Phylogenetic diversity, host specificity and geographic distribution of avian malaria in Africa

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ABSTRACT

Three genera of haemosporidians malaria parasites (*Plasmodium, Haemoproteus,* and *Leucocytozoon*) are known to infect birds across the globe. Molecular-based research on avian malaria parasites have rapidly increased over recent years. As a consequence, databases such as MalAvi have been constructed as a research platform to promote data accessibility. Centralization of data now allows for metanalyses of data to identify broad scale continental patterns. In this thesis I aim to review all published work on molecular-based avian malaria research in Africa. In Chapter 2 this thesis explores the geographic extent of studies in Africa and highlights important research gaps. Using sequence data available for Africa, in Chapter 3 I tested if the cytochrome *b* gene, used to detect the presence of parasite DNA within avian host blood, could also be used to delimit different species of parasite. Using the established phylogenetic species, in Chapter 4 I aimed to identify the distribution of these parasite lineages across Africa and examine the extent of their host ranges. Focusing on avian malaria prevalence among hosts distributed in fragmented forests of South-Eastern South Africa, in Chapter 5 I tested for presence of the three genera of parasites in four sympatric bird species (*Camaroptera brachyura*, *Cossypha dichroa*, *Phylloscopus ruficapilla* and *Pogonocichla stellata*). A total of 460 birds were tested for the three malaria genera using PCR methods. Phylogenetic methods were then used to identify host specificity and the geographic distribution of parasites. This provides important information for how habitat fragmentation affects host-parasite relationships.

By reviewing all avian malaria studies in Africa, I found that research effort was not uniform across the continent. Most avian malaria research was conducted on *Plasmodium*, with most research conducted in the Canary Islands, the Gulf of Guinea, Madagascar and South Africa. Using cytochrome *b* sequence data available for Africa from MalAvi, I tested for the difference between inter- and intraspecific genetic distances within each of the three genera of avian malaria. A distinct "gap" was recorded in *Plasmodium* and *Haemoproteus*, allowing me to divide sequences into OTUs analogous to species. For *Leucocytozoon*, the gap used came from a suggested distance threshold by previous studies. Using the OTUs and additional information on geography and avian hosts, I tested the hypothesis that species belonging to *Haemoproteus* are more host specific than *Plasmodium* which is more generalist infecting a large taxonomic diversity of birds. This was found to be true as well as OTUs which had greater host ranges also had larger geographic ranges. Focusing on a smaller spatial scale I investigated the prevalence of avian malaria in the four sympatric forest bird species occurring in the naturally fragmented forests of South-Eastern South Africa. My research revealed that three of the four avian hosts had low levels of parasite prevalence (10.4-12.1%). The fourth avian host, *C. dichroa*, had a remarkably higher parasite prevalence (55.6%). The phylogenetic structure of the parasites revealed that there was a higher diversity of host-specific parasite species and lineages that were geographically isolated. In contrast, the most abundant *Haemoproteus* species was found in all forest types and all four host species. My research has shown that there are still large gaps in our knowledge on these parasites in particular, a wider range of hosts should be examined in geographical regions that are understudied. This study provides a valuable large-scale review of all current knowledge on avian malaria in Africa and contributes significantly towards the field of host-parasite research.

PREFACE

The data described in this thesis were collected in Pietermaritzburg, Republic of South Africa from February 2020 to October 2022. Experimental work was carried out while registered at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Sandi Willows-Munro.

This thesis, submitted for the degree of Master of Science in the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, School of Life Sciences, Pietermaritzburg campus, represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged in the text.

Sam van Zwieten

October 2022

I certify that the above statement is correct and as the candidate's supervisor I have approved this thesis for submission.

……..

Professor Sandi Willows-Munro

Supervisor

October 2022

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION 1 - PLAGIARISM

I, Sam van Zwieten, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: ……

Sam van Zwieten

October 2022

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Figure 5.5. Maximum likelihood phylogeny of *Haemoproteus* from four sympatric forest bird species (*Camaroptera brachyura*; *Cossypha dichroa*; *Phylloscopus ruficapilla*; and *Pogonocichla stellata*). The dotted line differentiates MalAvi lineages. OTUs are labelled HI and HII. The symbols represent the different host species; Circle = chorister robin-chat, Triangle = green-backed camaroptera, Square = white-starred robin, Star = yellow-throated woodland warbler. Symbols are coloured by forest type; Blue = Albany Forest, Green = Amatole Mistbelt Forest, Red = Eastern Scarp Forest, Purple = Transkei Mistbelt Forest, Yellow = Pondoland Scarp Forest, Orange = Eastern Mistbelt Forest.

Figure 5.6. Maximum likelihood phylogeny of *Leucocytozoon* from four sympatric forest bird species (*Camaroptera brachyura*; *Cossypha dichroa*; *Phylloscopus ruficapilla*; and *Pogonocichla stellata*). The dotted line differentiates MalAvi lineages. OTUs are labelled LI – LIV. The symbols represent the different host species; Circle = chorister robin-chat, Triangle = green-backed camaroptera, Square = white-starred robin, Star = yellow-throated woodland warbler. Symbols are coloured by forest type; Blue = Albany Forest, Green = Amatole Mistbelt Forest, Red = Eastern Scarp Forest, Purple = Transkei Mistbelt Forest, Yellow = Pondoland Scarp Forest, Orange = Eastern Mistbelt Forest.

TABLES

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Three genera of haemosporidians parasites (*Plasmodium, Haemoproteus,* and *Leucocytozoon*) are responsible for malaria infections in mammals, reptiles, and birds (Cornet et al., 2014; Roberts et al., 2013; Valkiūnas, 2005). Avian malaria has been found on every continent except Antarctica, infecting about 68% of examined bird species (Atkinson and Van Riper III, 1991; Clark et al., 2014). These parasites use invertebrate vectors from the order Diptera to infect their avian hosts (Roberts et al., 2013; Valkiūnas, 2005). Traditionally, microscopy has been the preferred method for identifying the presence of these parasites within blood and other tissues from avian hosts and vectors (Richard et al., 2002; Rivero and Gandon, 2018). However, molecular-based research on avian malaria parasites have rapidly increased over recent years. As a consequence, databases such as MalAvi (Bensch et al., 2009) have been constructed as a research platform to promote data accessibility. Centralization of data now allows for meta-analyses of data to identify broad scale continental patterns.

1.2 Structure of this thesis

In **Chapter 2**, all published molecular research on avian malaria conducted in Africa was compiled. In this chapter, several trends in research were highlighted including the geographic distribution of research and key knowledge gaps. The results from this chapter highlight areas that future avian malaria research should prioritize moving forward.

Using the compiled research from the previous chapter, **Chapter 3** aims to determine if the cytochrome *b* sequence data currently available in databases such as MalAvi can be used to delimit parasite species. To this end, I tested if there is a "gap" between inter- and intraspecific genetic distances in two genera (*Plasmodium* and *Haemoproteus*). This method adapted from the field of DNA barcoding is more statistically rigorous than the method currently used to identify parasite lineages on MalAvi. Using the phylogenetic species concept and the position of the "gap" I was able to statistically identify a number of parasite species using cytochrome *b* sequences.

In **Chapter 4**, using the phylogenetic species delimited in Chapter 3, I aimed to reveal the geographic distribution and host ranges of parasite species. This will provide a better understanding on the variations of host specificity observed within these malaria genera, as well as the manner of host specificity for these parasites.

Chapter 5 has a similar approach to Chapter 4 but focuses on a smaller spatial scale. This chapter looks at the diversity of avian malaria parasites within four sympatric forest bird species in south-eastern South Africa. This chapter not only looks at how habitat fragmentation of these forests may affect the species of parasites and their relationship with their hosts, but also any distribution and host specific patterns at a small spatial scale level.

The final chapter, **Chapter 6**, aims to synthesize findings for the entire study by highlighting the research presented here. In this thesis, I aim to provide a better understanding of the processes driving avian malaria research in Africa.

CHAPTER 2

A REVIEW OF MOLECULAR RESEARCH ON AVIAN MALARIA IN AFRICA

Abstract

Avian malaria is caused by three genera of Haemosporidian parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*). Historically, these parasites were detected in avian host blood using microscopy. In recent years molecular approaches have been developed. In this review, I aim to summarize published molecular-based avian malaria research in Africa. In particular, this chapter examines the geographic spread of research and I highlight key knowledge gaps. Using data from 76 studies conducted in 34 African countries and 8 foreign territories (African regions governed by non-African countries), I found that avian malaria research was not evenly distributed geographically, but most research has been conducted in regional research hotspots. There were also key differences among studies which may affect continental-scale meta-analyses such as the number of parasite genera studied, the number of avian host species screened and the number of individuals per host species screened. These inconsistencies make it difficult to draw accurate cross-continental conclusions. This review found that of the three parasite genera causing avian malaria, *Plasmodium* has been the most studied genus, while *Leucocytozoon* has been the least studied. Earlier publications focus on a single genus of parasite, but as molecular diagnostic tools improved, recent publications now tend to include data on all three parasite genera. This review highlights geographic regions that are understudied and calls for a broader taxonomic range of avian hosts to be studied.

Keywords: Avian malaria, *Plasmodium*, *Haemoproteus*, *Leucocytozoon*

2.1 Introduction

The protozoan order Haemosporida include blood parasites that cause a malaria-like disease that infects a wide taxonomic array of animals including mammals, birds and reptiles (Cornet et al., 2014; Loaiza and Miller, 2013; Roberts et al., 2013; Valkiūnas, 2005). Avian malaria is associated with three different parasite genera namely *Plasmodium, Haemoproteus* and *Leucocytozoon* (Rivero and Gandon, 2018; Videvall, 2019). Not all research is in agreement over which parasites are responsible for malaria. Some may refer to *Plasmodium* alone as the cause of malaria, while others rebuke *Leucocytozoon* as causing malaria infection due to lack of a production of the malarial pigment hemozoin (Roberts et al., 2013; Valkiūnas, 2005). For the following literature review, all three genera will be included when referring to avian malaria.

Avian malaria has been recorded in approximately 68% of all bird species examined and is present on every continent except Antarctica (Atkinson and Van Riper III, 1991; Clark et al., 2014). Of the three genera, *Haemoproteus* is the most prevalent, being recorded in approximately 67% of screened bird species, with *Plasmodium* found in 41.5% and *Leucocytozoon* in 39% of screened bird species (Atkinson & van Riper, 1991). These parasites make use of invertebrate vectors mainly from the order Diptera, although each genus makes use of different Dipteran families to transmit to avian hosts (Roberts et al., 2013; Valkiūnas, 2005). Female mosquitos (Culicidae) are linked to the spread of *Plasmodium* in birds, with the genera *Culex* and *Aedes* responsible for the largest proportion of transmission (Rivero and Gandon, 2018; Valkiūnas, 2005; Valkiunas and Iezhova, 2018). *Haemoproteus* is not restricted to a single family of vector but can be transmitted by biting midges (Ceratopogonidae) as well as louse flies (Hippoboscidae) (Roberts et al., 2013; Valkiūnas, 2005). Biting midges are also responsible for transmitting one species of

382 *Leucocytozoon* (*L. caulleryi*), but all other *Leucocytozoon* species are spread by female blackflies (Simuliidae) (Roberts et al., 2013; Valkiūnas, 2005).

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The three parasite genera undergo a similar heteroxenous lifecycle with some key differences 386 (Remple, 2004; Valkiūnas, 2005). The sporozoites of the haemosporidian parasites will cross over to avian host's blood stream through the vector's saliva while the vector is obtaining a blood meal 388 (Atkinson and Van Riper III, 1991; Roberts et al., 2013; Valkiūnas, 2005). These sporozoites will make their way to target host tissue (often liver or lung), where a form of asexual reproduction 390 called exoerythrocytic schizogony takes place within the tissue resulting in the production of merozoites (Roberts et al., 2013; Valkiūnas, 2005). With the exception of *Haemoproteus*, the other 392 genera undergo an additional reproductive phase which occurs within the blood stream of the host, called erythrocytic schizogony which produces more merozoites (Roberts et al., 2013; Valkiūnas, 394 2005). After several generations of merozoites being produced, some of these merozoites undergo gametogony, forming macrogametocytes and microgametocytes within the erythrocytes of the 396 host (Atkinson and Van Riper III, 1991; Roberts et al., 2013; Valkiūnas, 2005). When an invertebrate vector feeds from an infected host, these gametocytes may inadvertently be ingested 398 along with the blood meal (Roberts et al., 2013; Valkiūnas, 2005). Within the invertebrate host, the gametocytes fuse, resulting in the formation of an ookinete (Atkinson and Van Riper III, 1991; 400 Roberts et al., 2013; Valkiūnas, 2005). Due to sexual reproduction of these parasites occurring in the invertebrate vector, it is important to note that the invertebrate vector is in fact the definitive 402 host (Roberts et al., 2013). The ookinete will then form an oocyst within the epithelial cells of the midgut of its hosts, where it undergoes its final reproductive stage, sporogony (Valkiūnas, 2005). 404 This asexual reproduction will result in sporozoites which, when released, make their way through

the haemocoel of the invertebrate towards the salivary glands, where the cycle can repeat in a new host when the vector takes its next blood meal (Roberts et al., 2013; Valkiūnas, 2005).

Charles Louis Alphonse Laveran was the first to identify the cause of malaria in 1880, by identifying parasites under a microscope (Cox, 2010). Shortly after, in 1885, the first evidence of malaria presence in birds was presented and since then microscopy has been the primary method used to identify malarial parasite infection (Richard et al., 2002; Rivero and Gandon, 2018). Currently, morphological identification is still used (Valkiūnas, 2005). Although microscopy is effective in identification and diagnosing malaria presence, it does have drawbacks. These include the time-consuming process of analysing individual slides and the potential for overlooking low intensity malaria infections. The morphological delimitation of malaria species also requires taxonomic knowledge, and it may not be possible to correctly identify parasite taxa to species level (Richard et al., 2002; Rivero and Gandon, 2018; Valkiūnas, 2005). Although alternative methods can be used to determine malaria presence, such as an antibody ELISA (Graczyk et al., 1994), a molecular PCR-based approach is currently favoured.

Using primate *Plasmodium* and *Haemoproteus* sequences, Bensch et al. (2000) designed primers in the conserved regions of the cytochrome *b* genes of these parasites. Additional primers were then designed by Hellgren et al. (2004) to amplify *Leucocytozoon* together with *Plasmodium* and *Haemoproteus*. This nested PCR assay can detect the presence of malaria in a wide taxonomic diversity of avian hosts leading to an acceleration in avian malaria research (Bensch et al., 2009). As a result, databases such as MalAvi (Bensch et al., 2009) have been established as a research platform to promote data accessibility and also provide a standardised naming system for parasite

lineages. As of 2021, the MalAvi database includes avian malaria data from six continents, 120 countries, over 2100 avian host species and more than 4600 unique avian malaria lineages (Bensch et al., 2009).

This chapter aims to review all published molecular-based avian malaria studies in Africa between 2000 and 2020. In particular, this study will examine the geographic distribution of studies to identify regional research hotspots. I also review the 489 avian host species found to be infected with these parasites.

2.2 Materials and methods

A systematic review of the literature was conducted. Published studies spanning 20 years (between the years 2000 and 2020) were downloaded from MalAvi and Google Scholar using the key words: "avian", "malaria", "*Plasmodium*", "*Haemoproteus*", "*Leucocytozoon*", "haematozoa", "haemosporidian", in turn with a combination of location-based keywords such as "Africa", "Indian Ocean", "Atlantic Ocean". I also searched using the name of each African country/territory. For the latter search, older names were also included for example both Swaziland and Eswatini were used when searching. In addition to continental African countries, the island nations of Cape Verde, Comoros, Madagascar, Mauritius, Seychelles, and São Tomé and Príncipe were also included. Foreign dependencies and territories were also included such as: Europa Island, Mayotte and Réunion (France), Madeira and the Savage Islands (Portugal), Canarias (Spain), Saint Helena, Ascension and Tristan da Cunha (United Kingdom), and Socotra (Yemen). The information collected from each article included the year of publication, avian malaria

genus/genera investigated, country/region the research was conducted in, and avian hosts infected with *Plasmodium*, *Haemoproteus* or *Leucocytozoon*.

2.3 Results and discussion

2.3.1 Summary of temporal and geographic distribution of avian malaria studies

In total, 76 published articles on avian malaria were recovered with a list available at https://figshare.com/articles/dataset/Supplementary_material_-_Articles_used/21629438. These studies were conducted in 34 African countries and eight regions. Figure 2.1 shows how molecular-based detection of avian malaria has steadily increased since Bensch et al. (2000) published the cytochrome *b* primers widely used to detect avian malaria.

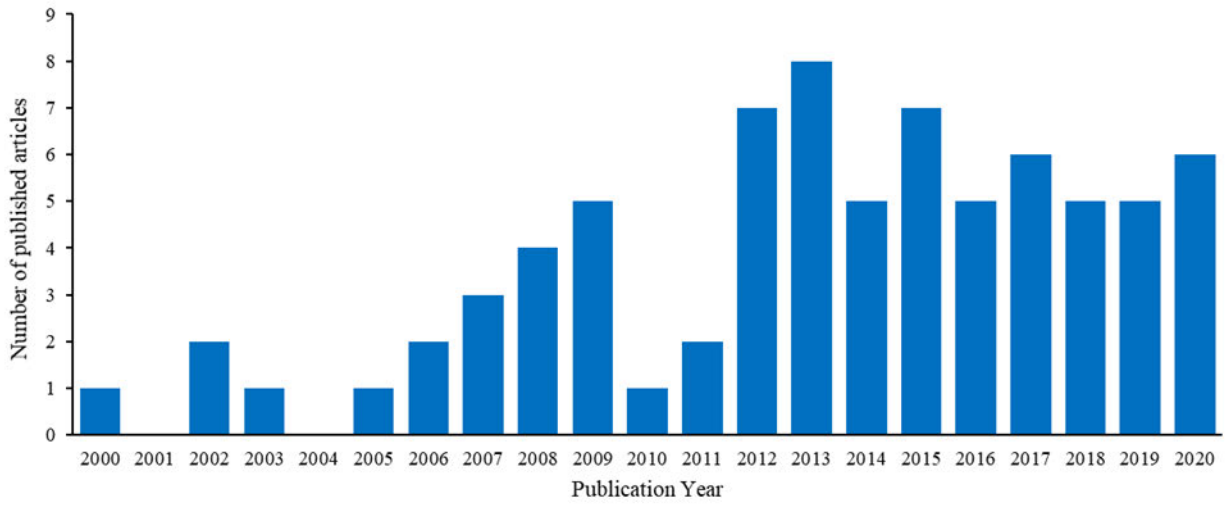

Figure 2.1. Bar graph showing the growth in molecular-based avian malaria studies in Africa since Bensch et al. (2000) published cytochrome *b* primers used to detect the presence of avian malaria genera.

