Diversity and distribution of avian haemosporidians in sub-Saharan Africa: an inter-regional biogeographic overview

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SUMMARY

The diversity of avian malaria parasites is much greater than 20th century morphologists realized and virtually every study in this field in the last 15 years has uncovered previously undocumented diversity at multiple levels within the taxonomic hierarchy. Despite this explosion of knowledge, there remain vast sampling gaps, both geographically and host-taxonomically, which makes characterizing patterns of diversity extremely challenging. Here, we summarize the current state of knowledge of sub-Saharan African avian malaria parasite diversity, focusing on avian hosts endemic to Africa. The relative proportions of the parasite genera included here, *Plasmodium, Haemoproteus* (including *Parahaemoproteus*) and *Leucocytozoon*, varied between regions, in part due to habitat preferences of the insect vectors of these genera, and in part we believe due to sampling bias. Biogeographic regions of sub-Saharan Africa harbour about the same proportion of endemic to shared parasite lineages, but there appears to be no phylogenetic structuring across regions. Our results highlight the sampling problem that must be addressed if we are to have a detailed understanding of parasite diversity in Africa. Without broad sampling within and across regions and hosts, using both molecular tools and microscopy, conclusions about parasite diversity, host–parasite interactions or even transmission dynamics remain extremely limited.

Key words: Haemosporida, Africa, birds, malaria.

INTRODUCTION

The ecosystems of the African continent collectively harbour some of the highest continental bird diversity in the world; only the Neotropics and tropical Asia are more diverse. Not surprisingly, the malaria parasites of these birds appear to be as diverse as their avian hosts (see also Clark *et al*. 2014), as reflected in a number of regional studies conducted over the past decade (e.g. Smith *et al*. 2011; Loiseau *et al*. 2012; Okanga *et al*. 2014; Lauron *et al*. 2015; Lutz *et al*. 2015). While this diversity is to some extent driven by regional ecosystem differences including host distributions (see also Lauron *et al*. 2015), the underlying method of transmission is likely also a factor (Santiago-Alarcon *et al*. 2012). Although all three major genera of avian malaria parasites are vectored by dipteran arthropods, the current understanding is that *Plasmodium* is vectored by mosquitoes, whereas *Haemoproteus* is transmitted by louse flies or biting midges, and *Leucocytozoon* is transmitted by black flies (Valkiūnas, 2005). The different ecological requirements of these dipteran vectors (largely related to water availability, water flow and temperature) play a major role in the overall distribution of each haemosporidian genus. Our goal here is to use existing genetic data on parasite lineages to provide a synthesis of known malaria parasite diversity across sub-Saharan Africa. To accomplish this, we have characterized patterns of diversity in African avian malaria parasites to address three basic questions: (1) How diverse are malaria parasites within the biogeographic regions of Africa?, (2) How are parasite lineages distributed geographically, i.e. what are the proportions of endemic parasites in each region and are some regions more likely to share lineages than others?, and (3) What are the host preferences and host breadths of these parasites?

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The state of the continent

From very early microscopy analyses of ~11 500 blood smears, 70 morphological *Haemoproteus* (including *Parahaemoproteus* and *Haemoproteus*) and 13 morphological *Plasmodium* species have been found in birds in Africa (Garnham, 1950; see Valkiūnas, 2005 for summary). Yet of these, just

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15 *Parahaemoproteus*, 2 *Haemoproteus* and 2 *Plasmodium* morpho-species were endemic to Africa. From the 1970s through early 1990s, surveys based on microscopy of blood smears collected from a taxonomically broad spectrum of host species were conducted in sub-Saharan Africa, and prevalence (percentage of birds with detectable parasites in the blood) ranged from similar levels of 11·5% in Senegal, 13% in Cameroon and 19·1% across sub-Saharan Africa (collectively), to a comparatively high level of 37% from East African savannah regions (Bennett and Herman, 1976; Bennett *et al*. 1978; Kirkpatrick and Smith, 1988; Bennett *et al*. 1992). In 2005, two very detailed studies, one focused on western African rainforests (Sehgal *et al*. 2005) and one from Uganda (Valkiūnas *et al*. 2005), highlighted the diversity of haemosporidian parasites in Africa using microscopy techniques. Combined, these two studies included 1276 individual birds, and haemosporidian parasites were identified in 27 avian families, with prevalence ranging from 28·6 to 61·9%, with the highest numbers from Uganda.

