthly averages of precipitation and temperature, and were collected from 1970 to 2000 at $\sim$1 km^2 resolution. We included monthly variables such as precipitation, water vapor pressure, wind speed, and temperature maximum and minimum for the second quarter (April, May and June) as this is reflective of vector emergence, infection, and resulting host infection for the collecting period in which we sampled (Supplementary Table S1). From the EVIREM data set, we included additional variables associated with measures of potential evapotranspiration, climate moisture index, and an aridity index, and additional climate and topographic data. ENVIREM data was collected from $\sim$1960 to 1990 and also at $\sim$1 km^2 resolution. Our initial climate data set contains 53 variables (Supplementary Table S1).

We used partial least squares regression (PLSR) to model the relationships of bioclimatic variables (as predictor variables) to haemosporidian richness and prevalence recovered across localities (response variables). PLSR multivariate regression is best suited for a high number of predictor variables and a small number of sampled response variables, responding favorably to highly correlated data (Mevik and Wehrens, 2007). The high number of predictor variables ($n = 53$) are transferred from linear correlations of the factors to latent variables (LV or components), for which the covariance between response and predictor variables is maximized. We conducted the PLSR as implemented in the *pls* package (Mevik and Wehrens, 2007) in R software version 3.2.2 (R Core Team, 2016) and examined our initial bioclimatic data set of 53 predictor variables together with measures of richness and prevalence for each haemosporidian genus across sampling localities as response variables. The SIMPLS algorithm was used in PLSR, given the high number of predictor variables (de Jong, 1993). The resulting number of LVs were reduced, using a leave one out cross-validation method. Prevalence was calculated as the proportion of individuals infected for each genus across each sampling locality. Richness was calculated as the number of unique lineages recovered across each sampling locality. Bioclimatic data were $\log(x + 1)$ transformed. Response variables were square root transformed for counts and logit transformed for proportions. We used the resulting root mean squared error of prediction (RMSEP) validation results to determine the number of LVs as most predictive for each response variable (Supplementary Fig. S1). We then used the regression coefficients for each response variable across LVs and determined the most significant predictors for each association. All bioclimatic variables (predictors) with significant values were selected for the number of components with the corresponding lowest RMSEP value across all response variables, resulting in $n = 37$ predictor variables (Supplementary Table S2). The significance of the predictors was determined by taking the squared value of the coefficient and determining those greater then $1/k$ (where k is the number of predictor variables).

2.5. Phylogenetic analysis

We created the avian host phylogeny using birdtree.org, which uses the comprehensive avian phylogeny recovered by Jetz et al. (2012) together with a fossil calibrated backbone phylogeny (Hackett et al., 2008), which constricts sampled species to their respective clades to create distribution trees (Jetz et al., 2014). From the 9,000 distribution trees generated, we created a consensus phylogeny. Specific avian epithets listed follow Howard and Moore (Howard and Dickinson, 2003). Species were then grouped into taxonomic clades for descriptive purposes, by order, family, or a combination of both where necessary.

The Cyt *b* haemosporidian Bayesian phylogeny was constructed in MrBayes 3.2. We selected the most appropriate model of nucleotide substitution as the GTR + I model, as determined by both jModelTest 2.1. (Guindon et al., 2003; Darriba et al., 2012) and PartitionFinder 2.1.1 (Lanfear et al., 2017), and ran 10 million generations, sampling every 1000 generations. A 20% percent burn-in of trees was discarded before creating a majority rule consensus tree. An outgroup was not specified and the final tree was rooted to the *Leucocytozoon* clade.

2.6. Prevalence heat map analysis

Heat maps were created in R 3.3.2 (R Core Team, 2016) using the Superheat package (Barter and Yu, 2018). For the avian host taxonomy, we clustered species phylogenetically, and associated this with the prevalence of haemosporidian lineages (unscaled data), also clustered phylogenetically. We included duplicates of each haemosporidian lineage if recovered from multiple localities, to characterize associations with avian hosts across localities.