The geographic distribution of studies was not uniform across Africa (Figure 2.2). Most molecular-based avian malaria research has been conducted in Southern Africa and the Gulf of Guinea. South Africa had the most research conducted, with a total of 19 published articles, followed by Cameroon with 14 published articles. Madagascar and the Canary Islands had 10 published articles each. In contrast, limited research has been conducted in Northeast Africa (with the exception of Egypt with three published articles). Worryingly, over a third of the continent's countries (20 countries) as well as several territories (Western Sahara and French Southern Territories etc.) have had no molecular avian malaria research conducted within them. Many songbird species breed in Europe and migrate across North Africa to overwinter in tropical Africa. North Africa is an important transition node for many migrating species. Assessing the prevalence of infectious disease in these regions is of considerable importance. Lack of studies in countries with high avian species richness, such as the Democratic Republic of the Congo, is also highlighted as an important knowledge gap (BirdLife International, 2022a).

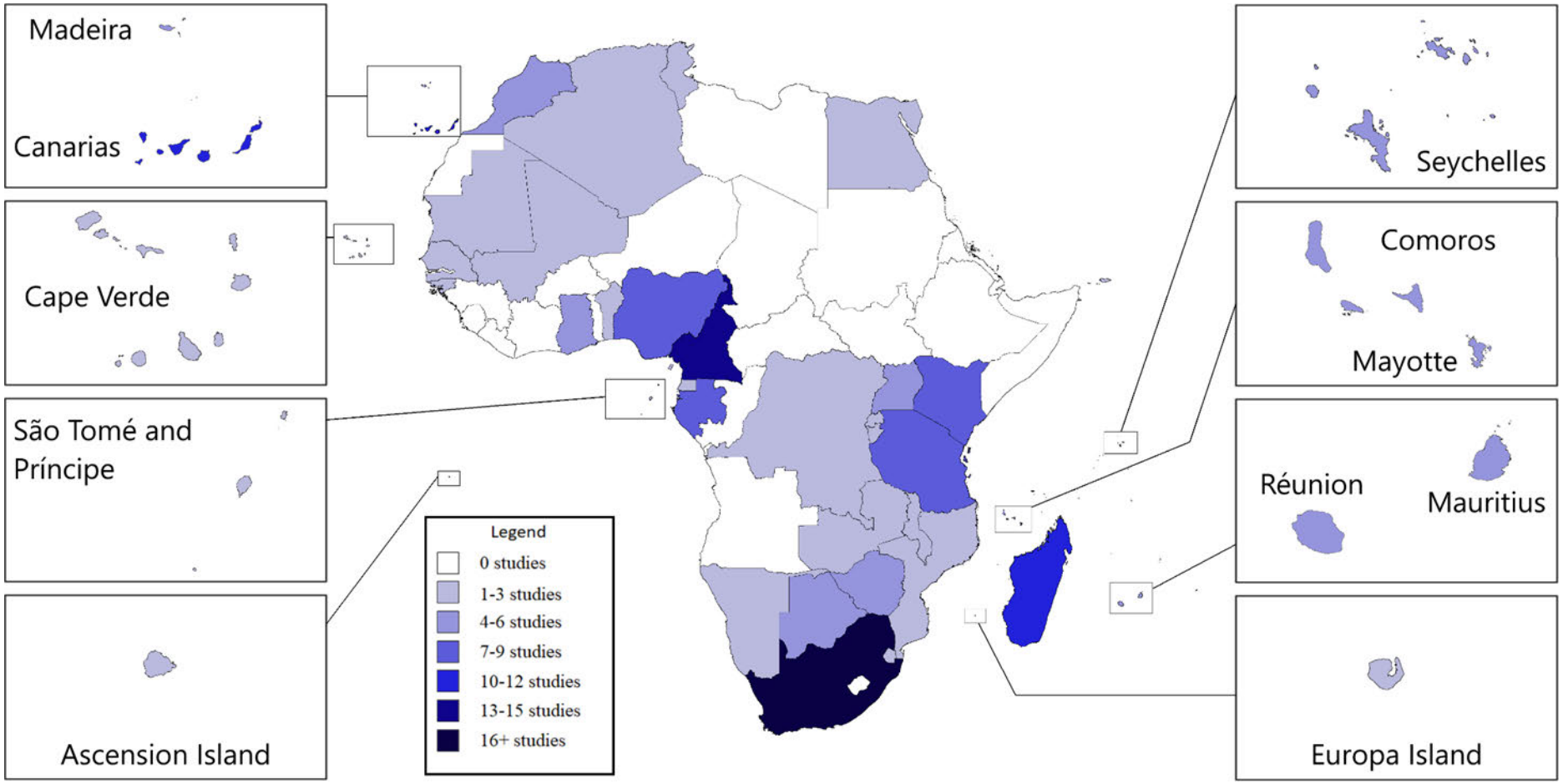

Figure 2.2. Map showing the number of avian malaria (*Plasmodium*, *Haemoproteus* or *Leucocytozoon*) studies conducted per African country/region. The colour of each country/region indicates the number of studies conducted within each country/region.

2.3.2 Summary of avian hosts infected with avian malaria

There are different sampling strategies used by avian malaria investigators. Some researchers investigated the prevalence of avian malaria in a large taxonomic spread of bird species but included only a few representatives of each host species (for example Beadell et al., 2009, 2006; Drovetski et al., 2014; Harvey and Voelker, 2017; Tchoumbou et al., 2020). Other studies focus on a single host species but include many individuals belonging to the same species (for example Gonzalez-Quevedo et al., 2014; Hammers et al., 2016; Nebel et al., 2020). African avian malaria literature also included studies describing new parasite species (for example Iezhova et al., 2011; Valkiunas et al., 2013). These different sampling strategies make it difficult to compare results across studies. In addition, some studies may not report negative results i.e., bird species examined that did not show infection. Nonetheless below I report some general trends and highlight key knowledge gaps.

Avian malaria was found in 489 African bird species, belonging to 219 genera, 76 families and 20 avian orders. Avian malaria was reported in *Acrocephalus sechellensis* more than any other host species in Africa, with a total of 1103 individuals found to be infected with avian malaria (Figure 2.3a). Passeriformes recorded the highest incidence of avian malaria with 88.98% of recorded infections were isolated from passerine hosts (Figure 2.3d). This also supports hypotheses that passerine birds are more susceptible to avian malaria infections or are most easily captured and sampled (Rivero and Gandon, 2018).

Of the estimated 2500 bird species occurring in Africa (BirdLife International, 2022b), less than 20% (N = 489) of Africa's bird species have been found to be infected with avian malaria. It has

been estimated that avian malaria is present in 68% of bird species that have been screened for avian malaria (Atkinson and Van Riper III, 1991). The low number of African host species found to be infected is probably biased by under sampling.

Malawi has recorded the highest diversity of avian hosts species infected with malaria (108 bird species). Countries within the Gulf of Guinea also had a large number of host species infected with avian malaria with Cameroon, Gabon and Nigeria having avian malaria recorded in 83, 77 and 60 host species respectively. Interestingly, South Africa, which had the highest number of publications, only recorded avian malaria in 56 host species. Similarly, the Canary Islands, which also has a higher number of publications, had only 13 host species identified with avian malaria infections. It is difficult to make any biological inference from this given that of the research conducted on the Canary Islands, the majority only conducted research focusing on one host species (Armstrong et al., 2019; Gangoso et al., 2016; Gonzalez-Quevedo et al., 2014; Gutiérrez-López et al., 2015; Hellgren et al., 2007b; Pérez-Rodríguez et al., 2013; Spurgin et al., 2012).

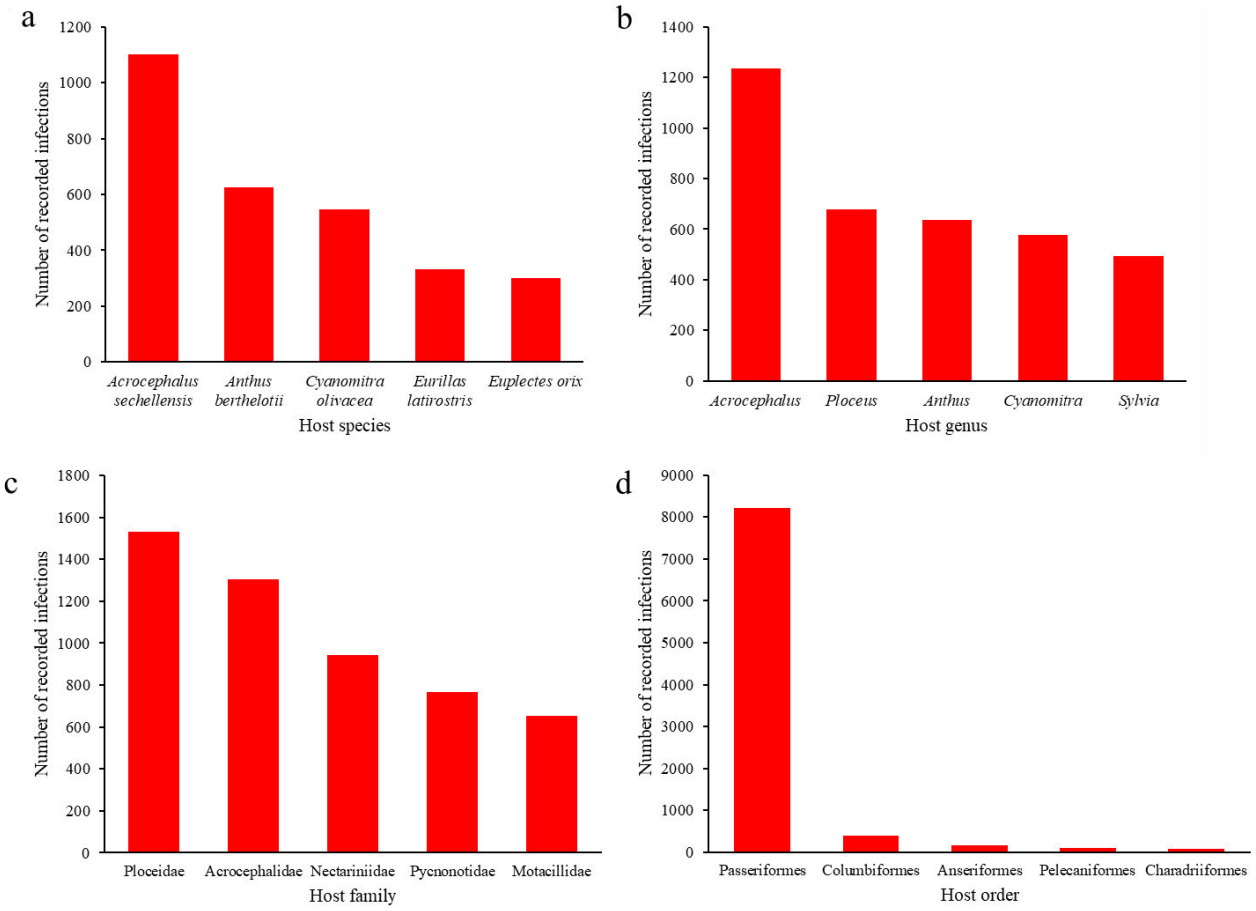

Figure 2.3. Bar graphs showing a) the five most frequently recorded host species infected with avian malaria in Africa, b) the top five host genera recorded to have avian malaria in Africa, c) the top five host families recorded to have avian malaria in Africa and d) the top five host orders recorded to have avian malaria in Africa.

2.3.3 Plasmodium, Haemoproteus and Leucocytozoon representation in literature

Of the three parasite genera, *Plasmodium* has been reported most frequently in African birds, with records for this parasite making up 46.66% of the over 9400 avian malaria records. *Haemoproteus* (34.43% of records) and *Leucocytozoon* (15.05% of records) is reported less frequently. In 3.86% of recorded infections, parasites were not classified to genus. Not all publications assessed all three parasite genera and so it is difficult to draw biological conclusions from the number of records available in public databases such as MalAvi and GenBank.

Of all 76 articles published, only 30 screened for all three genera of avian malaria, while 27 only reported on two genera and 19 only conducted research on a single avian malaria genus (Figure 2.4a). *Plasmodium* and *Haemoproteus* were the most frequently studied of the three parasite genera and were reported in 67 and 64 of the 76 research articles reviewed in this study respectively. The lack of data for *Leucocytozoon* could be because there is still some debate if this genus is a malarial parasite (Roberts et al., 2013; Valkiūnas, 2005). However, studies conducted including all three genera show a steady increase in publications between 2006 and 2020 (Figure 2.4b).

Plasmodium was reported to infect birds in 38 countries/territories making it the most geographically widespread of the three genera (Figure 2.5). *Haemoproteus* was reported in 31 countries/territories (Figure 2.6) and *Leucocytozoon* in 22 countries/territories (Figure 2.7). Unsurprisingly, higher numbers of *Plasmodium* infections were found in countries which have had more research conducted within them. A similar trend is also seen when considering *Haemoproteus* and *Leucocytozoon* infections with some notable exceptions. For example, the high rate of *Haemoproteus* infections seen in the Seychelles is surprising given the low number of studies conducted in this country. Instead, the studies conducted by Fairfield *et al.*, (2016) and Hammers *et al.*, (2016) included a very high number of individuals examined (3888 and 2454 *Acrocephalus sechellensis* sampled respectively). Similarly, Réunion, a region with only a few published studies, recorded one of the highest levels of *Leucocytozoon* infection. This is largely attributed to the large sample size used in Cornuault *et al.* (2012) which involved screening over 900 birds solely for *Leucocytozoon* infections.

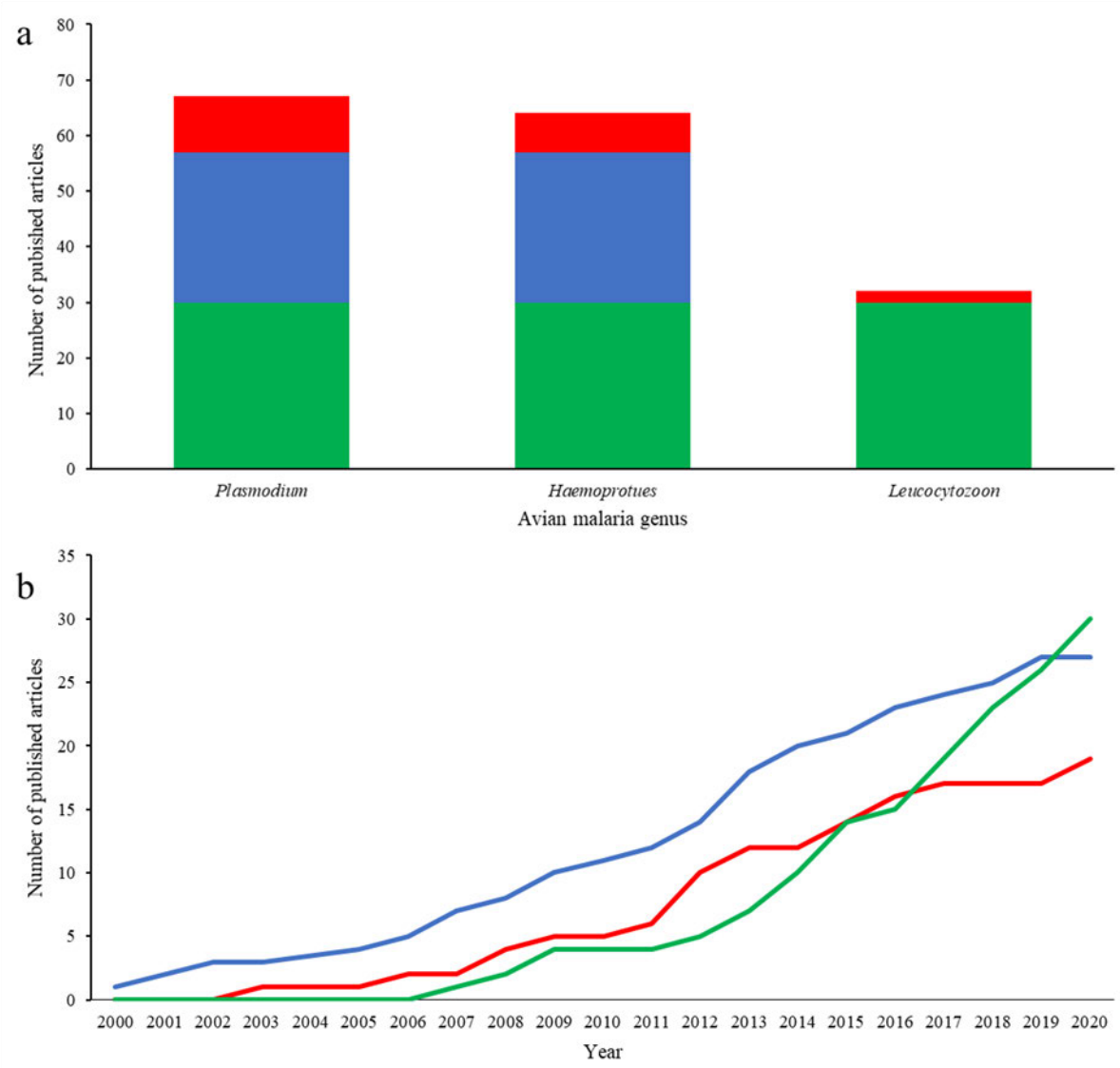

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Figure 2.4. a) Stacked bar graph representing the number of published articles investigating each malarial genus. The
556 different colours represent the number of genera studied in the literature. Green represents three genera investigated, blue represents two genera and red represents single genus studies. b) Cumulative line graph showing the increase in
558 avian malaria studies from 2000 to 2020 in respect to the number of avian malaria genera investigated, matching the colours in Figure 2.4a.