Surveys of molecular phylogenetic relationships generally return higher diversity and prevalence estimates than microscopy, although these can be inflated due to circulating sporozoites. A recent survey of parasites in Western Congolian rainforests (Møller *et al*. 2011) identified haemosporidians from 25 host families from just 527 individual birds; *Plasmodium* infections alone were detected in 45% of individuals (Beadell *et al*. 2009). And indeed, most molecular examinations of parasite diversity in birds have focused on just a few families (primarily sunbirds [Nectariniidae] and bulbuls [Pycnonotidae]) from the Western African or Western Congolian rainforests (Bonneaud *et al*. 2009; Chasar *et al*. 2009; Loiseau *et al*. 2010; Iezhova *et al*. 2011; but see Hellgren *et al*. 2007 and Lauron *et al*. 2015). Recent exceptions to these taxonomically limited, rainforest-based studies are from the Vwaza Marsh in Malawi (Lutz *et al*. 2015) and wetlands in the Western Cape of South Africa (Okanga *et al*. 2014). The only study to assess diversity in the Saharan region used targeted sampling in northeastern Nigeria for a limited number of avian species ($n = 9$) thereby not reflecting the overall malaria diversity of that region (Waldenström *et al*. 2002).

In a broader study of global parasite diversity, Clark *et al*. (2014) include sub-Saharan Africa as a biodiversity hotspot for haemosporidian parasites, but do not explicitly discuss parasite distributions on the continent. In another study focusing on *Plasmodium* parasites from African sunbirds, Lauron *et al*. (2015) show that there is a great deal of variation in the distributions of *Plasmodium* lineages, with some being geographically limited and others very widespread. Likewise, some *Plasmodium* lineages from sunbirds are probably more specialized on hosts than others.

State of the sampling: limitations

Studies of haemosporidian diversity in Africa, like those in virtually every other part of the world, have been inconsistent with collection and with laboratory protocols to detect parasites. Much of the variation that we see across Africa may be in part due to these research biases. The overwhelming majority of studies have focused on *Haemoproteus* and *Plasmodium* (Ricklefs and Fallon, 2002; Waldenström *et al*. 2002; Durrant *et al*. 2007; Pérez-Tris *et al*. 2007; Beadell *et al*. 2009; Bonneaud *et al*. 2009; Ishtiaq *et al*. 2012; Karamba *et al*. 2012), and sometimes only on *Plasmodium* (Beadell *et al*. 2006; Valkiūnas *et al*. 2009; Loiseau *et al*. 2012). Of the 15 studies, the data for which are included in our analyses, only three include data from *Leucocytozoon* (Hellgren *et al*. 2007; Ishak *et al*. 2008; Lutz *et al*. 2015), because these other studies did not look for this parasite. Further restricting the potential diversity is that most studies include only passerines (songbirds, representing roughly ½ of avian diversity), or at least the majority of the samples came from passerines. And, finally, most studies have been geographically limited, as we discuss above.

We point out also that previous studies of African malaria parasites (see above) did not systematically sample across parasite lineages in their molecular techniques. In particular, primer bias issues in commonly used polymerase chain reaction (PCR) techniques exist such that some primers target primarily *Plasmodium* or *Plasmodium* and *Haemoproteus* but are not well suited for *Leucocytozoon*. Thus, any regional differences could be partially attributable to primer bias, rather than differences related to vectors and their life histories or to host specialization.