We used the reduced data set of associated bioclimatic variables (Supplementary Table S2 1, $n = 37$) resulting from the PLSR analysis as bioclimatic input for heat map association with phylogenetically clustered haemosporidian lineages (based on the MrBayes analysis). The bioclimatic data were scaled from zero to one using a quantile preserving scale. We then used a hierarchical clustering analysis with pairwise Euclidean distances and a Ward's algorithm for the linkage method. Our haemosporidian lineages included duplicates of each lineage only if recovered from multiple localities, to characterize all environmental associations of each lineage. All data and R code are available on GitHub (DOI: https://doi.org//10.5281/zenodo.1299983).

3. Results

3.1. Parasite lineage diversity

We recovered a total of 85 unique haplotypes (hereafter referred to as lineages); 40 (47.1%) of these lineages were previously recorded in MalAvi/GenBank, while 45 (52.9%) were novel lineages. Of the 85 unique lineages, 41 (48.2%) were *Haemoproteus* haplotypes (28 of which were novel lineages), 33 (38.8%) were *Plasmodium* haplotypes (11 of which were novel), and 11 (13%) were *Leucocytozoon* haplotypes (six of which were novel lineages).

Pairwise genetic distance within each genus varied; *Haemoproteus* reached 10.9%, *Plasmodium* reached 10.1%, and *Leucocytozoon* reached 7.3% (Supplementary Fig. S2). Between group mean distance across genera was 9.6% for *Haemoproteus* and *Plasmodium*, 14.9% between *Haemoproteus* and *Leucocytozoon*, and 15.5% between *Plasmodium* and *Leucocytozoon*.

Co-infections, PCR recoveries of more than one genus of haemosporidian (*Haemoproteus*, *Leucocytozoon*, and/or *Plasmodium*) were recovered for 23 individuals (Supplementary Tables S3, S4). *Haemoproteus* and *Leucocytozoon* co-infections were recovered in three individuals. *Haemoproteus* and *Plasmodium* co-infections were recovered across 14 individuals. *Leucocytozoon* and *Plasmodium* co-infections were recovered in six individuals. Co-infections of more than one lineage of the same genus in an individual were recovered in 21 individuals. Co-infections of *Haemoproteus* lineages were recovered in seven individuals. Co-infections of *Plasmodium* were recovered in 14 individuals (Supplementary Table S4).

We recovered 105 lineages which were unique across different sampling localities, meaning that 20 lineages were recovered from multiple sampling localities (found across two to five different localities). These were included in the analysis to characterize environments and host associations for all recoveries of lineages (Figs. 2, 3).

The most highly recovered haemosporidian lineage LAMPUR03 (within clade P4), previously recorded in MalAvi, was here recovered a total of 38 times across 17 host species, across six avian families, and across all five localities (Figs. 2, 3). The majority of LAMPUR03 host associations from our sampling were recovered in Nectariniidae ($n = 26$). The lineage LAMPUR03, as known from four previous MalAvi recoveries is widespread, recovered once from Gabon for a Sturnidae species, once in Bulgaria, and twice in Sweden. The three latter recoveries were from a European-African migrant *Ficedula* spp. Lineage GRW09 is the most highly recovered MalAvi lineage across Africa (77 recorded MalAvi recoveries across eight countries), yet in this study it was only recovered three times, twice in Pycnonotidae and once in Cisticolidae, all from the Lama Forest locality (Fig. 1).

3.2. Parasite host associations

Avian sampling was diverse and included representatives of seven orders, 27 families, and 77 species, with most sampling consisting of Passeriformes (77%) (Supplementary Fig. S2; Supplementary Table S3). We recovered positive PCR amplifications for haemosporidians from 113 of the total 222 individual avian hosts sampled (50.9%) (Table 1). We detected *Haemoproteus* in 48 of 222 individual hosts sampled (21.6%), *Plasmodium* in 74 of 222 individual hosts (33.3%), and *Leucocytozoon* in 14 of 222 individual hosts (6.3%).