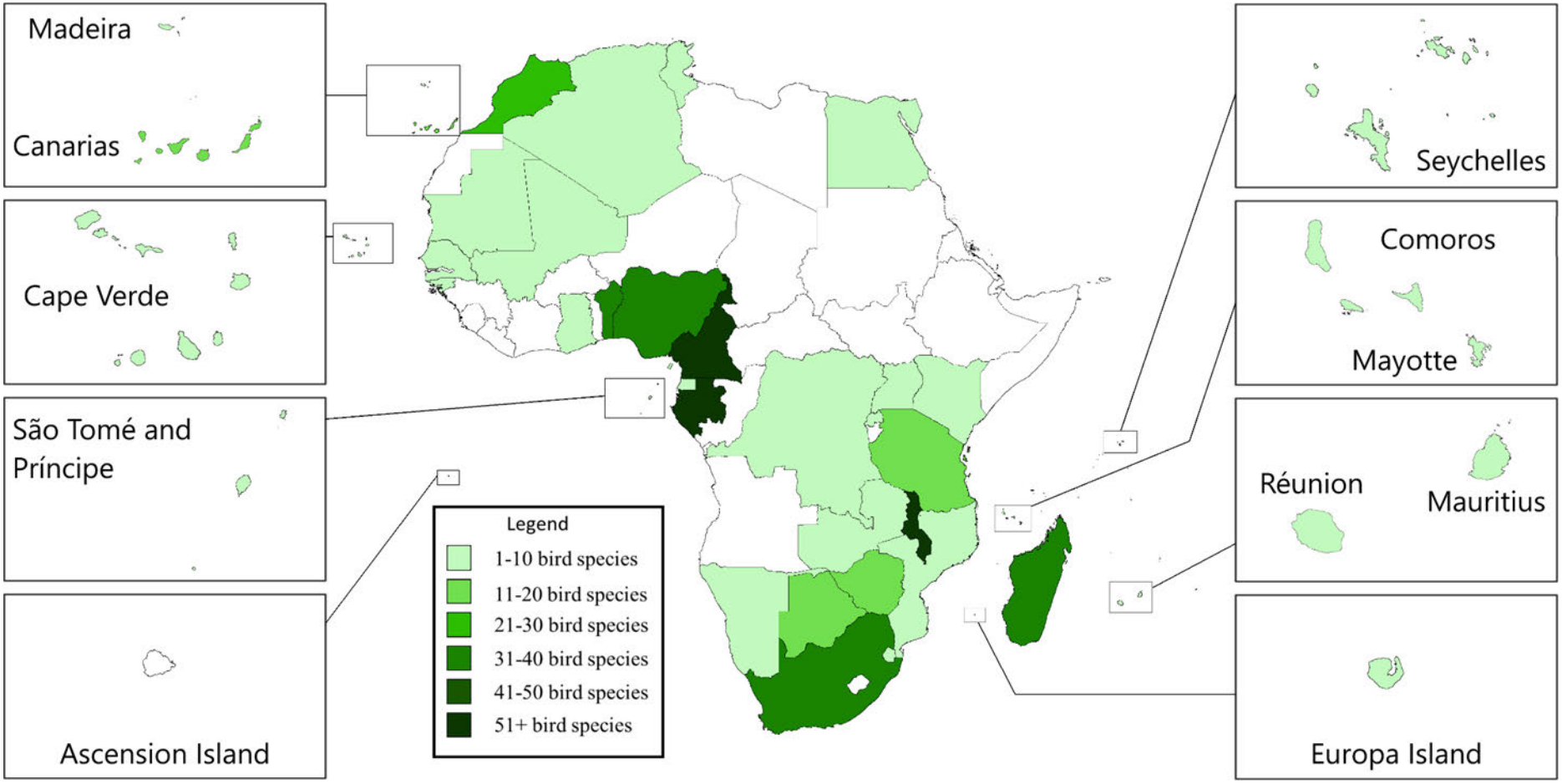
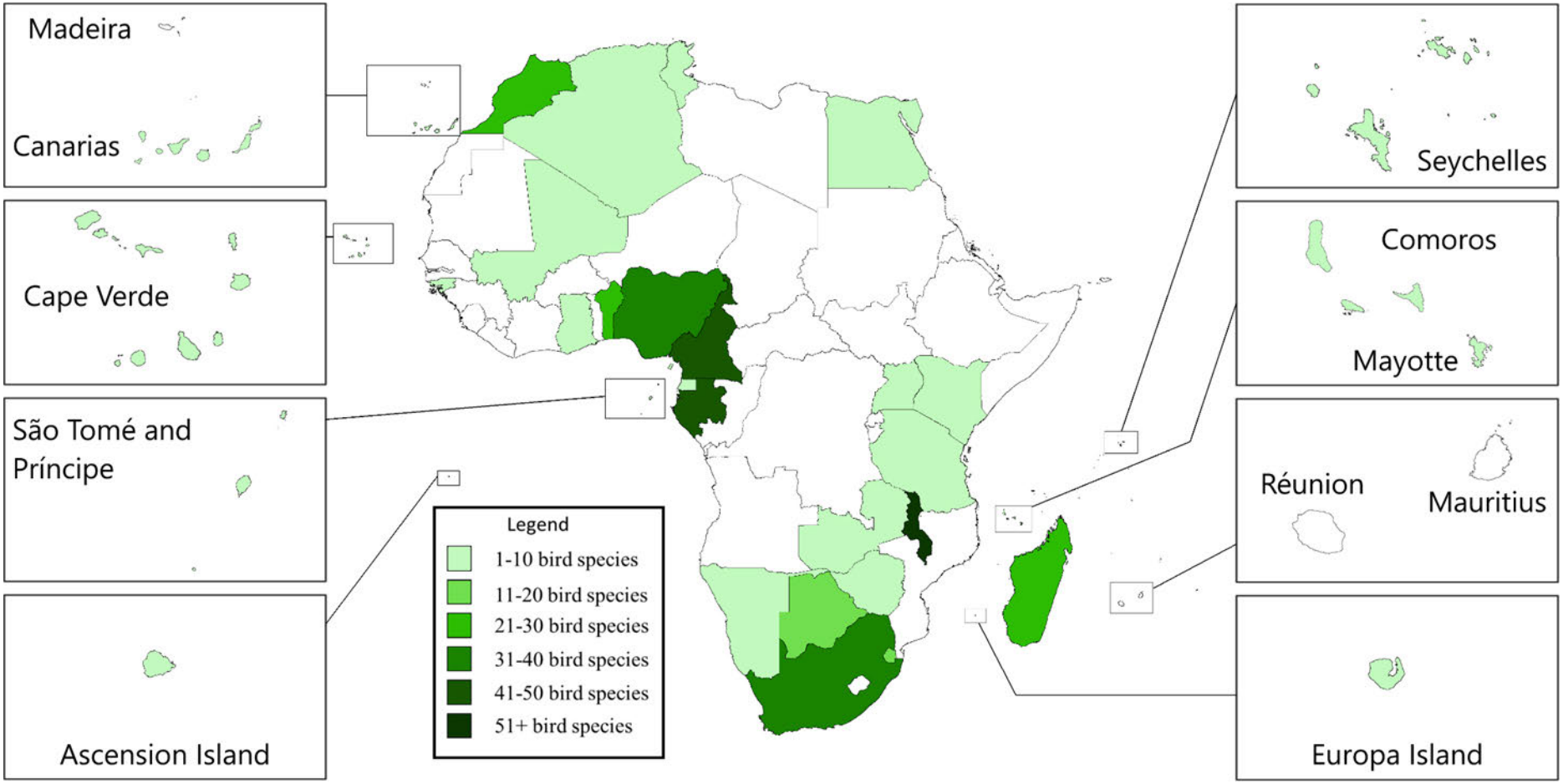


Figure 2.5. Map showing number of avian host species reported to be infected with *Plasmodium* per African country/region. The colour of each country/region indicates the number of bird species reported to be infected in each country/region.



564 Figure 2.6. Map showing number of avian host species reported to be infected with *Haemoproteus* per African country/region. The colour of each country/region indicates the number of bird species reported to be infected in each country/region.

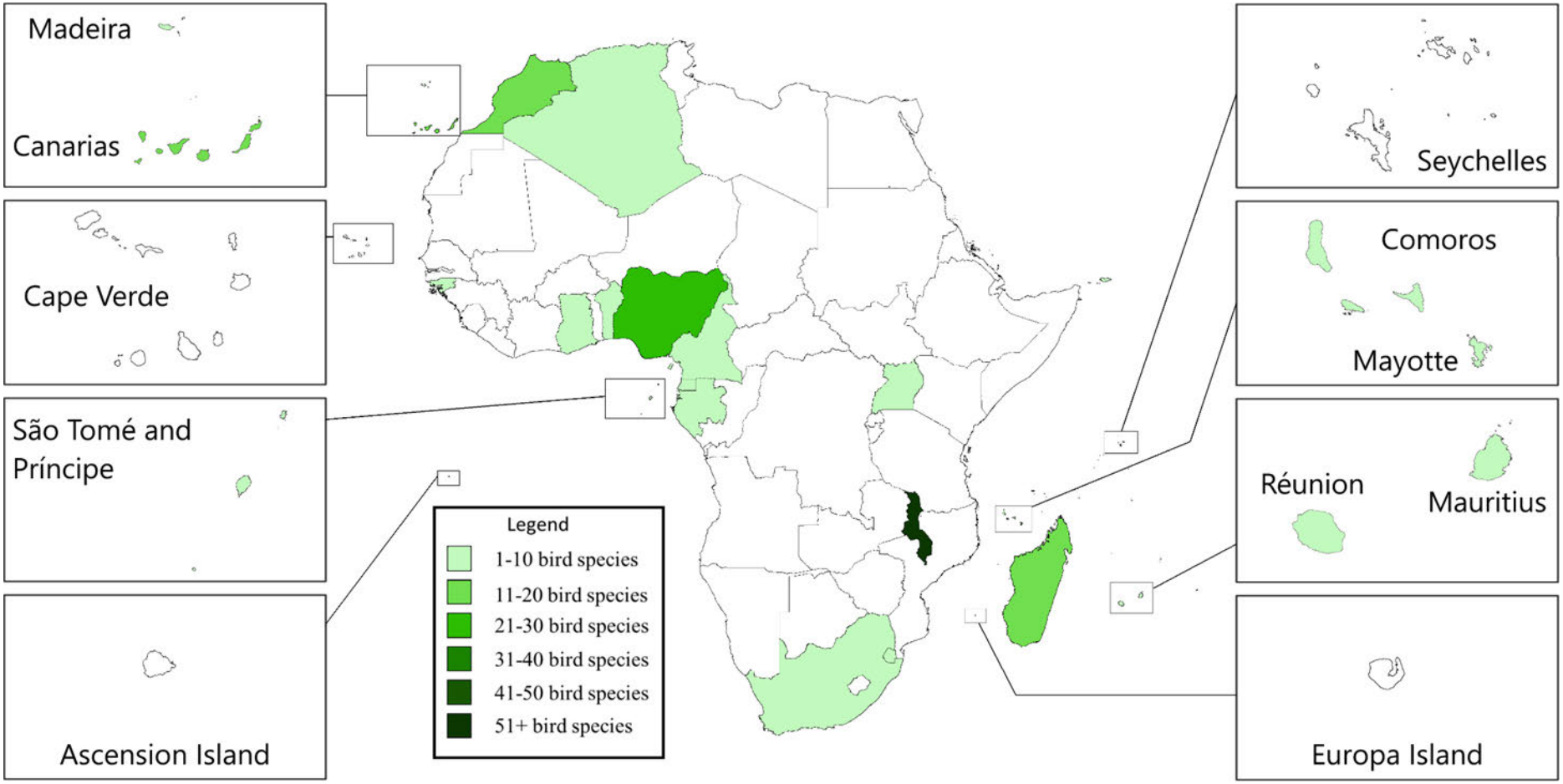


Figure 2.7. Map showing number of avian host species reported to be infected with *Leucocytozoon* per African country/region. The colour of each country/region indicates the number of bird species reported to be infected in each country/region.

When looking at the diversity of host species for each parasite, these high research areas did reveal high avian host diversity. *Plasmodium* had the highest host diversity in Gabon with 59 recorded host species, followed by Cameroon (58 host species) and Malawi (57 host species) (Figure 2.5). However, the majority (65.79%) of countries/regions did not record more than 10 host species infected with *Plasmodium*. *Haemoproteus* had high host diversity in Malawi with 52 recorded host species, followed by Gabon and Cameroon with 47 and 44 host species (Figure 2.6). Similar to *Plasmodium*, the majority of African countries/regions (67.74%) reported less than 10 host species for *Haemoproteus*. However, for *Leucocytozoon*, 17 of the 22 countries (77.27%) reported less than 10 host species infected with *Leucocytozoon* parasites (Figure 2.7). The highest host diversity for *Leucocytozoon* was found in Malawi with 56 host species, followed by Nigeria with 23 reported host species. Although high research areas may reveal high levels of reported parasite infections, we do see variations in host diversity. The countries within the Gulf of Guinea appear to be a research hotspot (Figure 2) and showed high host diversity for the parasites (Figures 2.5-7). However, South Africa also had high levels of research and parasite infections but in comparison reported lower host diversity. Malawi on the other hand was not a high research country but had some of the highest host diversity. Tropical climates like those found in some African countries provide a favorable habitat to the vectors of these parasites, leading to show an expected higher level of infections (Githeko et al., 2000; Roberts et al., 2013).

From the articles obtained for this chapter, a total 327 bird species were found to be infected by species belonging to *Plasmodium*, 282 bird species infected with *Haemoproteus* and 172 were found to be infected with *Leucocytozoon*. Some of the most frequently reported host species have already been mentioned (Figure 2.3). Of all reported infections, *Plasmodium* and *Leucocytozoon*

parasites were recorded at high frequency (93% of *Plasmodium* infections and 93.95% of *Leucocytozoon* infections) in passerine hosts. Interestingly, *Haemoproteus* was reported at lower frequency (81.20%) in passerines. This could be a biological difference, or the data could have been biased by a high rate of *Haemoproteus* infection (11.40%) reported in Columbiformes. This order of birds had the highest number of reported avian malaria infections after passerine birds. This may be attributed to *Haemoproteus* having one of its two subgenera (also named *Haemoproteus*) found exclusively in pigeons (Valkiūnas, 2005).

2.4 Conclusion

The development of molecular-based diagnostics has led to an acceleration in avian malaria research. This in turn has led to the establishment of public databases for the storage and analysis of molecular data linked to the three genera of parasites (Bensch et al., 2009). Despite this, avian malaria research in Africa has lagged behind than that produced by the rest of the world. Research is concentrated in "research hotspots" such as the Gulf of Guinea and Southern Africa. In addition, only 20% of African bird species have been reported to be infected with these parasites. This limits using the continental scale data to test hypotheses such as host susceptibility to avian malaria and the geographic distribution of infectious lineages. The need for integrative molecular/morphological studies to increase the availability of avian malaria parasite data is particularly emphasized in this study. In particular, research should focus on broadening the number of avian host species screened in regions that are understudied to better understand the effect avian malaria has on African avifauna.

CHAPTER 3

MOLECULAR DELIMITATION OF PARASITE SPECIES CAUSING AVIAN MALARIA (*PLASMODIUM*, *HAEMOPROTEUS* AND *LEUCOCYTOZOON*)

Abstract

Traditionally the detection and identification of parasites responsible for avian malaria has been based on microscopy. The development of molecular methods of detecting the three parasite genera responsible for avian malaria (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) has led to an accumulation of cytochrome *b* sequence data in databases such as MalAvi. In this study I determine if this cytochrome *b* data can be used to delimit parasite species. Although MalAvi does assign sequence data to lineages, some MalAvi lineages may belong to the same morphospecies of avian malaria. In this study I apply a more statistically robust method of species delimitation by testing for the difference between inter- and intraspecific genetic distances within the three genera of avian malaria. A distinct statistically verified "gap" between inter- and intraspecific genetic distances was recorded in *Plasmodium* and *Haemoproteus*. For *Plasmodium* the gap fell between 1.0% and 1.5%, and for *Haemoproteus* between 2.0% and 3.5%. Unfortunately, too few *Leucocytozoon* species were attributed to sequence data, so I was unable to perform gap analyses on this genus, but I used the 2.0% genetic distance threshold suggested by previous studies. Using all the sequence data available for Africa and the position of gap as a threshold, I recovered 18 *Plasmodium* Operational Taxonomic Units (OTUs), 19 *Haemoproteus* OTUs, and 19 *Leucocytozoon* OTUs. When comparing the number of OTUs to MalAvi lineages and morphological species there was no clear trend. In some cases, many MalAvi lineages were

associated to a single OTU, in contrast some OTUs were only associated with a single MalAvi lineage. The lack of associated morphospecies to many OTUs may be associated to a lack of expertise in morphological identification of these parasites coupled with few diagnostic characters and conserved morphology.

Keywords: Avian malaria, *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, species delimitation

3.1 Introduction

The parasites known to cause avian malaria (species belonging to *Plasmodium*, *Haemoproteus* and *Leucocytozoon*) infect a large number of bird species around the world (Atkinson and Van Riper III, 1991; Clark et al., 2014). These parasites are maintained in populations as avian malaria tends to only have a higher mortality rate in young immunologically naive individuals and those with an underlying disease (Atkinson et al., 2008; Grim et al., 2003; Valkiūnas, 2005). Due to immunological naivety having an effect on mortality, some bird populations have also shown to be more susceptible to the disease, such as Hawaii's honeycreepers and penguins, due to these species and populations have been isolated from malaria vectors (Atkinson and Van Riper III, 1991; Gracyzk et al., 1995; Grim et al., 2003; Van Riper III et al., 1986). In many other bird species, most individuals will have limited or no clinical signs, although clinical signs such as anaemia, necrosis of infected tissue, reduced reproductive success, reduced lifespan or a combination of these effects may impact some individuals (Asghar et al., 2015; Atkinson et al., 2008; Delhaye et al., 2018; Ishtiaq and Barve, 2018; Kilpatrick et al., 2006; Knowles et al., 2010; Valkiūnas, 2005). Healthy birds may be able to immunologically suppress the parasitaemia of these haemosporidians, resulting in chronic low levels of parasitaemia or a full recovery and

660 immunity to further infection (Atkinson et al., 2008; Atkinson and Van Riper III, 1991; Botes et al., 2017; Rivero and Gandon, 2018; Valkiūnas, 2005).

662

Microscopy is still the favoured method for species identification for avian malaria parasites 664 (Richard et al., 2002; Rivero and Gandon, 2018). But species delimitation based on microscopy alone is difficult for non-experts (Richard et al., 2002; Rivero and Gandon, 2018; Valkiūnas, 666 2005). Avian malaria infection may also be difficult to detect by microscopy in individuals with low parasite load (Richard et al., 2002; Valkiūnas, 2005). Also parasites found in different bird 668 hosts are often described as different species, although the same parasite species can infect a wide taxonomic range of hosts (Beadell et al., 2004; Valkiunas and Iezhova, 2018). This has led to wide 670 synonymy within the three genera of parasites (Valkiūnas, 2005). In contrast, some parasite species had been lumped together and reported as a single species if found to infect birds from the same 672 geographic region (Valkiūnas, 2005).