Biogeographic regional parasite diversity

Linder *et al*. (2012) developed statistical models of faunal distributions across sub-Saharan Africa and here we use that which they developed and defined for birds (i.e. the hosts). Virtually all African avifauna cluster into these seven statistically-defined sub-Saharan biogeographic regions (rather than geographic as in Lauron *et al*. 2015): Saharan (to include Mauritanian), Sudanian, Ethiopian, Somalian, Congolian, Zambezian and Southern African. Although each region does have some number of endemic bird species, areas with a larger proportion of endemics are the Ethiopian, Congolian and Zambezian regions; these three regions also have the highest levels of species richness (Jetz and Rahbek, 2002; Fjeldså, 2003). For the Ethiopian

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region, endemism and species richness are tied to distinctive high elevation mountains, whereas endemism and species richness in the Congolian region are tied to lowland tropical forest habitat. And, in the Zambezian region, endemism and species richness are largely associated with Afromontane forests in the East Arc Mountains of Tanzania and Kenya. Much of the remainder of the Zambezian region comprises semi-arid savannah and deciduous dry forests, and as such, much of the avifauna found in the Zambezian region is also found in the different and varying habitats found in the Southern African region as well (see Sinclair and Ryan, 2010 for avian distributions).

Similarly, the desert and semi-arid northern savannahs found in the Saharan, Sudanian and to some extent the Ethiopian and Somalian regions also tend to share avian species. The Sudanian region is unique however in hosting a significant number of wintering European migrant species (see Sinclair and Ryan, 2010). And finally, avifaunal assemblages to the north of the Sahara, for example those found in the Atlas Mountains of Morocco and Algeria, tend to have closer ties to the European avifauna than to sub-Saharan ones. As a broad generalization then, avian species tend to be restricted to one or more northern regions, the Congolian region, or to either or both of the southern regions (Zambezian and Southern African). Because of the regional variation in avian assemblages, we sought to evaluate whether these partitions are also apparent in avian malaria parasites.

Geographic distribution of parasite lineages

With the large number of endemic bird species within each of the biogeographic regions, we were interested in evaluating whether this regional variation is also reflected in their malaria parasites. Here, we define an endemic parasite by whether the lineage was only recovered from one region but was found in two or more host individuals. We also wanted to know how many lineages are shared between regions, i.e. whether a parasite lineage was found in two or more regions. Because the genera of malaria parasites use different insect vectors (see above), we also evaluated the proportions of each parasite genus within each region. For an evolutionary context, we conducted a phylogenetic analysis of all lineages to determine whether phylogenetic structure was linked to biogeographic regions in any way, i.e. do one or more regions harbour distinct clades of malaria parasites as you might expect if parasites were host specific?

Host preferences

Recent research has shown that there may be as many avian malaria parasite species as there are avian hosts (see Ricklefs *et al*. 2014), so parasites should be the most diverse in regions with the greatest proportions of endemic host species. To address this, we determined the number of host species in which each parasite lineage was detected and how host families are parasitized by genus. Five of the best sampled passerine families (Cisticolidae, Estrildidae, Nectariniidae, Ploceidae and Pycnonotidae) have their greatest diversity in Africa. However, because these families are widespread across Africa, their distributions may homogenize parasite communities across African regions to some extent due to the Abundance–Occupancy Relationship (Drovetski *et al*. 2014). Finally, we used the Host Specificity Index (S_{TD}; Poulin and Mouillot, 2003) to determine if, at the broad scale of genera (due to the limitations of 'positive-only' data in MalAvi; Bensch *et al*. 2009), parasite genera demonstrate different strategies of parasitism. Without prevalence data by species and region, we cannot eliminate the possibility of spillover infections into non-preferred or dead-end hosts confounding potential patterns; this is a major limitation of these data included here.