We recovered associations for haemosporidian lineages across all avian taxonomic clades including non-passerines and passerines (Supplementary Table S3). All orders, with the exception of Charadriiformes (nested within the Glareolidae/Ardeidae clade (grouped for description purposes), were positive for haemosporidian detection. Of the families sampled ($n = 27$), 20 of 27 had one or more haemosporidian recoveries; the remaining seven families resulted in no recoveries, including one family within Charadriiformes, two families within Piciformes, and four families within Passeriformes (Fig. 2). Overall, of the 77 total avian species sampled, 55 species were positive for haemosporidian detection. *Haemoproteus* was recovered in all avian clades except for Glareolidae/Ardeidae. *Plasmodium* was recovered in all but four avian clades (Glareolidae/Ardeidae, Piciformes, Pellorneidae/Leiothrichidae, Passeridae). *Leucocytozoon* was taxonomically restricted to associations within the Glareolidae/Ardeidae, Campephagidae, Pycnonotidae, Pellorneidae/Leiothrichidae, and Nectariniidae clades.

We recovered several haemosporidian clades that were highly associated with avian host clades (Fig. 2). Nectariniidae had the highest haemosporidian prevalence, with a total of 53 lineages (*Haemoproteus* $n = 11$, *Plasmodium* $n = 39$, *Leucocytozoon* $n = 3$) recovered across 28 individuals. Nectariinidae exhibited three primary haemosporidian clades, one in *Haemoproteus* (H1), which was recovered from one northern (western Sudanian savanna) and two southern (Guinean forest-savanna) localities, and two *Plasmodium* clades (P3 and P4), which were recovered across all five localities. Clade P4 had numerous recoveries from other taxonomic host groups (Cisticolidae, Muscipadae, and Monarchidae) (Fig. 2). Other strong associations were found in Pycnonotidae, with 19 lineages (*Haemoproteus* $n = 5$, *Plasmodium* $n = 7$, *Leucocytozoon* = 7) recovered from 24 individuals, which were recovered from four of five localities. The majority of Pycnonotidae infections were recovered from a single host species (*Pycnonotus barbatus)* which is one of the most common and widespread species across sub-Saharan Africa (Fishpool and Tobias, 2017). Pycnonotidae exhibited a predominant association with parasite the *Plasmodium* clade (P1), where all lineages were recovered from *P. barbatus* and from both northern localities and a single southern locality (the northernmost Dogo Forest locality). Cisticolidae had 15 lineages (*Haemoproteus* $n = 5$, *Plasmodium* $n = 13$) recovered from 23 individuals sampled and was found across four of five localities. Ploceidae had 20 lineages (*Haemoproteus* $n = 12$, *Plasmodium* $n = 8$) recovered from 19 individuals which were found across all localities with a distinctive *Haemoproteus* association (H3) which was found with detections across two of the southern localities (Dogo Forest and Lake Toho). The Viduidae/ Estrildidae avian host clade recovered 16 lineages from 22 individuals (*Haemoproteus* $n = 8$, *Plasmodium* $n = 8$). The Estrildidae/Viduidae clade exhibited a dis-