674 Recently molecular-based methods of avian malaria detection have been developed (Bensch et al., 2000; Hellgren et al., 2004). These methods are more sensitive than traditional microscopy-based 676 methods at detecting low levels of infection (Valkiūnas, 2005; Videvall, 2019). This molecular sequence data used in combination with microscopy could be useful into accurately delimiting 678 parasite species (Outlaw and Ricklefs, 2014). The accumulation of avian malaria sequence data (in particular cytochrome *b* amplified from the parasite genome) has led to the development of 680 databases such as MalAvi (Bensch et al., 2009), which provides a unique resource for avian malaria research. MalAvi assigns sequences to lineages which may be analogous to species (Bensch et al., 682 2009) however many recognized morphospecies have been split into several MalAvi lineages

(Rivero and Gandon, 2018). While it has also been noted that many MalAvi lineages are synonymous to others and have incorrectly been attributed multiple MalAvi lineage names (Outlaw and Ricklefs, 2014). Current research practice involves providing, where possible, both the morphospecies and the MalAvi lineage of sequences obtained (Rivero and Gandon, 2018).

Another method of species delimitation from sequence data is followed by the DNA barcoding community (Hebert et al., 2003; Hebert and Gregory, 2005). This method involves testing for the presence of a "gap" between inter- and intraspecific genetic distances (Hebert et al., 2003). The position of the gap is then used to assign sequences to species clusters (Govender and Willows-Munro, 2019; Hanzen et al., 2020). Given that in this method species are delimited based on genetic distances inferred from sequence data, this method follows the phylogenetic species concept (Cracraft, 1982). In this study I aim to determine if there is a distinct statistically verifiable "gap" between inter- and intraspecific genetic distances generated from the cytochrome *b* data available for African taxa in MalAvi. The species inferred using this method will then be compared to the MalAvi lineage assignment to compare these two species delimitation methods.

3.2 Materials and methods

Cytochrome *b* sequence data from African taxa were collected using information available on the MalAvi database (Bensch et al., 2009) and from published articles found using Google Scholar. Sequences were downloaded from GenBank between April 2020 and June 2021 using ascension numbers provided by MalAvi and published articles found. Articles were searched for using the key words: "avian", "malaria", *Plasmodium*", "*Haemoproteus*", "*Leucocytozoon*", "haematozoa", "haemosporidian", in turn with a combination of location-based keywords such as "Africa",

706 "Indian Ocean", Atlantic Ocean", and "name of African country/territory". For the last keyword, the name of each African country was used, including older names that have now changed. For
708 example, both Swaziland and Eswatini were used in search of articles. In addition to continental African countries, the island nations of Cape Verde, Comoros, Madagascar, Mauritius, Seychelles,
710 and São Tomé and Príncipe were included. Foreign dependencies and territories were also included such as: Europa Island, Mayotte and Réunion (France), Madeira and the Savage Islands (Portugal),
712 Canarias (Spain), Saint Helena, Ascension and Tristan da Cunha (United Kingdom), and Socotra (Yemen). Referencing the sequences to MalAvi, most of the sequences collected were not
714 morphologically assigned to an avian malaria species but were assigned to a MalAvi lineage.

716 For the alignments, any duplicates of sequences were removed. Sequences belonging to *Plasmodium*, *Haemoproteus* and *Leucocytozoon* were aligned separately using Clustal W
718 (Thompson et al., 1994) in BioEdit version 7.0.5.3 (Hall, 1999). The alignments were manually optimized to ensure homology. The sequences that were associated with specimens that were
720 morphologically identified were then grouped by their species for measuring genetic distances. This was not possible for *Leucocytozoon* as too few morphological species were attributed to the
722 sequences collected. Sequences were only assignable to *L. macleani* and *L. toddi*. Gap analyses was performed for *Plasmodium* and *Haemoproteus* but for *Leucocytozoon* a threshold of 2%
724 sequence divergence was used (Bensch et al., 2004; Galen et al., 2018). Inter- and intraspecific uncorrected genetic distances were calculated using MEGA 11.0.9 (Tamura et al., 2021) and the
726 position of the gap compared.

The "gap" was determined by plotting inter- and intraspecific genetic distances on the same graph and by subtracting the maximum intraspecific distance from the minimum interspecific distance (Meier et al., 2006). To determine if the inter- and intraspecific distance classes were statically separable, the Jefferies-Matusita test was performed (Dabboor et al., 2014).

Phylogenetic trees for all three genera were constructed using maximum likelihood and Bayesian inference. A best-fit model for each alignment was first determined using the AIC criteria in jModelTest2 on XSEDE (Darriba et al., 2012). Maximum likelihood analyses were conducted using GARLI 2.10 on XSEDE (Zwickl, 2006). Nodal support was assessed using 1000 bootstrap replications. MrBayes 3.2.7a on XSEDE (Ronquist et al., 2012) was used to conduct the Bayesian inference. Two Bayesian runs were simultaneously conducted, each consisting of four Markov chains run for 25 million generations. Trees were sampled every 1000th generation. Convergence was determined when Effective Sampling Size (ESS) values were above 200 for all parameters (Drummond et al., 2006). ESS values were generated in Tracer 1.7 (Rambaut et al., 2018). The first 20% of trees were removed as burn-in and 50% majority rule consensus trees were created in the CONSENSE module of PHYLIP 3.698 (Felsenstein, 2005). All trees were midpoint rooted in Figtree 1.4.4 (Rambaut, 2018). Bootstrap and Bayesian posterior probability values were annotated onto the maximum likelihood tree. Using the species delimitation thresholds calculated in the gap analyses for *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* parasite species estimated were highlighted on the phylogenies.

3.3 Results

3.3.1 Defining the gap between inter- and intraspecific genetic distances

For *Plasmodium*, the intraspecific genetic distances ranged between 0.000 and 0.015 and the interspecific genetic distances ranged from 0.005 to 0.100. Although there was some overlap, there was an observable gap in the distribution of the genetic distances between genetic distances 0.010 and 0.015 (Figure 3.1a). The Jefferies-Matusita statistic for this data set was 1.984, exceeding the threshold value, confirming that the two genetic distance classes are statistically separable. In the case of *Haemoproteus* (Figure 3.1b) intraspecific genetic distances ranged from 0.00 to 0.02. The interspecific genetic distances ranged from 0.02 to 0.18. The Jefferies-Matusita statistic for this data set was 1.724, exceeding the threshold value, confirming that the inter- and intraspecific distance classes are statistically separable for this genus (Trigg and Flasse, 2001).

The topologies produced by the maximum likelihood and Bayesian inference were similar, subsequently posterior probabilities ≥0.95 and bootstrap values ≥0.75% are presented on the most likely trees (Figure 3.2, 3.3 and 3.4). Using the position of the gap between the inter- and intraspecific genetic distances, parasite species were defined as Operational Taxonomic Units (OTUs), which were annotated onto phylogenetic trees (Figure 3.2, 3.3 and 3.4). For *Plasmodium* 11 of the 18 OTUs were monophyletic and supported by high bootstrap (≥75%) and posterior probability values (≥0.95) (Figure 3.2). For *Haemoproteus* 10 of the 19 OTUs were monophyletic and supported by high bootstrap (≥75%) and posterior probability values (≥0.95) (Figure 3.3). Finally, for *Leucocytozoon* 10 of the 19 OTUs were monophyletic and supported by high bootstrap (≥75%) and posterior probability values (≥0.95) (Figure 3.4). The remaining seven *Plasmodium*

772 OTUs, nine *Haemoproteus* OTUs, and nine *Leucocytozoon* OTUs, were monophyletic but were not supported by high bootstrap (≥75%) and posterior probability values (≥0.95).

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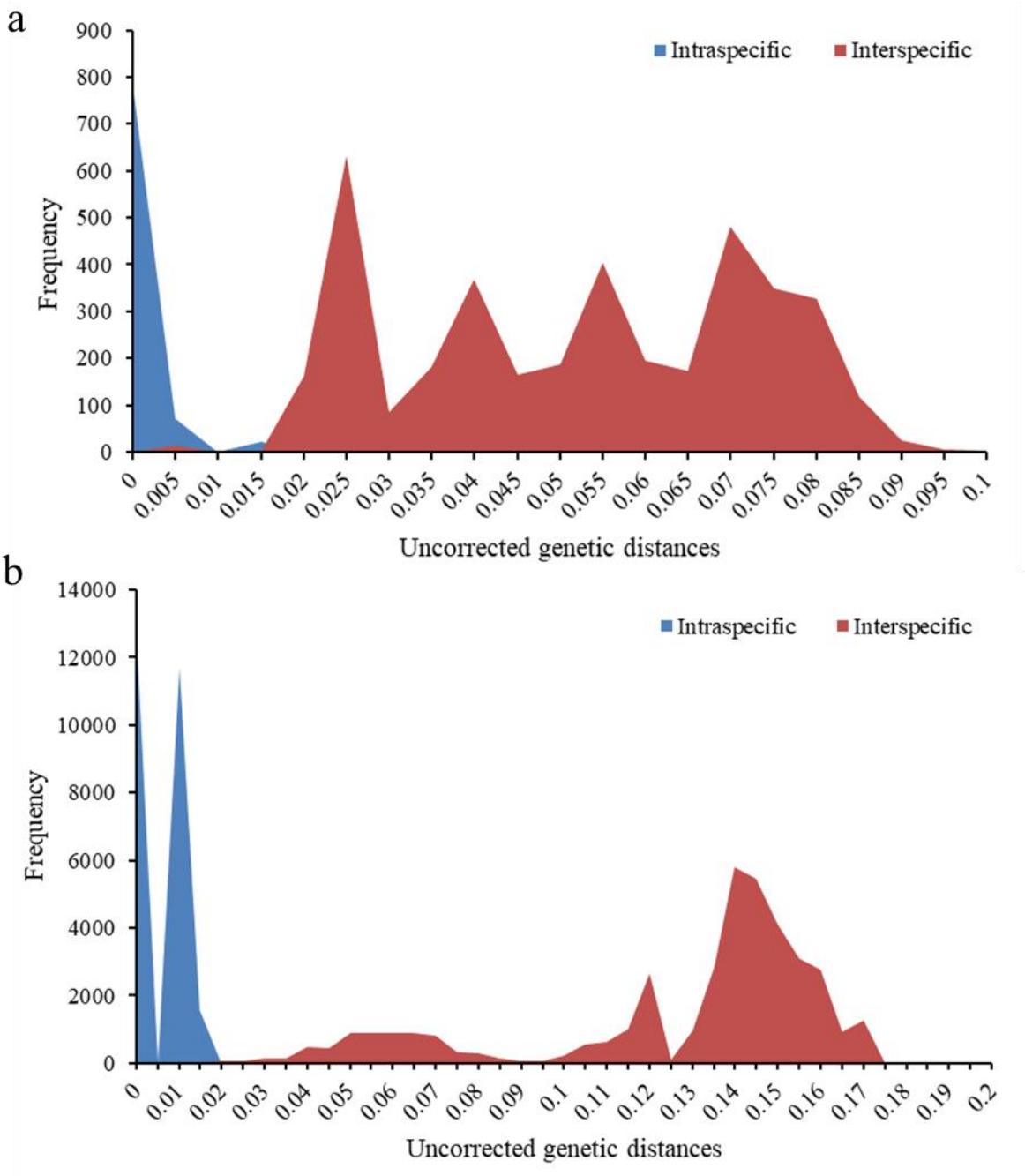


776 Figure 3.1. Frequency of pairwise uncorrected genetic distances for a) *Plasmodium*, and b) *Haemoproteus.* Intraspecific distances are in blue and interspecific distances are in red.

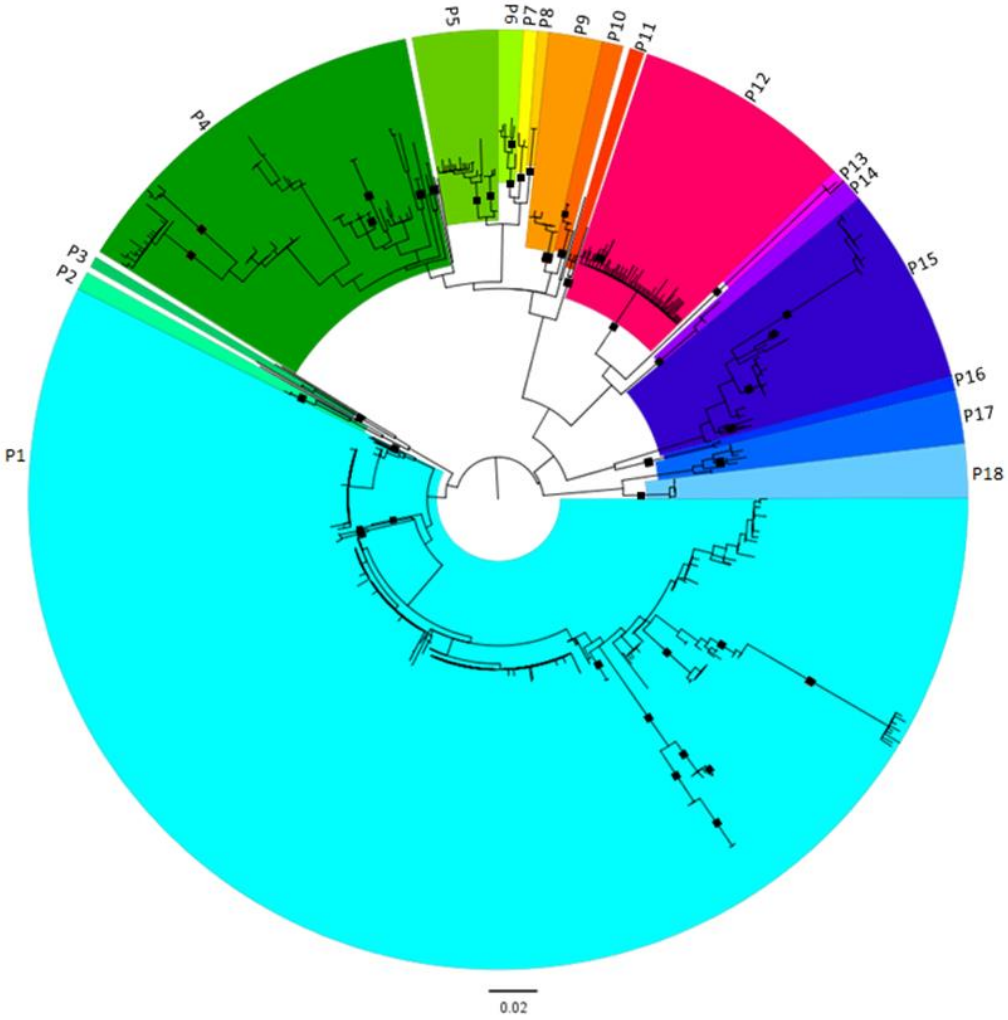


Figure 3.2. Mid-point rooted most-likely phylogeny constructed using cytochrome *b* for *Plasmodium* collected from African birds. Parasite species estimated comparing inter- and intraspecific genetic distances are highlighted and labelled P1 to P18. Branch support values ≥75% bootstrap values and ≥0.95 posterior probabilities are indicated by ■.

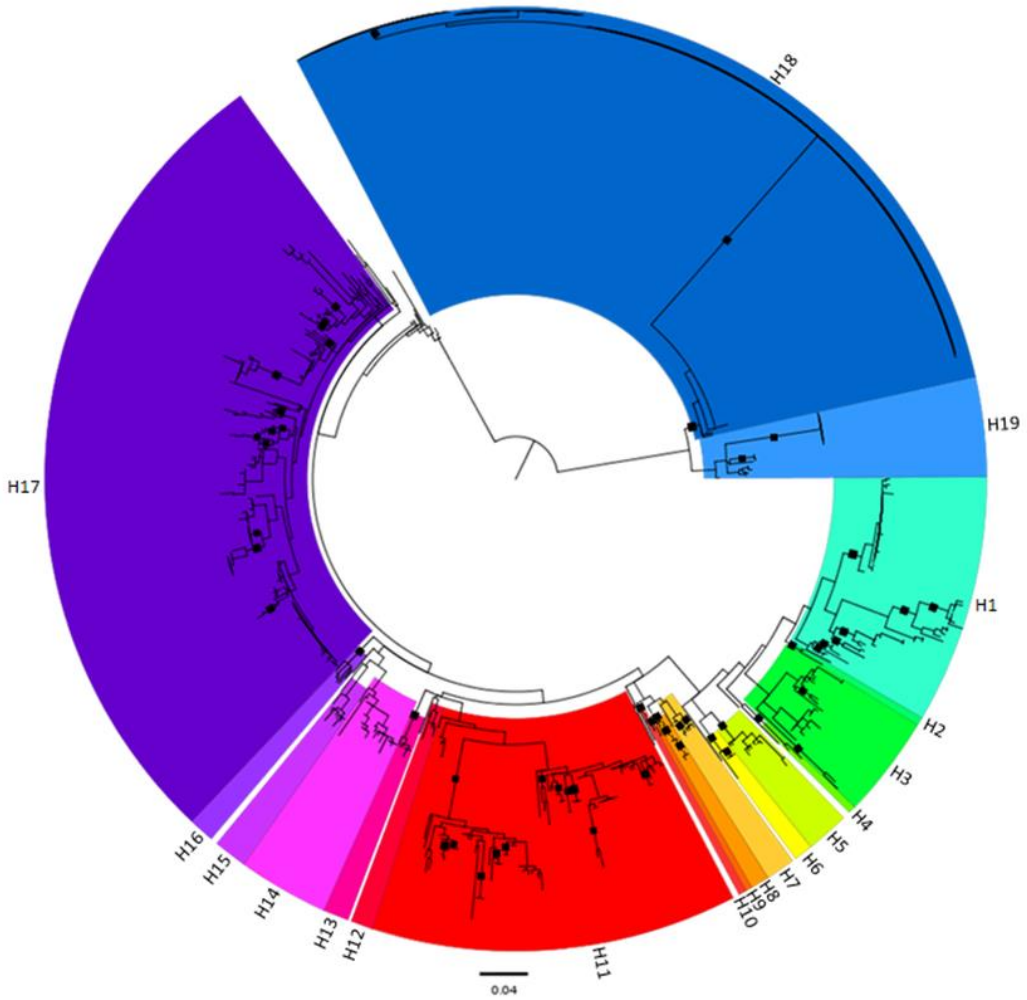


Figure 3.3. Mid-point rooted most-likely phylogeny constructed using cytochrome *b* for *Haemoproteus* collected from African birds. Parasite species estimated comparing inter- and intraspecific genetic distances are highlighted and labelled H1 to H19. Branch support values ≥75% bootstrap values and ≥0.95 posterior probabilities are indicated by ■.