MATERIALS AND METHODS

Data compilation

We downloaded all malaria lineage data from MalAvi (April 2015; http://mbio-serv2.mbioekol.lu.se/Malavi/) and pruned the dataset to include only those lineages sampled from resident birds from continental sub-Saharan Africa (1068 cytochrome *b* sequences in 410 lineages [unique sequences]). Limiting our sampling to sub-Saharan Africa allowed us to make use of the statistically-derived African bird faunal regions from Linder *et al*. (2012) for inter-regional comparisons and assessments of regional endemism. We assigned each malaria lineage to one or more of four regions: Congolian, South African, Sudanian and Zambezian (Table 1). Somalian and Ethiopian regions were excluded from the analyses because no data from MalAvi exist from these regions, and Saharan samples were excluded because just 13 parasite sequences have been found there (i.e. too few for meaningful comparisons). For some analyses, we also excluded South African samples because only ten lineages were found there.

Data analyses

We compiled the numbers of lineages that were found in only one region (hereafter, 'endemic') or were found in two, or more regions (hereafter, 'shared'), but excluded lineages found only once (i.e. lineages represented by only one sequence) (Table 2). Using a general linear model (SPSS

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Table 1. Lineages found only within one region, by genus ($P = 0{\cdot}0002$).

Region/genus	*Haemoproteus*	*Plasmodium*	*Leucocytozoon*	Total
Congolian	8	19	0	27
Sudanian	6	4	7	17
Zambezian	15	11	19	45

Table 2. Number of lineages in each region that are endemic to that region and number of lineages found in each region that are shared with other (1 or more) regions ($P = 0{\cdot}0617$).

Region	Endemic	Shared
Congolian	27	27
Sudanian	17	31
Zambezian	45	34

v24), we tested whether biogeographic regions varied in their proportions of hosts infected by parasite genera (*Haemoproteus, Plasmodium* and *Leucocytozoon*). Using a chi-square test (vassarstats.net), we calculated whether biogeographic regions differed in their proportions of endemic and shared lineages. Host specificity was measured using taxonomic distance between host species infected with the same malaria lineage by using the host specificity index S_{TD} and VarS_{TD}, which measures taxonomic evenness (Clarke and Warwick, 2001; Poulin and Mouillot, 2003). Host species are arranged into six broadly accepted taxonomic levels using class, superorder, order, family, genera and species (Dickinson and Remsen, 2013). These taxonomic steps provide the maximum value of S_{TD} where 5 is the maximum steps needed to reach a common ancestor (Class Aves) when all other taxonomic classes are different and 1 being the minimum value when host are sister species; lower specificity values indicate increased host specificity. On the other hand, VarS_{TD} values are indicative of symmetry in taxonomic structure (or evenness), such that a low score would indicate equal taxonomic distances (steps in the taxonomic hierarchy employed) across host species, whereas high values would indicate taxonomic asymmetry or unequal taxonomic distances across host species. Parasite lineages recovered once were removed from S_{TD} and VarS_{TD} analyses as no comparisons can be made.

Phylogenetic analysis

We reconstructed 10 000 000 trees of all lineages using a Bayesian framework (BEAST v1.6.2 with HKY + I + Γ model of nucleotide substitution with estimated nucleotide frequencies and a Yule Process [speciation]) sampling every 1000 trees (Drummond *et al*. 2012). After evaluating tree log-likelihood scores using Tracer (v1.6.0; Rambaut *et al*. 2014) we calculated the maximum clade credibility tree from 10 000 trees with TreeAnnotator (v1.6.2). We coded each terminal branch by the biogeographic region from which it was found (or black for widespread lineages).