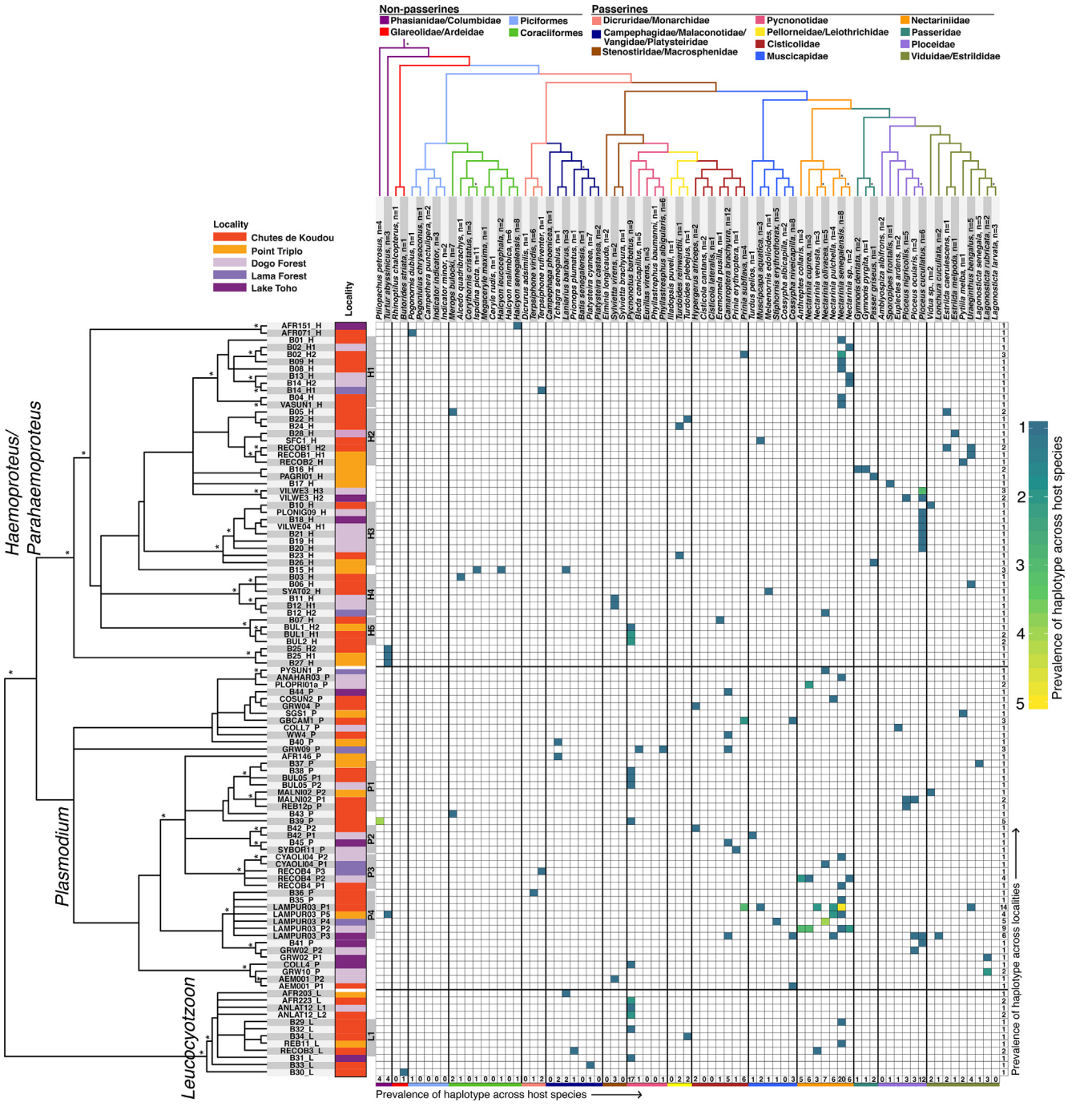

Fig. 2. Haemosporidian phylogeny and heat map prevalence across avian host phylogeny. The heat map shows haemosporidian lineages (left, shown in phylogenetic order) with prevalence as indicated by color gradient (Prevalence scale = 1–5; zeroes noted with a white square) across avian host taxonomy. Prevalence totals for each species sampled are shown at bottom (ranging from 0 to 20). The prevalence of each lineage (across recovered localities) is shown on right side (ranging from 1 to 14). Asterisks indicate posterior probabilities >95. (For full colour resolution, please see the online version.)

crete clade in *Haemoproteus* (H2) found primarily in northern sites as well as the one recovery in the most northern southern locality, the Dogo Forest. Leucocytozoon L1, was detected in four passerine families Vangidae, Pycnonotidae, Leiothrichidae and Nectariniidae.

3.3. Environmental associations

Hierarchical clustering of environmental variables resulted in five main clusters, where the variance in the variables was most similar. We categorized these five clusters as: (1) precipitation 1, (2) dryness (which includes primarily PET variables that measure the potential amount of evapotranspiration that would occur if water was available), (3) T1 and T2 (temperature related variables), and (4) temperature 3, (5) P2 (precipitation variables) (Fig. 3; Supplementary Table S1). We recovered varying degrees of clustering within the haemosporidian lineages across localities and environmental variables. We recovered *Haemoproteus* and *Plasmodium* from all five sampling localities. *Leucocytozoon* was recovered from four of five sampling localities, with none was recovered from the Lama Forest. We observed higher associations of clustering of