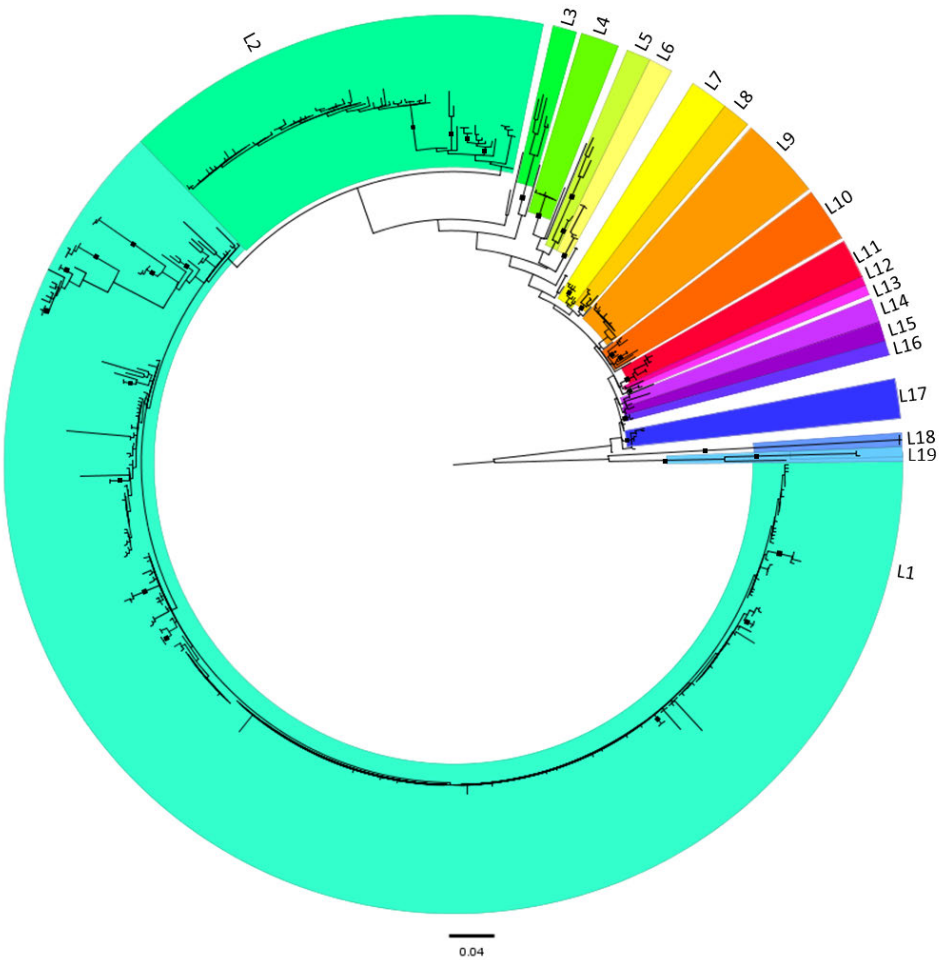


Figure 3.4. Mid-point rooted most-likely phylogeny constructed using cytochrome *b* for *Leucocytozoon* collected from African birds. Parasite species estimated comparing inter- and intraspecific genetic distances are highlighted and labelled L1 to L19. Branch support values ≥75% bootstrap values and ≥0.95 posterior probabilities are indicated by ■.

3.3.2 Comparison between MalAvi lineages and species defined using gap analysis

3.3.2.1 *Plasmodium*

Plasmodium OTUs P1, P4, and P15 were associated with seven, four and two morphological species respectively (Table 3.1). OTU P1 had the highest diversity of *Plasmodium* morphological species associated with it, with a total of seven different morphological species assigned to this OTU. The most frequently associated morphological species to P1 is *P. relictum*, making up 55.41% of reported P1 infections associated to a morphological species. There was one sequence in this OTU that was associated to *P. elongatum*, however this is more than likely a misidentification, as all remining *P. elongatum* sequences are found in OTU P15. The remining five morphological species associated with OTU P1 are *P. cathermerium*, *P. circumflexum*, *P. lucens*, *P. megaglobularis*, and *P. motacillae*. Figure 3.5 represents an expanded version of the *Plasmodium* phylogenetic tree (Figure 3.2), showing only the phylogeny for OTU P1. On this tree, all sequences associated to morphological species were highlighted on the tree. The sequences representing *P. relictum* in Figure 3.5 show that this morphological species is either polyphyletic or many of these other associated morphological species have been misidentified or are incorrectly assigned to separate species.

The OTUs P4 and P15 had fewer associated morphological species. The four morphological species found within P4 are *P. globularis*, *P. multivacuolaris*, *P. parahexamerium* and *P. vaughani*. These morphological species were all monophyletic unlike *P. relictum* in P1. This could mean that these four species have been unnecessarily split. While all sequences associated with morphological species in OTU P15 belonged to *P. elongatum*, except for one sequence which was named *P. matutinum* in the database. There were three defined OTUs that are matched to

morphological species. OTU P7 is *P. rouxi*, P14 is *P. ashfordi* and P16 is *P. homocircumflexum*. However, there were a total of 12 OTUs for which no morphological data was available.

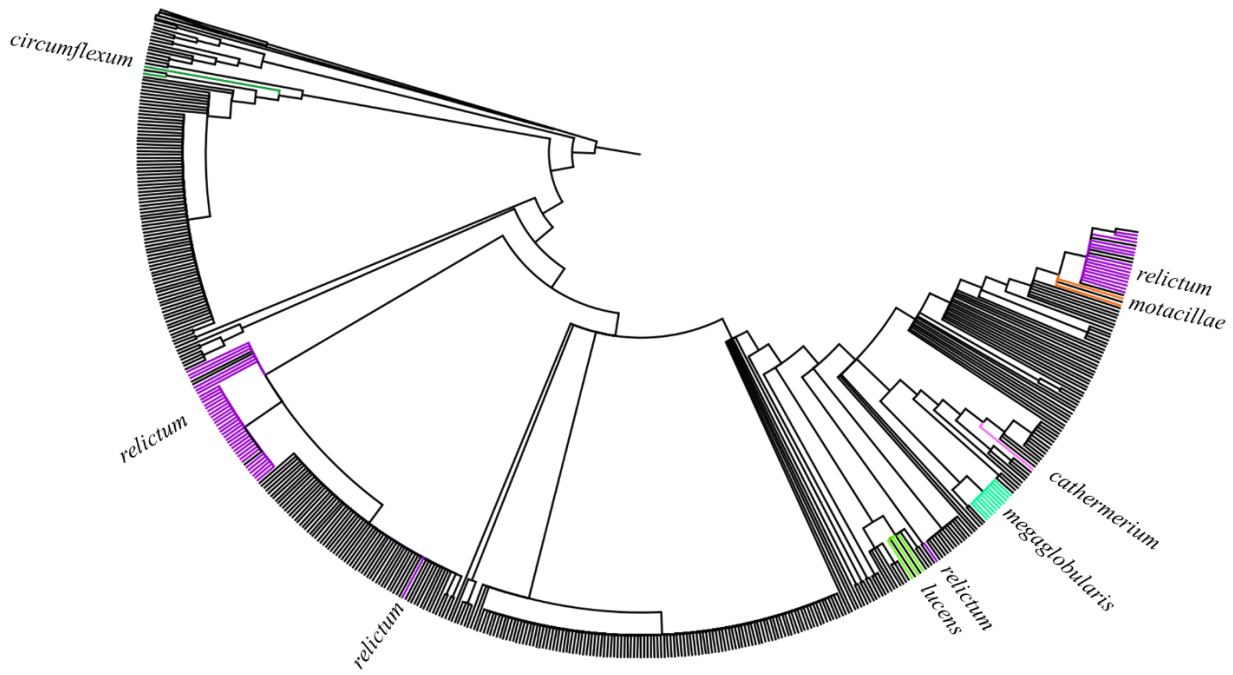


Figure 3.5. Midpoint rooted maximum likelihood phylogeny of *Plasmodium* OTU P1 from avian hosts across Africa. P1 was inferred using the gap analysis method. Sequences linked to morphological species are indicated on the phylogenetic tree: pink for *P. cathermerium*, dark green for *P. circumflexum*, light green for *P. lucens*, cyan for *P. megaglobularis*, orange for *P. motacillae*, and purple for *P. relictum*.

In general, my approach using the gap between inter- and intraspecific genetic divergences as species delimitation threshold was more conservative than the method used by MalAvi, with many MalAvi lineages falling within the same OTU. For example, P1 included 73 different MalAvi lineages. In contrast, in some cases there was a good match between species defined using the gap analyses and MalAvi lineages, for example OTUs P7, P8, P13 and P18 were all only associated with single MalAvi lineages (Table 3.1). In Table 3.1, the MalAvi lineages that were most

frequently associated with each species delimited using the gap analyses are provided. This has been included for the OTUs which have no associated morphological species as a form of representation for these OTUs. A list of all morphospecies and MalAvi lineages associated to each OTU can be found at https://figshare.com/articles/dataset/Supplementary_material_xlsx/21346779.

Table 3.1. OTUs defined for *Plasmodium*. The most frequently associated morphological species within each OTU is also noted. The number of MalAvi lineages and the most frequently associated MalAvi lineage per OTU is also indicated.

OTU name	Number of associated morphological species	Most frequently associated morphological species	Number of associated MalAvi lineages	Most frequently associated MalAvi lineage
P1	7	*Plasmodium relictum*	73	PLOVEL01
P2	0	No data available	5	NEWAM06
P3	0	No data available	2	AFR117 TCHSEN01
P4	4	*P. multivacuolaris*	45	PHICT01
P5	0	No data available	7	PSEGRI01
P6	0	No data available	7	ALEFUE01
P7	1	*P. rouxi*	1	PAGRI02
P8	0	No data available	1	PBPIP1
P9	0	No data available	2	RECOB4
P10	0	No data available	4	LK6
P11	0	No data available	3	AFR006 AFR040 AFR047
P12	0	No data available	63	BULIBP15
P13	0	No data available	1	LAMPUR03
P14	1	*P. ashfordi*	2	GRW2
P15	2	*P. elongatum*	24	GRW6
P16	1	*P. homocircumflexum*	2	COLL4
P17	0	No data available	4	RTSR1
P18	0	No data available	1	ACCTAC01

3.3.2.2 *Haemoproteus*

Of the 19 *Haemoproteus* OTUs identified, five (H1, H9, H11, H17, and H18) were found to be associated with multiple morphological *Haemoproteus* species. OTU H11 had the highest number of *Haemoproteus* morphological species assigned to it, with a total of eleven morphological species assigned to this clade (Figure 3.6). The most frequently associated morphological species to H11 is *H. vacuolatus*, making up 31.82% of reported occurrences of H11. The remaining morphological *Haemoproteus* species found within this OTU (in decreasing order of frequency of reported H11 infections) are *H. sanguinus*, *H. palloris*, *H. pallidus*, *H. zosteropis*, *H. fuscae*, *H. homominutus*, *H. syrnii*, *H. pastoris*, *H. minutus*, and *H. enucleator*. The expanded phylogenetic tree of *Haemoproteus* showing just OTU H11 (Figure 3.6) shows the sequences associated to *Haemoproteus* morphological species. The majority of these morphological species were recovered as small monophyletic clades. However, one morphological species, *H. pallidus* is either polyphyletic, or morphological species *H. minutus* was misidentified or incorrectly delimited into a separate morphological species.

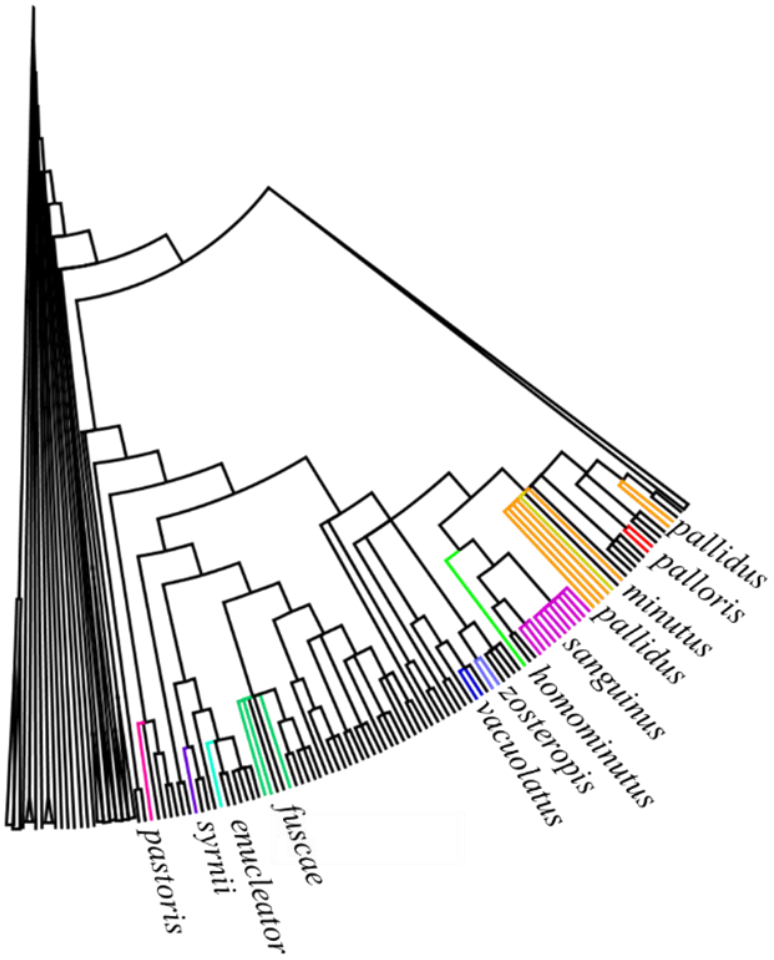


Figure 3.6. Midpoint rooted maximum likelihood phylogeny of *Haemoproteus* OTU H11 from African birds. Morphological species associated with sequences are highlighted on the tree. H11 was inferred using the gap analysis method. Sequences linked to morphological species are indicated on the phylogenetic tree: cyan for *H. enucleator*, dark green for *H. fuscae*, green for *H. homominutus*, yellow for *H. minutus*, orange for *H. pallidus*, red for *H. palloris*, pink for *H. pastoris*, light purple for *H. sanguinus*, dark purple for *H. syrnii*, dark blue for *H. vacuolatus*, and blue for *H. zosteropis*.

OTU H17 had the second highest number of *Haemoproteus* morphological species associated to it, with a total of nine morphological species. These morphological species are listed in the supplementary material. However, the most frequently associated morphological *Haemoproteus*

species to this OTU is *H. cyanomitrae*. Each morphological species was recovered in small monophyletic clades. This likely indicates that these morphological variants are over-split into separate species. The OTU H1 was also associated with two morphological species and may also be a case of over-splitting. The OTU H18 was associated with morphological species *H. columbae* and *H. multivolutinus*. This is likely a misidentification with the overwhelming majority of reports (99.56%) belonging to *H. columbae* and only a single sequence assigned to *H. multivolutinus*. A similar observation was seen in OTU H9, which was associated with morphological species *H. attenuatus* and *H. balmorali*. This is also a possible misidentification with all other *H. balmorali* sequences found within H8 and only one sequence associated to this species in H9. There was a total of eight OTUs that represent single morphological *Haemoproteus* species. These are *H. payevski* (OTU H2), *H. belopolskyi* (OTU H3), *H. hirundinis* (OTU H4), *H. lanii* (OTU H6), *H. balmorali* (OTU H8), *H. minchini* (OTU H12), *H. paranucleophilus* (OTU H14), and *H. iwa* (OTU H19). In total only four OTU were not associated with any morphological described *Haemoproteus* species.

Similar to what was observed in *Plasmodium*, using the gap between inter- and intraspecific genetic divergences as a threshold for *Haemoproteus* was more conservative than the method used by MalAvi, with many MalAvi lineages falling within the same gap species as defined by the gap analyses. OTU H17 included 110 different MalAvi lineages, the highest for any *Haemoproteus* gap species. While the OTU with the lowest amount of included MalAvi lineages were H2, H4, H9, and H10, including two MalAvi lineages each. The list of all OTUs and the number of included MalAvi lineages in each OTU is found in Table 3.2, as well as the most frequently included MalAvi lineage for each OTU. This has been included for the OTUs which have no associated

morphological species as a form of representation for these OTUs. A list of all morphospecies and MalAvi lineages associated to each OTU can be found at https://figshare.com/articles/dataset/Supplementary_material_xlsx/21346779.

Table 3.2. OTUs defined for *Haemoproteus.* The most frequently associated morphological species within each OTU is also noted. The number of MalAvi lineages and the most frequently associated MalAvi lineage per OTU is also indicated.

OTU name	Number of associated morphological species	Most frequently associated morphological species	Number of associated MalAvi lineages	Most frequently associated MalAvi lineage
H1	2	*Haemoproteus killangoi*	40	ZOSMAD01
H2	1	*H. payevski*	2	HIICT2 RW1
H3	1	*H. belopolskyi*	22	SW1
H4	1	*H. hirundinis*	2	DELURB2
H5	0	No data available	12	CCF6
H6	1	*H. lanii*	2	RBS4
H7	0	No data available	7	SFC10 SFC11
H8	1	*H. balmorali*	4	SFC1
H9	2	*H. attenuatus*	2	ROBIN1
H10	0	No data available	2	MONSHA01
H11	11	*H. vacuolatus*	63	ANLAT02
H12	1	*H. minchini*	6	ISPIC02
H13	0	No data available	4	AGNIG02
H14	1	*H. paranucleophilus*	16	FOUMAD02
H15	0	No data available	9	PAHIS2
H16	0	No data available	7	NEWAM04
H17	9	*H. cyanomitrae*	110	CYAOLI03
H18	2	*H. columbae*	7	HAECOL1
H19	1	*H. iwa*	10	COQUI06

3.3.2.3 *Leucocytozoon*

Gap analysis on sequences belonging to this genus was not possible as only two sequences were linked to morphologically delimited species. Instead, I used the 2.0% threshold suggested by Bensch et al. (2004). This threshold was very similar to that estimated for *Plasmodium* and

Haemoproteus using gap analyses. Using this threshold, 19 OTUs were defined, but only one of these were linked to a morphologically defined species, *L. toddi* (OTU L18). A single sequence was obtained for morphologically defined species, *L. macleani*, and could not be defined within an OTU. Not all sequences obtained were attributed to an OTU, as only monophyletic clades made up of two or more sequences were attributed to an OTU. This was the case for *L. macleani*. However, this could reveal that the OTU diversity in Africa for *Leucocytozoon* may in fact be greater than what we have found as there may be more morphological species that have not been studied or reported in a molecular sense. However, many of these *Leucocytozoon* OTUs are not supported by high bootstrap (≥75%) and posterior probability values (≥0.95). This indicates that further research and collection of sequences are needed to strengthen or correct these proposed OTUs for *Leucocytozoon*, as well as those for *Plasmodium* and *Haemoproteus*.