RESULTS AND DISCUSSION

Biogeographic regional parasite diversity

A total of 983 infections were reported in resident birds from sub-Saharan Africa. The Congolian region had 249 infections (71 *Haemoproteus*, 175 *Plasmodium*, 3 *Leucocytozoon*); the South African had 76 infections (19 *Haemoproteus*, 57 *Plasmodium*, 0 *Leucocytozoon*); the Sudanian had 167 infections (61 *Haemoproteus*, 62 *Plasmodium*, 44 *Leucocytozoon*); and the Zambezian had 491 infections (121 *Haemoproteus*, 216 *Plasmodium*, 154 *Leucocytozoon*). Focusing on lineages rather than infections, the number of lineages by genus found within only one region varied (chi-square, $P < 0{\cdot}0002$, D.F. = 6); notably, when excluding lineages reported only once, the Congolian region harbours no *Leucocytozoon* lineages in resident birds. However, migratory birds collected there have been infected with this parasite genus (see Hellgren *et al*. 2007).

The different proportions of parasite genera between regions may be due to the ecological distributions of their vectors or to uneven sampling, or to a combination thereof. For example, *Plasmodium* and its *Culex* mosquito vectors (Garnham, 1966; Valkiūnas, 2005) tend to be broadly distributed (Clark *et al*. 2014), but *Culex* are limited by temperature and precipitation, and require standing water (often stagnant) for breeding (Patz and Olson, 2006; other mosquito genera utilize floodwater areas). The genus *Haemoproteus* and its subgenera *Haemoproteus* and *Parahaemoproteus* are vectored by several species of hippoboscid flies (Hippoboscidae) and biting midges (Culicoides), respectively (Atkinson and van Riper, 1991). Both hippoboscids and biting midges tend to range from semi-moist to more arid regions but require moist soil or water for breeding (Meiswinkel *et al*. 2004). Finally, *Leucocytozoon* is vectored by black flies (Simuliidae), which require clean, flowing water to lay their eggs (Carlsson, 1967).

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These different environmental preferences of vectors could explain some of the parasite genera proportions we found within regions. In the comparatively moist/humid Congolian region for example, *Plasmodium* dominated the infections (Fig. 1) with a moderate level of *Haemoproteus*, and *Leucocytozoon* was virtually absent. Importantly however, *Leucocytozoon* was found in many migrants from the Congolian region, the data for which were excluded from our analyses (see MalAvi; Bensch *et al*. 2009). This same pattern held for the Sudanian region, which seems anomalous given that region is far more arid than the Congolian. However the specific localities sampled in the Sudanian region were close to the Congolian region. Thus, the higher rates of infection by *Plasmodium* and *Haemoproteus* would be expected. The same was also true for the Zambezian, a region also drier than the Congolian. Given that the bulk of the Zambezian sampling comes from a single study focused on the Vwaza Marshes of Malawi (Lutz *et al*. 2015), the high levels of *Plasmodium* and *Haemoproteus* are also expected. We would anticipate that sampling in the Sudanian and Zambezian regions, away from extensive water sources, would result in lower regional prevalence of *Plasmodium* and *Haemoproteus*. Finally, the Southern African region (which has the poorest sampling to date; Fig. 1) is dominated by *Plasmodium* infections, with a lower level of *Haemoproteus* infection and no *Leucocytozoon* infections. Given the broad range of habitat variation across this region, particularly in South Africa, we would expect that more intensive and systematic sampling will yield not only higher levels of *Haemoproteus* and *Leucocytozoon* infections, but also strong intra-regional differences in genus level infections.