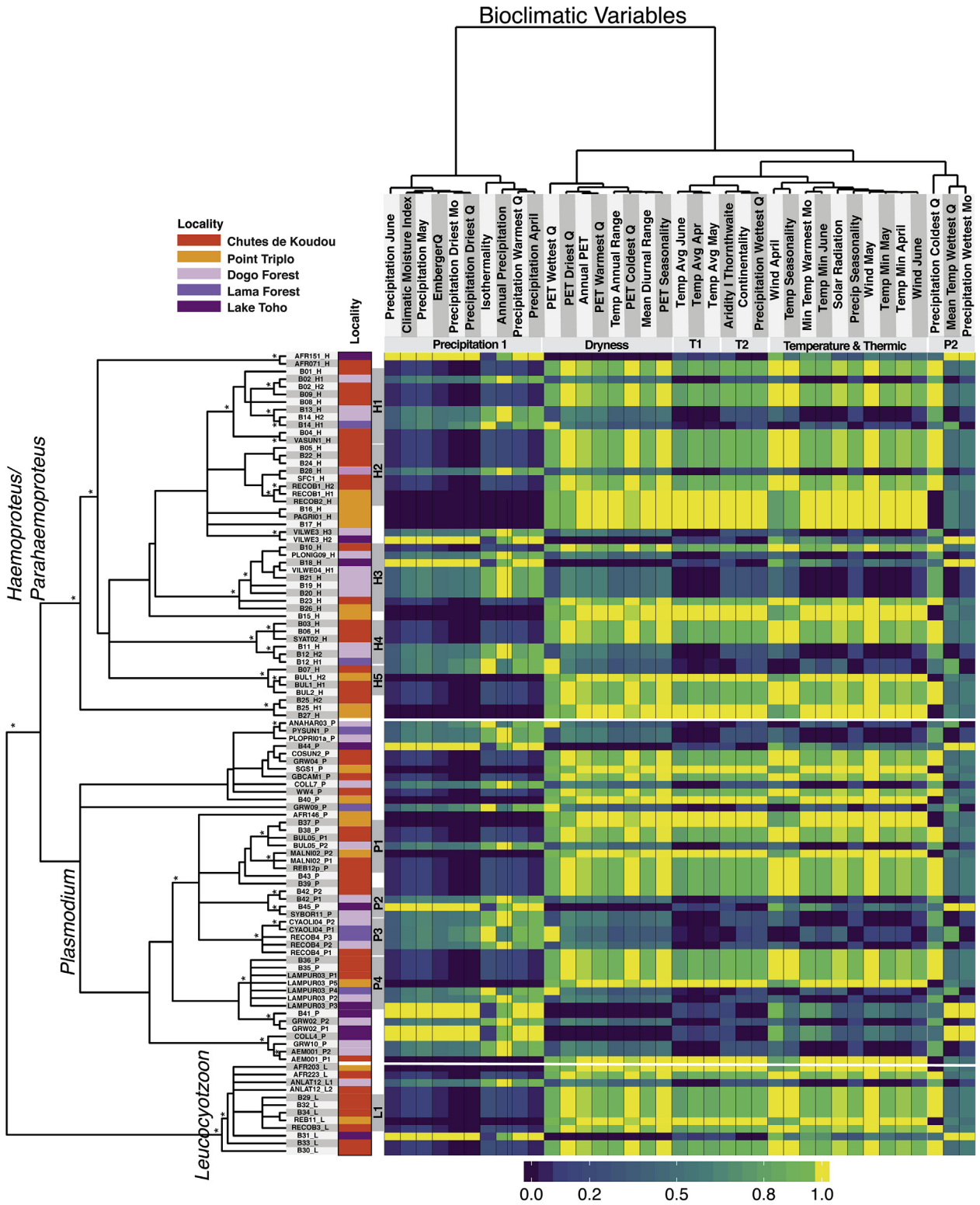

Fig. 3. Dendrogram of hierarchically clustered bioclimatic variables (WorldClim V2 and ENVIREM) using Euclidean distances and Wards linkage method. Bioclimatic variables are scaled from zero to one using a quantile preserving scale. The dendrogram associated with parasite phylogeny shows unique lineages across localities (if lineages are recovered from multiple localities then the lineage is listed for each). Asterisks indicate posterior probabilities >95. (For full colour resolution, please see the online version.)

intra-generic clades in *Haemoproteus* (H2, H4 and H5) compared with *Plasmodium* (P2, P3, P4), where clades clustered in bioclimatically similar localities. In *Haemoproteus* clade H3, clustering of variables was for high precipitation and lower temperature and aridity measures, whereas for Plasmodium clade P1 we saw the opposite and clustering was for low precipitation and high temperature and aridity measures.