Although few *Leucocytozoon* morphological species had been associated with OTUs, there were several MalAvi lineages. However, again the gap between inter- and intraspecific genetic divergences was more conservative than the delimitation used by MalAvi, and many MalAvi lineages fell within the same OTU as defined by the gap analyses. OTU L1 contained the highest number of MalAvi lineages (N = 148; Table 3.3). However, none of the sequences belonging to OTU L4 were associated with a MalAvi lineage. OTUs L8, L12, L13, L16 and L18, were each associated with two MalAvi lineages. Unlike the *Plasmodium* and *Haemoproteus* OTUs, five OTUs (L3, L5, L7, L18 and L19) did not have one MalAvi lineage that was reported more frequently than other lineages. Instead, each associated MalAvi lineage was reported only once. These Malavi lineages maybe unnecessarily split into separate lineages. This has been included for the OTUs which have no associated morphological species as a form of representation for these

OTUs. A list of all morphospecies and MalAvi lineages associated to each OTU can be found at https://figshare.com/articles/dataset/Supplementary_material_xlsx/21346779.

Table 3.3. OTUs defined for *Leucocytozoon.* The most frequently associated morphological species within each OTU is also noted. The number of MalAvi lineages and the most frequently associated MalAvi lineage per OTU is also indicated.

OTU name	Number of associated morphological species	Most frequently associated morphological species	Number of associated MalAvi lineages	Most frequently associated MalAvi lineage
L1	0	No data available	148	REB11
L2	0	No data available	61	ZOBOR04
L3	0	No data available	5	AFTRU2 AFTRU3 TUROLI09 TUVIS01 TUVIS02
L4	0	No data available	0	No data available
L5	0	No data available	5	CCF13 CCF15 GLSJO02 OTSCO03 OTSCO06
L6	0	No data available	3	TUMER01
L7	0	No data available	8	AFR176 AFR214 AFR225 AFR226 AFR237 AFR241 ANLAT18 SYBOR23
L8	0	No data available	2	HIRUS07
L9	0	No data available	10	CIAE02
L10	0	No data available	8	CALMAD01
L11	0	No data available	6	PARUS22

Table 3.3. OTUs defined for *Leucocytozoon.* The most frequently associated morphological species within each OTU is also noted. The number of MalAvi lineages and the most frequently associated MalAvi lineage per OTU is also indicated. (continued)

OTU name	Number of associated morphological species	Most frequently associated morphological species	Number of associated MalAvi lineages	Most frequently associated MalAvi lineage
L12	0	No data available	2	HYPMA02
L13	0	No data available	2	BT2
L14	0	No data available	4	NEWAM03
L15	0	No data available	3	ANLAT16
L16	0	No data available	2	REB6
L17	0	No data available	6	SFC8
L18	1	*Leucocytozoon toddi*	2	ACCFRA01 ACCFRA02
L19	0	No data available	3	NENOT03 ZOSLUG02 ZOSLUG03

3.4 Discussion

Traditionally the identification of species causing avian malaria was done using microscopy. Recent advances in the molecular detection of these parasites (Bensch et al., 2000) has led to large amounts of sequence data accumulating in public repositories such as MalAvi and GenBank. Although cytochrome *b* sequences usually amplified from these parasites are sorted into MalAvi lineages, in this study I test if the methods used by the barcoding community (examining the gap between inter- and intraspecific genetic distances) could be used to provide a statistically robust method of delimiting parasite species. Unfortunately, not enough information was available to perform gap analyses on *Leucocytozoon*. A statistically definable gap was presented in *Plasmodium* (between 1.0% and 1.5% sequence divergence) and *Haemoproteus* (between 2.0% and 3.5% sequence divergence), which is similar to the sequence divergence of 2.0% used by

Bensch et al. (2004). Using these gaps as a threshold, OTUs were delimited: 18 *Plasmodium* OTUs, 19 *Haemoproteus* OTUs, and 19 *Leucocytozoon* OTUs.

In general, there was a good match between species defined using morphology (where available) and OTUs defined using the molecular data. Notable exceptions include sequences assigned to *P. elongatum, P. relictum, H. balmorali,* and *H. multivolutinus* which were placed in multiple OTUs. Given that the polyphyly of these species usually only involves single sequences, I suggest that the polyphyly is probably the result of misidentification. It is important that species delimitation is not just based on a single method (morphology versus molecular data), but rather the molecular data should be used to inform morphology identification and vice versa. This will aid in future research to understand why some OTUs included multiple morphological species and could aid this current research on the validity of OTUs defined that were not supported by high bootstrap and posterior probability values. For *Plasmodium*, 12 of the 18 OTUs had no associated morphological information, while *Haemoproteus* only had 6 of 19 OTUs without associated morphological data. In particular, *Leucocytozoon* only had one OTU with associated morphological data. This highlights the need for further morphological examination of *Leucocytozoon* species, this could lead to the description of many more avian malaria parasites in Africa.

It has been suggested that MalAvi lineages may be analogous to species (Bensch et al., 2009). This was concluded by evidence from nuclear genes suggesting that avian malaria lineages are reproductively isolated from one another (Bensch et al., 2004; Hellgren et al., 2007a). If this is the case, then I expect the number of MalAvi lineages to be similar to the number of OTUs. But in

970 general, I found many more MalAvi lineages per OTU. Only four OTUs (all belonging to *Plasmodium*) were matched to MalAvi lineages. I also noted that the more sequences available for
972 an OTU, the higher the number of MalAvi lineages, suggesting that MalAvi lineages may also be tracing below morphospecies variation. This disparity will need to be examined in more detail in
974 subsequent studies and I recommend that additional molecular markers including nuclear genes be included.

976

Leucocytozoon is the least well studied genus of avian malaria in Africa (Chapter 2). Lack of
978 research on species belonging to this genus was also clear in this study. Sequences were available for only two morphologically described species (*L. toddi* and *L. macleani*). Much more research
980 is needed on the genus before continental scale patterns can be elucidated.

982 **3.5 Conclusion**

This study highlights the need for future research on the three parasite genera that cause avian
984 malaria in Africa. In particular, focus on *Leucocytozoon* is needed as the knowledge on this genus falls short of what we have for *Plasmodium* and *Haemoproteus*. Although molecular data may be
986 a powerful species delimitation tool future studies should combine traditionally morphology-based methods with analysis of sequence data. Addressing possible misidentifications, synonymous
988 morphological species and the species delimitation threshold would allow a more accurate picture of the diversity of avian malaria species infecting African birds.

CHAPTER 4

USING PUBLICLY AVAILABLE DATA TO TEST AVIAN MALARIA HOST-SPECIFICITY ACROSS AFRICA

Abstract

Research on avian malaria has accelerated since the year 2000 due to the development of molecular tools to detect parasite DNA within host blood. Using the species delimitation method developed in Chapter 3 based on the gap between inter- and intraspecific genetic distances, I identified potential phylogenetic species for avian malaria parasites *Plasmodium*, *Haemoproteus* and *Leucocytozoon* using all previously published cytochrome *b* data available for Africa. Using the available host and locality of collection data I examine host-specificity and geographic distributions of parasite species. Across all three avian malaria genera, host specificity varied for the different species, with some parasite species being highly host specific infecting only one or a few closely related species, while other parasites were shown to be more generalist infecting a wide taxonomic spread of avian hosts. In general, *Plasmodium* contained the most generalist species of the three malaria genera, while *Haemoproteus* contained the most host-specific species. Although generalist parasite species infected a large number of avian hosts, there was still a trend for parasite species to be limited to specific avian families or orders. Unsurprisingly, parasite species with the widest host ranges were found to have larger geographic distributions. Although this study highlights some important trends, there are still large gaps in our knowledge on these parasites in Africa, in particular species belonging the genus *Leucocytozoon* are understudied. In addition, many regions in Africa have no records of avian malaria due to lack of research capacity,

this biases meta-analyses such as these. This study does provide an interesting summary of the current status of knowledge of avian malaria in Africa.

Keywords: *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, host-specificity, distribution

4.1 Introduction

Studies of avian malaria in birds have shown that the parasites that cause this disease are geographically heterogeneously distributed (Atkinson et al., 2008; Lapointe et al., 2012). Specialized associations between parasites, hosts and insect vectors may drive this heterogeneity (Ellis et al., 2015; Manzoli et al., 2021; Medeiros et al., 2015). Local coevolutionary relationships between parasites and their hosts may also influence geographic distributions of both host and parasite populations (Fallon et al., 2005; Ricklefs, 2010). The choice of host species by the parasite depends on several factors including what is required from the host and what part of the parasite's lifecycle the host plays a role in and these factors lead to host specificity (Gunn and Pitt, 2012; Roberts et al., 2013). Many parasites tend to be host-specific and have the ability to thrive in only one host species or a small number of closely related host species, often being referred to as specialist parasites (Rivero and Gandon, 2018; Ventim et al., 2012). In contrast, other parasites can have wide host ranges, infecting a set of taxonomically different host species, and are referred to as generalists (Gunn and Pitt, 2012; Rivero and Gandon, 2018; Ventim et al., 2012). Each strategy has its own advantages and disadvantages. Generalists are able to infect far more hosts and are generally more abundant and can have far larger geographic distributions than that of parasites that are more host-specific (Rivero and Gandon, 2018). Specialists, are able to exploit hosts more effectively than generalists (Rivero and Gandon, 2018).

Even if parasites are host-specific, infection of new hosts can occur accidentally. In some cases, this new host species is not compatible to the parasite, meaning it cannot exploit its host to acquire the nutrients it needs or is unable to continue its lifecycle within the new host (Roberts et al., 2013). But this is not always the case. Some parasites can persist in a host species different to those they are evolved to infect and this is often referred to as spill-over (Hellgren et al., 2009). This can be beneficial to the parasite, but a spill-over event to hosts that are immunologically naïve may result in highly adverse effects for the host (Jenkins et al., 2018; Verwey et al., 2018). Monitoring for these possible "spill-overs" is not only important for conservation or to monitor possible epizootics in agriculturally important animals, but for human health as well as most pandemics and the majority of emerging infectious diseases seen in humans are zoonotic in origin (Jones et al., 2008; Kruse et al., 2004; Morse et al., 2012). West Nile Virus and St. Louis encephalitis are examples of diseases which have expanded their host range from their natural avian hosts to humans via their mosquito vector, *Culex* (Campbell et al., 2002; Farajollahi et al., 2011). The mosquito genus *Culex* is responsible for a variety of other human diseases, but also some viruses and parasites that have not escaped from their normal host range to include humans (Farajollahi et al., 2011).

Avian malaria refers to a group of haemosporidian parasites from the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, which infect a wide range of bird species across the globe (Valkiūnas, 2005). Species belonging to *Haemoproteus* tend to be more host-specific while *Plasmodium* species are considered more generalist. Limited *Leucocytozoon* research indicates its host specificity may fall somewhere between that of *Plasmodium* and *Haemoproteus* (Beadell et al., 2009; Doussang et al., 2021; Reeves et al., 2015; Rivero and Gandon, 2018) although empirical data testing this is limited and abiological events, such as climate, may affect host-specificity in

these parasites (Fecchio et al., 2019; Valkiūnas, 2005). However, the host-specificity of these parasites may also be attributed to the host-specificity of their vectors, with *Plasmodium* parasites using a greater number of mosquito species than the number of biting midge species *Haemoproteus* use (Valkiūnas, 2005). Host-spill over has been observed where avian malaria parasites favour a single host species but can also occur in a broad range of other hosts (Hellgren et al., 2009). Understanding which of these parasites are likely to expand their host range is an important consideration as avian malaria has been shown to be lethal to avian host species that are immunologically naïve, for example penguins and Hawaiian birds (Bueno et al., 2010; Van Riper III et al., 1986).

In this chapter I aim to use all available data from molecular avian malaria research across Africa to test the hypothesis that parasite species belonging to *Haemoproteus* are more host-specific, while *Plasmodium* species are more generalist. I also aim to clarify if *Leucocytozoon* species are more host-specific or generalist. I will also test if there is any correlation between host-specificity and geographic distribution of parasite species.

4.2 Materials and methods

Cytochrome *b* sequence data from parasites collected in Africa were collected using information available on the MalAvi database (Bensch et al., 2009) and from published articles found using Google Scholar. Sequences were downloaded from GenBank between April 2020 and June 2021 using ascension numbers provided by MalAvi and published articles found. Articles were searched for using the key words: “avian”, “malaria”, *Plasmodium*”, “*Haemoproteus*”, “*Leucocytozoon*”, “haematozoa”, “haemosporidian”, in turn with a combination of location-based keywords such as

"Africa", "Indian Ocean", Atlantic Ocean", and "name of African country/territory". For the last keyword, the name of each African country was used, including older names that have now changed for example both Swaziland and Eswatini were used in search of articles. In addition to countries part of continental Africa, the island nations of Cape Verde, Comoros, Madagascar, Mauritius, Seychelles, and São Tomé and Príncipe were included. Foreign dependencies and territories were also included such as: Europa Island, Mayotte and Réunion (France), Madeira and the Savage Islands (Portugal), Canarias (Spain), Saint Helena, Ascension and Tristan da Cunha (United Kingdom), and Socotra (Yemen). Associated information such as host species and collection location were also obtained. Parasite species belonging to *Plasmodium*, *Haemoproteus* and *Leucocytozoon* were delimited using the method outlined in Chapter 3. Briefly, for each genus the gap between inter-and intraspecific genetic distances was estimated. This is then used as threshold to assign sequences to different species. The thresholds used were between 1.0% and 1.5% for *Plasmodium*, between 2.0% and 3.5% for *Haemoproteus*, and 2.0% for *Leucocytozoon* based on Bensch et al. (2004). In this Chapter we will refer to these phylogenetic species as OTUs.

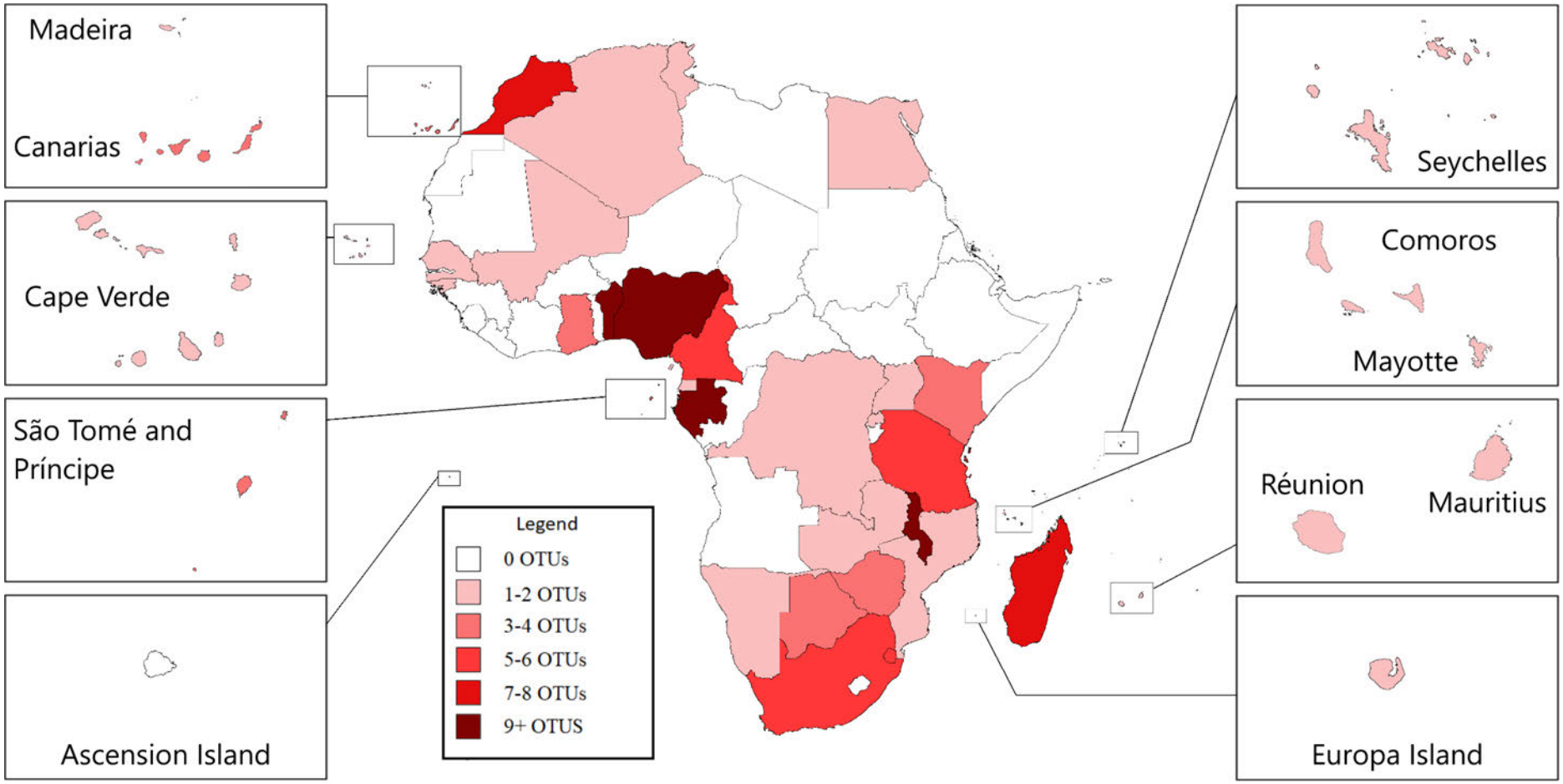
From this dataset, the OTU diversity within each country and region was determined by simply counting the number of OTUs reported within the region. Host-specificity trends for each OTU were described by recording the total number of avian host species, families and orders infected by each OTU. Finally the geographic distribution of each OTU was estimated by grouping the countries in which each species occurs into the five M49 standard geographic regions of Africa (United Nations, 1999). These geographic regions are Northern Africa, Eastern Africa, Middle Africa, Southern Africa, and Western Africa.