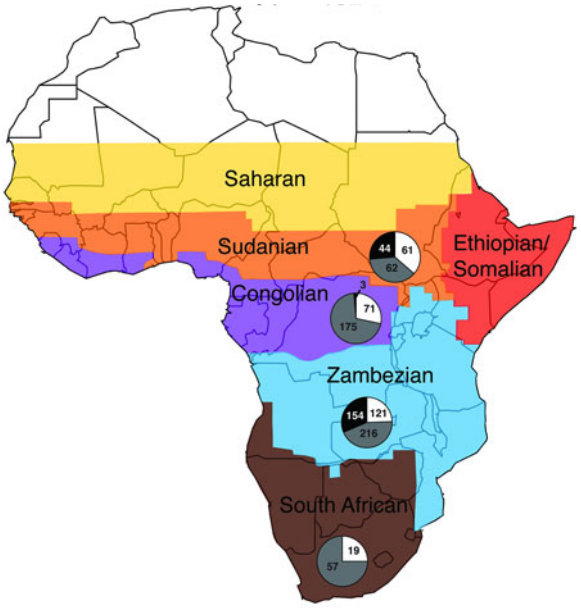

Fig. 1. Biogeographic regions of African avifauna (from Linder *et al*. 2012). Pie charts represent the relative proportions of parasite genera in each region (Grey: *Plasmodium*, Black: *Leucocytozoon*; White: *Haemoproteus*). Numbers in pie charts are the number of infections of each genus.

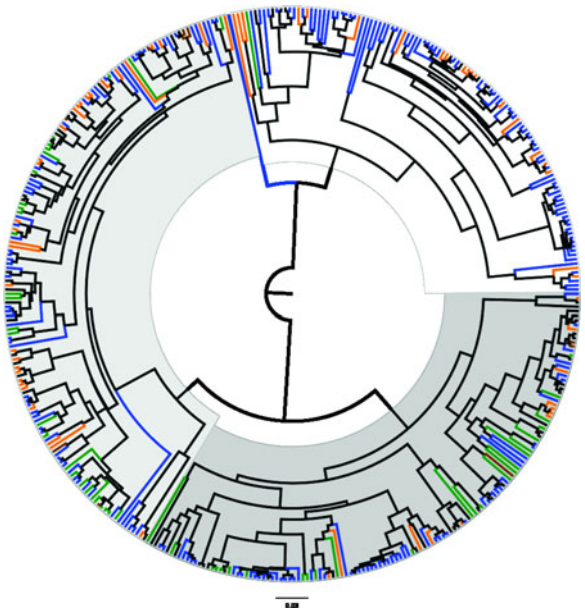

Fig. 2. Phylogenetic reconstruction African avian malaria parasites. Coloured terminal branches correspond to biogeographic regions: Congolian – purple, Southern African – brown, Sudanian – orange, Zambezian – blue, widespread – black. Clades are highlighted to genus: *Plasmodium* – light grey, *Leucocytozoon* – white, *Haemoproteus* – medium grey. Note that branch lengths are depicted as in a cladogram for simplification.

Geographic distribution of parasite lineages

There was no phylogenetic structuring of lineages by biogeographic region (Fig. 2), despite the differences in broad scale distributions of parasites at the generic level (Fig. 1) and the high proportions of endemics in several regions. This is probably due to the predominantly-sampled widespread avian families (largely Passeriformes, Table 3) that would homogenize, to some extent, parasite distributions across regions (see below). If this limited structure holds up to further sampling, this pattern would indicate the importance of other factors such as vector distributions and connectivity between regions due to host movements.

Host preferences

With a few exceptions based on geographic distributions, passerine families are infected by *Plasmodium* more than they are by *Haemoproteus* and *Leucocytozoon* (Table 3); however, very few studies sample all three genera at the same time, and these biases through 'lack of' sampling would almost certainly affect our interpretations. Weavers

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Table 3. Number of infections collected from hosts by host family, and number of lineages from each parasite genus by host family. Note that the number of lineages most often does not add up to the number of infected birds.