For *Haemoproteus* richness and prevalence, the environmental predictor variable which most effectively maximized the covariance was precipitation of the driest quarter (PDQ; Supplementary

Table 1
Haemosporidian detections across genera, including novel and previously recovered lineages.

	Haemoproteus	*Leucocytozoon*	*Plasmodium*	Total
Individuals infected, % infected	48, 21.62%	14, 6.31%	74, 33.33%	85
Novel lineages	28	6	11	45
Previously recovered lineages	13	5	22	40

Table S2). For *Leucocytozoon*, we also recovered PDQ as the most informative predictor variable for richness, yet precipitation of the wettest month was most informative for prevalence. The most informative environmental predictor variables for *Plasmodium* were precipitation of the coldest quarter and PDQ. Our first clade in *Haemoproteus* H2 was most clearly clustered into two groups by variables in the aridity cluster, whereas clade H3 was most clearly differentiated by extremes in the variables for precipitation 1 and P2 (Fig. 3). We saw lower fidelity to locality, and therefore bioclimatic clusters, across *Plasmodium.* We recovered a mix of climate, and thus localities, within clades P1, P2, and P4, with three to five localities represented within each clade. For Leucocytozoon our sole clade was highly clustered across bioclimatic variable and only recovered in both northern localities (Fig. 3).

4. Discussion

In this study, we addressed both the importance of host associations and environmental variables in the structuring of haemosporidian lineages to provide insights into the ways in which climate affects the distributions of parasites and their associated hosts. We recovered substantial numbers of previously documented lineages (47.1%) in our sampling, which serves to highlight the degree of connectivity that exists across African regions, as well as migration aided transmission along the Eurasian-African flyway. Importantly however, we also recovered equally substantial new haemosporidian diversity as represented by 52.9% novel haplotypes. The high number of novel haplotypes was not unexpected, given (i) that Benin has never been sampled for avian haemosporidians, and (ii) that other recent parasite studies (e.g. on avian lice) have also shown newly recovered African species (Takano et al., 2017). We also determined that in our Benin sampling, bioclimatic variables do have an impact on haemosporidian genera richness and prevalence, although this effect varies across genera. Results suggest that these characterizations are not genus-specific but instead specific at the intra-generic clade level. These intra-generic clades may represent more meaningful evolutionary units, although this determination was not the objective of this study.

This is the first known study addressing avian haemosporidians host and climate associations in the country of Benin. Sampling thus far remains low across the Sudanian biogeographic region (Outlaw et al., 2017), in which Benin is found, with sampling in only a few Sudanian countries (Supplementary Fig. S4). Nigeria has the highest haemosporidian number of lineages recovered (n = 201 MalAvi lineages); these recovered lineages are from both bioregions in which Nigeria is found, the Sudanian and Saharan (Dinerstein et al., 2017). A handful of other samples have been taken from other Sudanian bioregion countries (all from the Eastern Guinean Forest), for example Ghana (n = 38 MalAvi lineages) and Senegal (n = 2 MalAvi lineages). We recovered higher infections of *Haemoproteus* compared with a Sudanian bioregion meta-analysis of Malavi lineages (36.5% based on 167 lineages total Sudanian lineages; Outlaw et al., 2017); however, given the high world-wide diversity of biting midge species (Culicoides) this is not unexpected and results may have been due to limited sampling (Mellor et al., 2000). We recovered similar *Plasmodium* and lower *Leucocytozoon* infections compared with the broader Sudanian bioregion (Outlaw et al., 2017). We believe that our low recovery of *Leucocytozoon* is due to the general lack of streams and bodies of water typically used by black flies near many of our sampling sites, which would lower black fly vector abundance (Sutcliffe, 1986). While black flies are indeed present in Benin (current species inventories are reflective of medically relevant species primarily relating to cattle), vector diversity and abundance has not been well assessed across Africa (Adler and Crosskey, 2015).