4.3 Results

4.3.1 Plasmodium infection in Africa

Eighteen parasite OTUs were identified from the *Plasmodium* cytochrome *b* data. These OTUs were found in 36 African countries/regions. The countries that showed the highest diversity included Malawi (13 OTUs), Nigeria (11 OTUs), Benin (10 OTUs), and Gabon (9 OTUs) (Figure 4.1). High numbers of OTUs were found in high research regions and countries (Chapter 2) such as the Gulf of Guinea, South Africa, and Madagascar. In contrast only four OTUs were recorded in the Canary Islands even though a relatively large number of studies have been conducted here. In some countries only one OTU was reported, these countries included Democratic Republic of the Congo, Equatorial Guinea, Europa Island, Madeira, Namibia, Réunion, Seychelles, and Zambia.

The host range varied greatly between *Plasmodium* OTUs (Table 4.1). OTU P1 was found in 199 avian species, while P12 was only recorded in two bird species, with 98.51% of all reported infections recorded in *Bubulcus ibis*. Although P1 had a broad host range, 19.83% of all recorded P1 infections came from a single host species, *Cyanomitra olivacea*. The OTU P15 was most frequently found in *Cyanomitra olivacea*, however only 9.21% of recorded P15 infections were reported from this host species. For five OTUs, more than 50% of their recorded infections came from a single host species. While for seven OTUs, more than 50% of their recorded infections came from a single host family and three OTUs had over 90% of all reported infections coming from a single host family. This reveals an increase in host specificity at higher avian taxonomic levels.



1130 Figure 4.1. Map showing the number of *Plasmodium* OTUs per African country/region. The colour of each country/region indicates the number of OTUs present in each country/region.

1132

Table 4.1. *Plasmodium* OTUs. Number of host species, families and orders infected by the OTUs are given. The highest percentage of the reports of infection in a single avian host species, family, and order per OTU is given. The total number of infections and geographic distribution of each OTU is also provided.

OTU	No. of infections	No. of host species	Highest percentage of reported infection	No. of host families	Highest percentage of reported infection	No. of host orders	Highest percentage of reported infection	Distribution
P1	1620	199	*Cyanomitra olivacea* (19.83%)	39	Ploceidae (39.04%)	7	Passeriformes (98.95%)	
P2	8	5	*Newtonia amphichroa* (50.00%)	4	Vangidae (62.50%)	2	Passeriformes (87.50%)	
P3	3	3	*Batis dimorpha*, *Euplectes axillaris*, *Tchagra senegalus* (33.33%)	3	Malaconotidae, Platysteiridae, Ploceidae (33.33%)	1	Passeriformes (100.00%)	
P4	413	67	*Eurillas latirostris* (54.24%)	25	Pycnonotidae (68.28%)	3	Passeriformes (99.03%)	
P5	26	20	*Cinnyris mediocris*, *Saxicola torquatus* (11.54%)	11	Muscicapidae, Cisticolidae (26.92%)	3	Passeriformes (92.31%)	
P6	13	8	*Cinnyris chalybeus* (38.46%)	6	Nectariniidae (38.46%)	1	Passeriformes (100.00%)	
P7	26	4	*Passer domesticus* (88.46%)	2	Passeridae (96.15)	1	Passeriformes (100.00%)	

Table 4.1. *Plasmodium* OTUs. Number of host species, families and orders infected by the OTUs are given. The highest percentage of the reports of infection in a single avian host species, family, and order per OTU is given. The total number of infections and geographic distribution of each OTU is also provided. (continued)

OTU	No. of infections	No. of host species	Highest percentage of reported infection	No. of host families	Highest percentage of reported infection	No. of host orders	Highest percentage of reported infection	Distribution
P8	5	4	*Anthus leucophrys* (40.00%)	2	Motacillidae (80.00%)	1	Passeriformes (100.00%)	
P9	64	22	*Cyanomitra olivacea* (39.06%)	4	Nectariniidae (92.19%)	1	Passeriformes (100.00%)	
P10	691	13	*Anthus berthelotii* (75.40%)	9	Motacillidae (75.40%)	2	Passeriformes (99.71%)	
P11	3	3	*Amandava subflava*, *Emberiza flaviventris*, *Laniarius ferrugineus* (33.33%)	3	Emberizidae, Estrildidae, Malaconotidae (33.33%)	1	Passeriformes (100.00%)	
P12	67	2	*Bubulcus ibis* (98.51%)	2	Ardeidae (98.51%)	2	Pelecaniformes (98.51%)	
P13	18	17	*Chalcomitra senegalensis* (11.11%)	8	Nectariniidae (38.89%)	2	Passeriformes (94.44%)	
P14	19	11	*Calidris pugnax*, *Sylvia borin* (21.05%)	8	Sylviidae (31.58%)	3	Passeriformes (73.68%)	

Table 4.1. *Plasmodium* OTUs. Number of host species, families and orders infected by the OTUs are given. The highest percentage of the reports of infection in a single avian host species, family, and order per OTU is given. The total number of infections and geographic distribution of each OTU is also provided. (continued)

OTU	No. of infections	No. of host species	Highest percentage of reported infection	No. of host families	Highest percentage of reported infection	No. of host orders	Highest percentage of reported infection	Distribution
P15	76	44	*Cyanomitra olivacea* (9.21%)	22	Ploceidae (18.42%)	7	Passeriformes (88.16%)	
P16	6	4	*Foudia omissa*, *Terpsiphone rufiventer* (33.33%)	4	Monarchidae, Ploceidae (33.33%)	1	Passeriformes (100.00%)	
P17	21	15	*Quelea quelea* (19.05%)	7	Ploceidae (33.33%)	3	Passeriformes (90.48%)	
P18	16	15	*Ploceus cucullatus* (12.50%)	10	Turdidae, Ploceidae (18.75%)	4	Passeriformes (81.25%)	

Plasmodium occurs most frequently in passerines, with members of the order Passeriformes being the primary avian host in 17 of the 18 *Plasmodium* OTUs and seven OTUs were found exclusively in hosts from this order. A member of the order Pelecaniformes was found to be the primary host in P12. For most of the *Plasmodium* OTUs, there was a very high percentage of reported infections from passerine hosts. OTUs with a broader host range such as P1 and P15 were found across seven avian host orders, but still had a high proportion of reported infections from passerine hosts. After P12, P14 had the lowest proportion of recorded infections from passerine hosts, with 73.68% of reported infections found in passerines. There was also a high number of infections reported in members of the order Charadriiformes (21.05% of P14 infections).

Across Africa, Eastern and Western Africa had the highest OTU diversity with 15 OTUs found within each region, while Northern Africa had lowest diversity with only seven OTUs reported there (Table 4.1). A total of four OTUs were found in all five regions in Africa, while only one OTU (P11) was isolated to a single region (Eastern Africa).

4.3.2 Haemoproteus infection in Africa

Nineteen parasite OTUs were identified from the *Haemoproteus* cytochrome *b* data. These OTUs were found in 27 African countries/regions (Figure 4.2). Malawi (14 OTUs) and Morocco (11 OTUs) recorded the highest number of *Haemoproteus* parasites. Much like *Plasmodium*, the high research regions of South Africa, Madagascar and the Gulf of Guinea all had high *Haemoproteus* OTU diversity and once again the Canary Islands had a lower diversity than expected considering the number of studies conducted there. Many countries only had one identified OTU (Algeria,

Accession Island, Canary Islands, Cape Verde, Egypt, Europa Island, Mayotte, Zambia, and Zimbabwe).

The host range for *Haemoproteus* (Table 4.2) varied from 89 different host species (H17) to a single host species (*Delichon urbicum*) for H4. OTU H17 had 18.04% of all recorded infections found within a single host species, *Cyanomitra olivacea*. The *Haemoproteus* OTU which had the lowest proportion of reported infections from a single host species was H12, which was reported eight times and each report was from a different host species. For nine OTUs, more than 50% of their recorded infections came from a single host species. While for sixteen OTUs, more than 50% of their recorded infections came from a single host family and ten OTUs having over 90% of all reported infections coming from a single host family. This included six OTUs that were exclusively found within one host family. This reveals an increase in host specificity at higher avian taxonomic levels. The most obvious example of this was H3, where 16.19%, of recorded infections came from a single host species, *Acrocephalus schoenobaenus*, but 95.24% came from a single host family, Acrocephalidae.

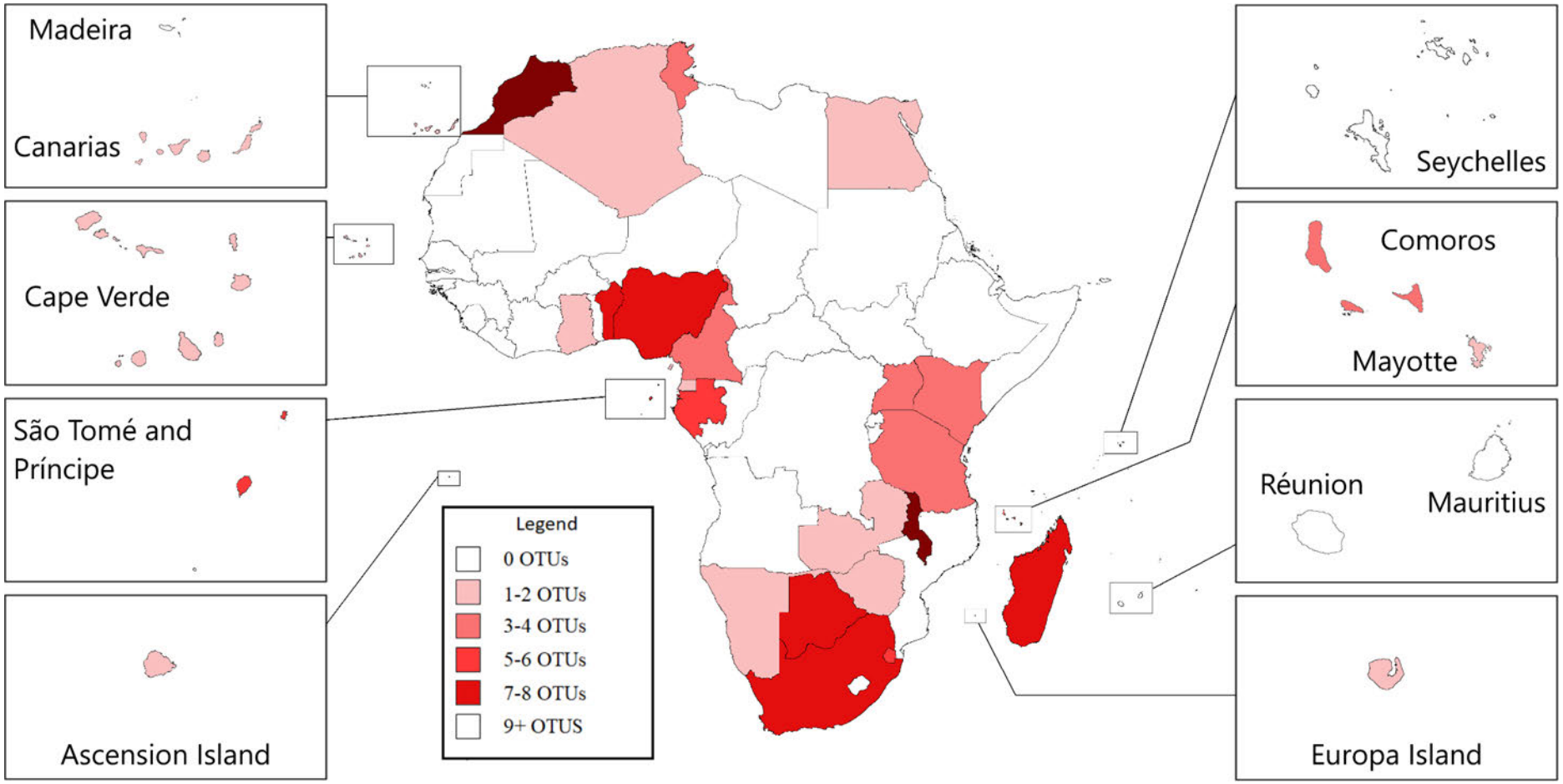


Figure 4.2. Map showing the number of *Haemoproteus* OTUs per African country/region. The colour of each country/region indicates the number of OTUs present in each country/region.

Table 4.2. *Haemoproteus* OTUs. Number of host species, families and orders infected by the OTUs are given. The highest percentage of the reports of infection in a single avian host species, family, and order per OTU is given. The total number of infections and geographic distribution of each OTU is also provided.

OTU	No. of infections	No. of host species	Highest percentage of reported infection	No. of host families	Highest percentage of reported infection	No. of host orders	Highest percentage of reported infection	Distribution
H1	185	29	*Zosterops maderaspatanus* (42.16%)	12	Zosteropidae (54.05%)	2	Passeriformes (99.46%)	
H2	2	2	*Hippolais icterina*, *Cisticola nigriloris* (50.00%)	2	Acrocephalidae, Cisticolidae (50.00%)	1	Passeriformes (100.00%)	
H3	105	18	*Acrocephalus schoenobaenus* (16.19%)	5	Acrocephalidae (95.24%)	2	Passeriformes (99.05%)	
H4	20	1	*Delichon urbicum* (100.00%)	1	Hirundinidae (100.00%)	1	Passeriformes (100.00%)	
H5	53	7	*Fringilla coelebs* (75.47%)	2	Fringillidae (98.11%)	1	Passeriformes (100.00%)	
H6	6	2	*Lanius collaris* (83.33%)	2	Laniidae (83.33%)	1	Passeriformes (100.00%)	
H7	9	5	*Muscicapa striata* (55.56%)	4	Muscicapidae (66.67%)	1	Passeriformes (100.00%)	
H8	11	4	*Muscicapa striata* (72.73%)	2	Muscicapidae (90.90%)	1	Passeriformes (100.00%)	

Table 4.2. *Haemoproteus* OTUs. Number of host species, families and orders infected by the OTUs are given. The highest percentage of the reports of infection in a single avian host species, family, and order per OTU is given. The total number of infections and geographic distribution of each OTU is also provided. (continued)

OTU	No. of infections	No. of host species	Highest percentage of reported infection	No. of host families	Highest percentage of reported infection	No. of host orders	Highest percentage of reported infection	Distribution
H9	3	3	*Erithacus rubecula*, *Ficedula hypoleuca*, *Saxicola rubetra* (33.33%)	1	Muscicapidae (100.00%)	1	Passeriformes (100.00%)	
H10	3	2	*Monticola sharpie* (66.67%)	1	Muscicapidae (100.00%)	1	Passeriformes (100.00%)	
H11	238	72	*Eurillas latirostris* (17.65%)	25	Pycnonotidae (31.51%)	6	Passeriformes (76.05%)	
H12	8	8	*Apaloderma vittatum*, *Batis dimorpha*, *Corythaeola cristata*, *Cossypha heuglini*, *Halcyon leucocephala*, *Ispidina picta*, *Laniarius barbarous*, *Onychognathus tenuirostris* (12.50%)	7	Alcedinidae (25.00%)	4	Passeriformes (50.00%)	
H13	7	4	*Agelastes niger* (42.86%)	3	Numididae (57.14%)	3	Galliformes (57.14%)	
H14	67	14	*Foudia omissa* (59.70%)	5	Ploceidae (94.03%)	4	Passeriformes (95.52%)	

Table 4.2. *Haemoproteus* OTUs. Number of host species, families and orders infected by the OTUs are given. The highest percentage of the reports of infection in a single avian host species, family, and order per OTU is given. The total number of infections and geographic distribution of each OTU is also provided. (continued)

OTU	No. of infections	No. of host species	Highest percentage of reported infection	No. of host families	Highest percentage of reported infection	No. of host orders	Highest percentage of reported infection	Distribution
H15	21	7	*Passer diffuses* (42.86%)	1	Passeridae (100.00%)	1	Passeriformes (100.00%)	
H16	13	3	*Newtonia amphichroa*, *Newtonia brunneicauda* (46.15%)	1	Vangidae (100.00%)	1	Passeriformes (100.00%)	
H17	510	89	*Cyanomitra olivacea* (18.04%)	21	Ploceidae (36.47%)	4	Passeriformes (92.55%)	
H18	233	4	*Columba livia* (97.42%)	1	Columbidae (100.00%)	1	Columbiformes (100.00%)	
H19	30	10	*Fregata minor* (41.38%)	2	Columbidae (55.17%)	2	Columbiformes (55.17%)	

Similar to *Plasmodium*, *Haemoproteus* was most frequently found in passerines with 16 of the 19 *Haemoproteus* OTUs found predominately in Passeriformes hosts. Of these 16 OTUs, 10 were found exclusively in passerine hosts. The three OTUs that didn't have passerines as its primary hosts were H13 (Galliformes), H18 and H19 (Columbiformes). *Haemoproteus* OTUs which infected a larger range of host species, such as H17 and H11, also infected a range of avian orders, but to a lesser degree than what was seen in *Plasmodium* OTUs P1 and P15. H17, which has the broadest range of host species was only found in four avian orders (Coraciiformes, Cuculiformes, Galliformes, and Passeriformes). OTU H11 which had a smaller a range of host species and was found in six avian orders (Columbiformes, Coraciiformes, Cuculiformes, Passeriformes, Piciformes, and Strigiformes).

The geographic distribution of these OTUs revealed that Eastern Africa had the highest *Haemoproteus* OTU diversity with 16 OTUs found in this region. While Middle Africa had the lowest *Haemoproteus* diversity with only seven OTUs present in this region. There were four OTUs found in all five regions in Africa while two OTUs were isolated to a single region (H4 in Northern Africa and H16 in Eastern Africa).

4.3.3 Leucocytozoon infection in Africa

Nineteen parasite OTUs were identified from the *Leucocytozoon* cytochrome *b* data. These OTUs were found in only 22 African countries/regions (Figure 4.3). This is far less than what was found for *Plasmodium* and *Haemoproteus*. The countries which showed the highest diversity of *Leucocytozoon* OTUs were Morocco (9 OTUs), Madagascar (8 OTUs), Nigeria (8 OTUs), and Malawi (7 OTUs). Once again, high numbers of OTU were found in high research regions and

countries (Chapter 2). However, unlike what was found in *Plasmodium* and *Haemoproteus*, the Canary Islands had a high OTU diversity for *Leucocytozoon*. The African countries and regions that only had a single *Leucocytozoon* OTU present were Algeria, Benin, Guinea-Bissau, Mauritius, Mayotte, and Socotra.