			Number of lineages		
Host order	Host family	*N* infections	*Haemoproteus*	*Plasmodium*	*Leucocytozoon*
Falconiformes	Accipitridae	5	1	4	
Passeriformes	Alaudidae	3		3	
Coraciiformes	Alcedinidae	14	9	2	1
Anseriformes	Anatidae	1		1	
Ciconiiformes	Ardeidae	2	1		
Bucerotiformes	Bucerotidae	3		1	2
Cuculiformes	Centropidae	2	1	1	
Passeriformes	Cisticolidae	43	4	18	7
Coliiformes	Coliidae	1		1	
Columbiformes	Columbidae	12	9	1	2
Coraciiformes	Coraciidae	1			1
Passeriformes	Corvidae	5	2	2	1
Cuculiformes	Cuculidae	1	1		
Passeriformes	Dicruridae	4	1	3	
Passeriformes	Estrildidae	55	11	17	11
Passeriformes	Eurylaimidae	1			1
Passeriformes	Fringillidae	27	7	13	5
Passeriformes	Hirundinidae	4	1	3	
Passeriformes	Indicatoridae	4	1	3	
Piciformes	Lybiidae	4	4		
Passeriformes	Malaconotidae	15		6	7
Coraciiformes	Meropidae	5	4	2	
Passeriformes	Monarchidae	8	2	3	
Passeriformes	Motacillidae	9	2	2	3
Passeriformes	Muscicapidae	68	11	24	17
Musophagiformes	Musophagidae	1	1		
Passeriformes	Nectariniidae	123	21	30	11
Galliformes	Numididae	2	2		
Passeriformes	Paridae	4		2	2
Passeriformes	Passeridae	16	6	8	
Galliformes	Phasianidae	7	1	1	3
Piciformes	Picidae	4	1	2	1
Passeriformes	Platysteiridae	21	3	10	8
Passeriformes	Ploceidae	116	29	21	24
Psittaciformes	Psittacidae	1		1	
Passeriformes	Pycnonotidae	126	12	28	30
Gruiformes	Rallidae	2		2	
Ciconiiformes	Scopidae	2	1		1
Passeriformes	Stenostiridae	6	1	2	3
Strigiformes	Strigidae	5	3		1
Passeriformes	Sturnidae	17	8	5	3
Passeriformes	Sylviidae	39	18	9	6
Passeriformes	Timaliidae	23	11	6	4
Trogoniformes	Trogonidae	3	1	1	1
Passeriformes	Turdidae	74	9	22	14
Passeriformes	Zosteropidae	15	6	3	4

(Ploceidae) are infected by all three parasite genera, but slightly more by *Haemoproteus*. Old World warblers (Sylviidae) are also infected by all three parasite genera, but again, more so by *Haemoproteus*. Sampling of bush-shrikes (Malaconotidae) is low, but none are infected by *Haemoproteus*. Old World sparrows (Passeridae) are seemingly not infected by *Leucocytozoon*. Sampling of non-passerine families has been geographically spotty and taxonomically biased, and therefore we need much more broad sampling to firmly establish patterns of host use.

Previous studies have shown how variable parasite distributions can be, and for example, that the distribution of malaria parasites across the landscape was dependent on habitat (distance from rivers), or age and sex of the bird (Wood *et al*. 2007). The differing number of lineages parasitizing a host species or family may be due to migratory behaviour, habitat (even microhabitat as different vectors may specialize in different strata), other environmental variables, host evasion and other behaviours (gregariousness; Atkinson and van Riper, 1991;

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Table 4. Host specificity indices by parasite genus.

Genus	*n*	*N* of host species	S_{TD}	VarS_{TD}
Haemoproteus	110	84	4·64	0·33
Leucocytozoon	108	61	4·12	0·00
Plasmodium	298	161	4·17	0·27
All	516	202	4·28	0·25

Loiseau *et al*. 2010; Rifkin *et al*. 2012; Garcia-Longoria *et al*. 2014; Olsson-Pons *et al*. 2015), as well as detection biases, i.e. abortive stages of development and PCR bias. For example, the somewhat higher levels of *Haemoproteus* infections in weavers could possibly be explained by the ease with which hippoboscid flies and midges could move between nests – many weaver species tend to nest in dense colonies. However, the former nesting behaviour is not characteristic of sylviids, which also showed higher *Haemoproteus* infections.