Host association and bioclimatic variables are likely critical factors structuring the distribution of haemosporidian lineages, and our analyses are an attempt to visually represent these relationships (Figs. 2, 3). The relationships recovered reflect a wide spectrum of lineage clusters associations, in which they are constrained to host taxonomy, by bioclimatic characteristics, or both. For example, clade H1 within *Haemoproteus* is strongly associated with the avian family Nectariinidae, with only two recoveries found outside this clade. Climatically, H1 is found in two of the southern sampling localities (Dogo Forest, Lama Forest), with multiple recoveries from only one of the northern localities (Chutes de Koudou). Chutes de Koudou is the most climatically similar to southern sites with respect to variables within Precipitation 1. Therefore while H1 is generally constrained by host taxonomy in our sampling, it is also structured geographically by precipitation, indicating vector constraint tied to precipitation. Alternatively, clade H2 lineages were recovered across four diverse host taxonomic groups and primarily recovered from climatically similar northern localities; the exception was a recovery from the Dogo Forest. Therefore, clade H2 distribution is apparently more impacted by climate variables than by host association and indicates broadly distributed vectors. This is supported by the fact that previously known haplotypes in this clade (MalAvi lineages SFC1, RECOB1, RECOB2) are recorded as occurring in the same families recovered in this study. In *Plasmodium,* we saw less host specificity for lineage associations as all clades are recovered broadly across two to six taxonomic groups. We saw varying levels of association across climatic variables, with lineage clusters tied to as few as three localities (P1, P2, and P3) and as many as all five (P4). The patterns of association for clades that clustered strongly for environmental extremes (such as clades H3 and P1; Fig. 3) indicated habitat specificity of the vectors.

While these visualizations provide insight into limitations of the distribution of phylogenetic sub-clades of haemosporidian lineages, we know there are other factors that shape distributions that are not represented here. These include, but are not limited to, competent host availability as well as non-competent host species densities (Keesing et al., 2006). Parasite transmission can be reduced if non-host density exceeds competent host density. Further, abiotic factors, which are difficult to measure, include water accumulation and distance to bodies of water. Given that the requirements of precipitation and standing water vary greatly among haemosporidian vector groups (from puddles of water to running streams) it is difficult to capture the broad variation needed to determine the distribution of vector groups and their subsequent presence in host populations. While the broad associations of *Plasmodium* lineages across sampled avian host taxonomy were as expected, the most abundant parasitism associations were

within fewer taxonomic groups (Fig. 1; *Haemoproteus*: H3 in Ploceidae, H2 in Estrildidae, and *Plasmodium*: P1 in Nectariniidae). Confirmation of this would require further quantification of parasitemia (real-time quantification PCR) as it is difficult to detect low or inactive infections using histology and microscopy (Valkiunas, 2005).

Disentangling distributional patterns in the avian haemosporidian system is complex and affected by several factors such as vector associations and bioclimatic constraints across ecoregions. We have limited knowledge of vector distributions and abundances, and bioclimatic data in this study were selected to take into account vector/parasite development requirements. Increasing the understanding of the ecological constraints of the various vector groups associated with each genus is a priority to inform parasite distribution modeling. Although we sampled across just two discrete ecoregions, our results indicate the importance of assessing bioclimatic variables when assessing haemosporidian distributions across regions and habitats. As such, we believe these types of across-ecoregion comparisons, which also include phylogenetic analyses, will be increasingly informative at a greater geographic scale, particularly with increased replicates across similar habitats. Future work should expand to examine haemosporidian diversity across the 110 diverse ecoregions of Africa (Fig. 1). The diversity of these ecoregions is well exemplified in the 18 ecoregions of South Africa. Increasing sampling localities will further allow the implementation of predictive modeling. To determine the effect of the host and bioclimatic characteristics, we need to assess the patterns at a larger geographic scale encompassing multiple ecoregions and diverse habitats.

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Appendix A. Supplementary data

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