Parasite genera are not host-specific. Values of the Host Specificity Index (S_{TD} ranged from 4·17 to 4·64; Table 4) reveal a low level of host specificity across all three parasite genera. When all the three genera are grouped the host specificity index remains high with a value of 4·28. Thus, parasite genera are widely distributed across Superorder and Orders of birds. VarSTD values were low, which indicates taxonomic structure was even across all parasite genera (individually and grouped) and further indicates that common ancestor distance of hosts were evenly distributed and reached at a high taxonomic level (in this case at the level of Order).

FUTURE DIRECTIONS

There are major sampling gaps of avian malaria parasites in Africa, and this is problematic for understanding a range of malaria-related topics, from simple assessments of lineage diversity to regional assemblages and endemism to broader ecological questions related to vector habitat preferences and how and why some lineages are broadly distributed while others are not (i.e. the Abundance–Occupancy Relationship; Drovetski *et al*. 2014; see also Lauron *et al*. 2015). Indeed, we find that when we sample a new region or a new host-taxonomic group, that we almost inevitably find new parasite lineages at both the species- and genus-level (Martinsen *et al*. 2016; Outlaw, unpublished data). The basic solution to all of these problems is simple: extensive geographic sampling is required and needs to include both molecular approaches and microscopy. And, sampling at each location should be as extensive as possible in terms of avian hosts (i.e. the broadest possible taxonomic diversity) and numbers of individual hosts sampled per species to convert singletons (or just a handful of birds of a given species) to reasonable frequencies for comparisons. Post-sampling, molecular assessments should be standardized in an effort to recover all the three malaria genera. Combined, these rather straightforward suggestions can provide an abundance of meaningful comparative data, even if just two regions are being compared (e.g. Mata *et al*. 2015).

Despite sampling issues (both taxonomic and molecular), currently available data from sub-Saharan Africa does suggest that there are a substantial number of endemic malaria lineages within avifaunal regions, and that this endemism may in turn be related to regional avian host endemism. Further, there does seem to be a general relationship between the type of habitat in which a host was sampled, and the prevalence of malaria parasite genera, as one would predict based on vector breeding-habitat preference, *Plasmodium* tends to be more prevalent in mesic habitats because mosquitoes require standing water, *Haemoproteus* tend to be more prevalent in xeric habitats because midges often only require moist soil, and *Leucocytozoon* is tied to running water, which its black fly hosts require for breeding. To extend our call for additional sampling, intra-regional microhabitats are another missing puzzle piece, as intra-regional dynamics may be as important and perhaps of more interest than coarse inter-regional studies. The Vwaza Marsh study by Lutz *et al*. (2015) is an excellent example of this. And, temporal variation may also play a role in malaria distribution in that temperature regimes may cause cyclical variation in prevalence; accounting for this should be considered, when and where possible (see Svensson-Coelho *et al*. 2013).

As we increase sampling across and within African bioregions, and indeed bioregions on any continent, we will be able to better decipher patterns of diversification across different scales (from inter-regional to micro-habitat). A broader knowledge of biogeographic patterns will not only help us better understand patterns and processes of malaria distributions, but will naturally lead to informative studies of vectors and how their natural history impacts malaria distribution. This knowledge can also provide insight into studies of why some avian hosts are more susceptible to infection, why some hosts are more likely to carry parasites between

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regions or continents, and why some host species tend to be more highly parasitized than others (see also Clark *et al*. 2014). An added benefit will be that the extensive sampling of avian hosts will allow for much finer scale phylogeographic/population genetic sampling of those hosts than is currently available for most species. This is particularly important in understory-dwelling host species found in Afrotropical forests, where recent work has uncovered extensive cryptic diversity (e.g, Huntley and Voelker, 2016). And together, extensive sampling of malaria parasites and their hosts will allow for powerful and meaningful assessments of the impacts of climate change over time. Ultimately, we do not know what is out there waiting to be discovered, and to expand our knowledge in these areas, it is all about the sampling.

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