The host range for *Leucocytozoon* OTUs were often very narrow, with only two OTUs being found in more than ten host species (Table 4.3). OTU L1 had the broadest host range, and was reported in 104 host species, followed by L2, which was reported in 43 avian host species. Although L2 did have a broad host range, 44.48% of reported infections came from a single host species (*Zosterops borbonicus*). In comparison, the host species that made up the highest proportion of recorded infections for L1 (*Cyanistes teneriffae*) only made up 10.71% of these reported infections. For the remaining *Leucocytozoon* OTUs, the lower host ranges observed may not be attributed to host specificity, but a result of too few representatives collected for each OTU, for example, L19 was only recorded three times in Africa. While the sequences associated with L4 did not have any annotated host species as the host species was not reported by Jones et al. (2018). This makes it difficult draw accurate conclusions. What was observed though was that in seven OTUs, more than 50% of their recorded infections came from a single host species. While for twelve OTUs, more than 50% of their recorded infections came from a single host family and five OTUs having over 90% of all reported infections coming from a single host family. This again reveals an increase in host specificity at higher avian taxonomic levels.

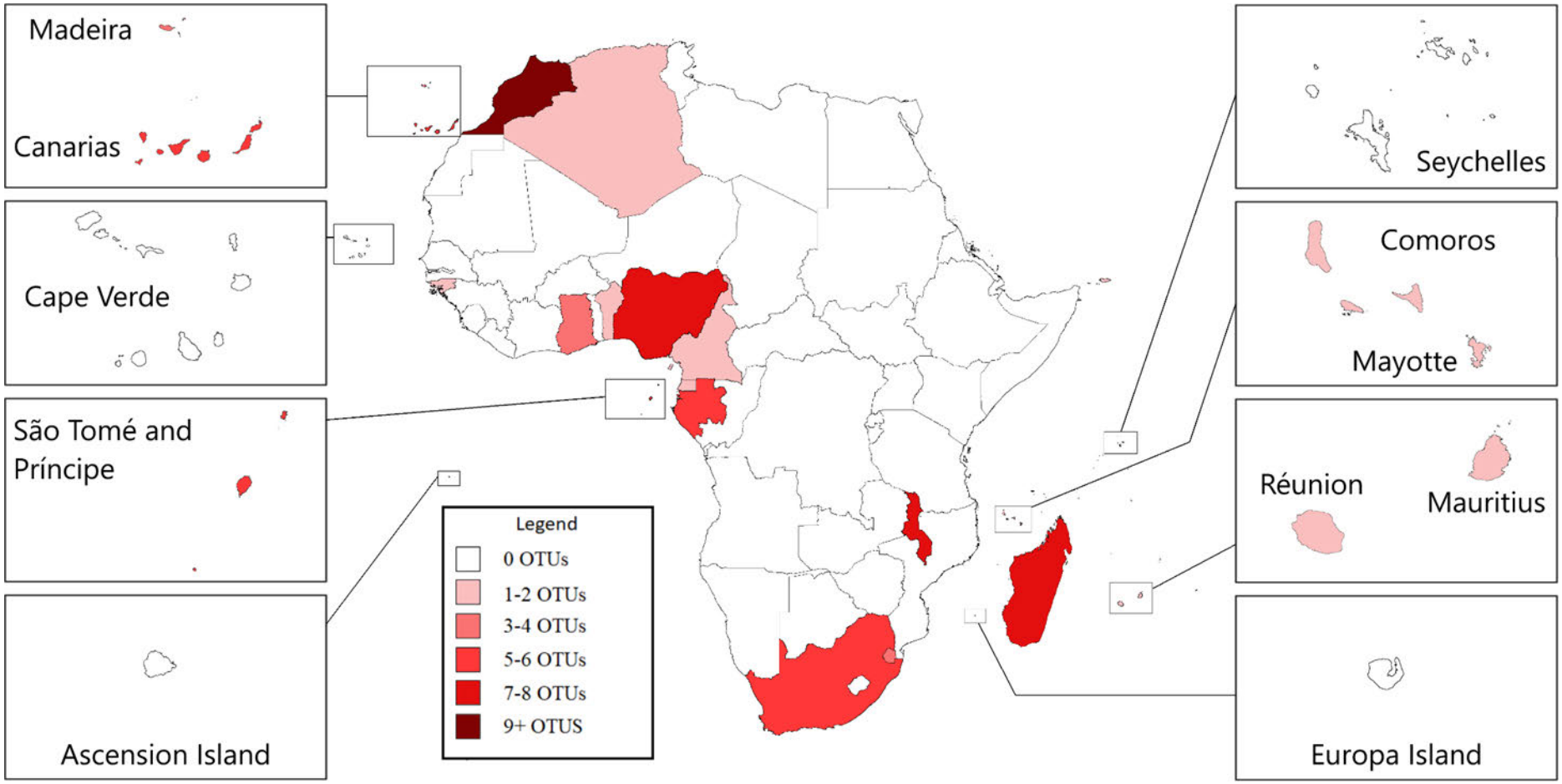


Figure 4.3. Map showing the number of *Leucocytozoon* OTUs per African country/region. The colour of each country/region indicates the number of OTUs present in each country/region.

Table 4.3. *Leucocytozoon* OTUs. Number of host species, families and orders infected by the OTUs are given. The highest percentage of the reports of infection in a single avian host species, family, and order per OTU is given. The total number of infections and geographic distribution of each OTU is also provided.

OTU	No. of infections	No. of host species	Highest percentage of reported infection	No. of host families	Highest percentage of reported infection	No. of host orders	Highest percentage of reported infection	Distribution
L1	696	104	*Cyanistes teneriffae* (10.71%)	26	Fringillidae (15.79%)	3	Passeriformes (99.25%)	
L2	358	43	*Zosterops borbonicus* (44.48%)	19	Zosteropidae (66.57%)	4	Passeriformes (99.13%)	
L3	5	3	*Turdus viscivorus*, *Turdus pelios* (40.00%)	1	Turdidae (100.00%)	1	Passeriformes (100.00%)	
L4	8	0	No data available	0	No data available	0	No data available	
L5	5	3	*Fringilla coelebs*, *Otus scops* (40.00%)	2	Strigidae (60.00%)	2	Strigiformes (60.00%)	
L6	8	3	*Turdus merula* (75.00%)	1	Turdidae (100.00%)	1	Passeriformes (100.00%)	

Table 4.3. *Leucocytozoon* OTUs. Number of host species, families and orders infected by the OTUs are given. The highest percentage of the reports of infection in a single avian host species, family, and order per OTU is given. The total number of infections and geographic distribution of each OTU is also provided. (continued)

OTU	No. of infections	No. of host species	Highest percentage of reported infection	No. of host families	Highest percentage of reported infection	No. of host orders	Highest percentage of reported infection	Distribution
L7	8	8	*Bradypterus cinnamomeus*, *Chloris chloris*, *Cryptospiza reichenovii*, *Euplectes albonotatus*, *Eurillas latirostris*, *Iduna natalensis*, *Phyllastrephus flavostriatus*, *Pogonocichla stellata* (12.50%)	7	Pycnonotidae (25.00%)	1	Passeriformes (100.00%)	
L8	14	9	*Delichon urbicum*, *Passer domesticus*, *Sylvia melanocephala* (16.67%)	9	Hirundinidae, Passeridae, Sylviidae (16.67%)	1	Passeriformes (100.00%)	
L9	20	9	*Bubulcus ibis* (47.37%)	6	Ardeidae (47.37%)	6	Pelecaniformes (47.37%)	
L10	11	7	*Calicalicus madagascariensis* (30.00%)	6	Pycnonotidae, Vangidae (30.00%)	2	Passeriformes (90.00%)	
L11	26	5	*Parus major* (53.85%)	3	Paridae (92.31%)	1	Passeriformes (100.00%)	

Table 4.3. *Leucocytozoon* OTUs. Number of host species, families and orders infected by the OTUs are given. The highest percentage of the reports of infection in a single avian host species, family, and order per OTU is given. The total number of infections and geographic distribution of each OTU is also provided. (continued)

OTU	No. of infections	No. of host species	Highest percentage of reported infection	No. of host families	Highest percentage of reported infection	No. of host orders	Highest percentage of reported infection	Distribution
L12	20	5	*Hypsipetes madagascariensis* (50.00%)	4	Pycnonotidae (50.00%)	1	Passeriformes (100.00%)	
L13	4	2	*Sylvia borin* (75.00%)	1	Sylviidae (100.00%)	1	Passeriformes (100.00%)	
L14	7	4	*Newtonia amphichroa*, *Philepitta castanea*, *Ploceus nigricollis* (28.57%)	3	Ploceidae (42.86%)	1	Passeriformes (100.00%)	
L15	4	3	*Cyanomitra olivacea* (50.00%)	2	Nectariniidae (75.00%)	1	Passeriformes (100.00%)	
L16	14	6	*Ploceus velatus* (42.86%)	2	Ploceidae (85.71%)	1	Passeriformes (100.00%)	
L17	14	9	*Sylvia atricapilla* (35.71%)	6	Sylviidae (57.14%)	1	Passeriformes (100.00%)	
L18	4	2	*Accipiter francesiae* (75.00%)	1	Accipitridae (100.00%)	1	Accipitriformes (100.00%)	
L19	3	2	*Zosterops lugubris* (66.67%)	2	Zosteropidae (66.67%)	1	Passeriformes (100.00%)	

Much like *Plasmodium* and *Haemoproteus*, *Leucocytozoon* occurs most frequently in passerines. Excluding L4, 15 out of 18 *Leucocytozoon* OTUs had members of the order Passeriformes as their primary host and 12 OTUs were exclusively found in passerine hosts. For the remaining OTUs, the primary host order for L15 was Strigiformes and L18 was found exclusively in Accipitriformes. The OTU L9 infected the broadest range of avian orders (N = 6). It had the highest proportion of reported infections in Pelecaniformes with 47.37% of recorded L9 infections within this host order. The remaining host orders L9 were reported in were Columbiformes, Coraciiformes and Cuculiformes, as well as Accipitriformes and Passeriformes but had only reported an L9 infection once each.

Across Africa, the diversity of *Leucocytozoon*'s OTUs were similar amongst most of the geo-regions with the highest diversity found in Eastern Africa (12 OTUs), followed by Middle Africa, Northern Africa and Western Africa with 10 OTUs each. Southern Africa had the lowest OTU diversity with only six OTUs reported in this region, however this is the smallest geo-region and that may play a role on the lower number of OTUs. Only two *Leucocytozoon* OTUs were found across all five geographic regions (OTUs L1 and L2), but five OTUs were found only in one region (L4 in Southern Africa, L11 in Northern Africa, L12 in Eastern Africa, L13 in Western Africa, and L18 in Eastern Africa).

4.3.4 OTU diversity within avian hosts

The highest number of *Plasmodium* OTUs found in a single bird species (*Ploceus cucullatus*) was seven (Table 4.4). The highest number of *Haemoproteus* OTUs found in a single bird species (*Ispidina picta*) was four. For *Leucocytozoon*, four host species (*Cyanistes teneriffae*, *Eurillas*

latirostris, *Ploceus cucullatus*, and *Sylvia borin*) had the highest number of OTUs found in a single bird species. Across all three avian malaria genera, the village weaver (*Ploceus cucullatus*) had the highest diversity of malaria OTUs with 13 OTUs, infecting this species; seven *Plasmodium* OTUs, two *Haemoproteus* OTUs and four *Leucocytozoon* OTUs.

Table 4.4. Number of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* OTUs per host species, family and order which had the highest numbers of OTUs reported.

Species	OTUs	Family	OTUs	Order	OTUs
Plasmodium					
Ploceus cucullatus	7	Estrildidae	9	Passeriformes	18
Cyanomitra olivacea	5	Muscicapidae	9	Accipitriformes	3
Hedydipna collaris	5	Ploceidae	9	Charadriiformes	3
Quelea quelea	5			Columbiformes	3
Sylvia borin	5			Coraciiformes	3
				Pelecaniformes	3
Haemoproteus					
Ispidina picta	4	Muscicapidae	9	Passeriformes	16
Ficedula hypoleuca	3	Alcedinidae	5	Columbiformes	5
Muscicapa striata	3	Cisticolidae	5	Coraciiformes	5
Passer domesticus	3	Columbidae	5		
Ploceus nigricollis	3				
Leucocytozoon					
Cyanistes teneriffae	4	Ploceidae	7	Passeriformes	17
Eurillas latirostris	4	Estrildidae	6	Columbiformes	3
Ploceus cucullatus	4	Pycnonotidae	6	Coraciiformes	2
Sylvia borin	4			Pelecaniformes	2
All					
Ploceus cucullatus	13	Muscicapidae	23	Passeriformes	51

The families Estrildidae, Muscicapidae and Ploceidae were all infected by nine *Plasmodium* OTUs. The highest number of *Haemoproteus* OTUs found in a host family was nine OTUs in Muscicapidae. For *Leucocytozoon* seven OTUs were found in the bird family Ploceidae. Other bird families with high numbers of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* OTUs are

shown in Table 4.4. Across all three avian malaria genera, the host family which had the highest number of OTUs reported to infect its members was Muscicapidae, with a total of 23 OTUs (*Plasmodium* = 9, *Haemoproteus* = 9, *Leucocytozoon* = 5).

Across all three avian malaria genera, the greatest OTU diversity was found in passerine birds with all *Plasmodium* OTUs infecting species belonging to this order. For *Haemoproteus*, only three OTUs were not found in passerines hosts (H13, H18 and H19) and for *Leucocytozoon*, only one OTU (L18) was not found in passerines. *Haemoproteus* also had the highest diversity of OTUs found outside of the passerine order, with five OTUs found to infect Columbiformes and Coraciiformes. This meant that passerines were infected with a total of 51 avian malaria OTUs.

4.4 Discussion

Plasmodium, *Haemoproteus* and *Leucocytozoon* have not been evenly studied over the last 20+ years (see Chapter 2). However, this research has been increasing since the development of a molecular approach to identifying these parasites within the blood of their avian hosts and occasionally other host tissue (Bensch et al., 2000; Hellgren et al., 2004). Due to this, it was possible to collect a large amount of information on avian malaria infections reported across African birds. Using the delimited OTUs from Chapter 3 and data for host species and geographic location of these parasites, this study examines the host-specificity and geographic distribution of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites. By doing so, I can observe if the patterns in data coincide with the hypotheses that *Haemoproteus* parasites are host-specific, while *Plasmodium* parasites are more generalist (Beadell et al., 2009; Rivero and Gandon, 2018). While limited research has been conducted on *Leucocytozoon*, this research also aims to unpack the host specificity of *Leucocytozoon* parasites.

What was found was that the host ranges for the OTUs varied within each avian malaria genus. Some OTUs had large host ranges (P1, P4, H11, H17, and L1) infecting over 50 different avian host species, with the broadest host range being 199 host species for P1. While some OTUs were almost completely isolated to a single avian host species (P12, H4, L13 and L18), with H4 found in only one host species (*Delichon urbicum*). In general, more *Plasmodium* OTUs (especially P1) were found to have larger host ranges than *Haemoproteus* and *Leucocytozoon* OTUs, suggesting that this genus seems to be the most generalist malaria genus and could explain the large number of bird species these parasites are reported in globally (Ricklefs and Fallon, 2002; Ventim et al., 2012). However, the vectors of *Plasmodium* may play an important role towards this host specificity, introducing these parasites to a broader range of host species than that of the vectors for *Haemoproteus* and *Leucocytozoon* (Valkiūnas, 2005). Thus, making the research on avian malaria's vectors vital to understanding these patterns. Although some *Haemoproteus* OTUs (such as H11 and H17) also had broad host ranges, 9 of the 19 *Haemoproteus* OTUs had more than 50% of their recorded infections coming from a single host species, revealing higher levels of host specificity. There were a fewer number of OTUs which had more than 50% of their recorded infections coming from a single host species for *Plasmodium* (N = 5 OTUs) and *Leucocytozoon* (N = 7 OTUs). This suggests that *Haemoproteus* parasites are more host specific and that *Plasmodium* parasites are more generalist, with *Leucocytozoon* parasites falling somewhere in between *Plasmodium* and *Haemoproteus*. However, it is difficult to draw conclusions for *Leucocytozoon* as far less research has been conducted on this genus of avian malaria. One such example is the low host ranges of many *Leucocytozoon* OTUs. Only two *Leucocytozoon* OTUs had a host range greater than 10 bird species (*Plasmodium* = 10 OTUs, *Haemoproteus* = 6 OTUs).

Although only seven OTUs showed patterns of host specificity with more than 50% of their recorded infections coming from a single host species. This leaves a total of 10 *Leucocytozoon* OTUs that are difficult to label as either host-specific or generalist due to too limited information. The recent increase in *Leucocytozoon* research using a molecular approach across Africa, as seen in Chapter 2, is promising and more data in future could be used to examine this hypothesis further.

What my results also showed was that host-specificity increased at the family level. (Bennett and Peirce, 1991; Pacheco et al., 2018; Rivero and Gandon, 2018). The number of OTUs that had 50% of reported infections come from a single host family reflected what was found earlier, that *Plasmodium* (N = 7 OTUs) are more generalist parasites, *Haemoproteus* parasites (N = 16 OTUs) are more host-specific, and *Leucocytozoon* parasites (N = 12 OTUs) fall in between them. The host specificity for these parasites increased again at higher taxonomic levels when looking at host orders. The data set seemed to confirm a well-documented hypothesis that avian malaria has a preference to avian hosts from the Passeriform order as only two OTUs were never reported in passerines (Valkiūnas, 2005). The *Haemoproteus* OTU H18, found exclusively in Columbiformes and the *Leucocytozoon* OTU L18, found exclusively in Accipitriformes.

Research on avian malaria across Africa has also been unevenly spread geographically, with some countries being studied more than others (Chapter 2). The high research regions, such as the Gulf of Guinea and Southern Africa did have a large amount of avian malaria OTUs but countries in these regions did not always have the highest number of OTUs. For *Plasmodium* the country with the highest number of *Plasmodium* OTUs was Malawi (N = 13 OTUs). Malawi also had the highest number of *Haemoproteus* OTUs (N = 14 OTUs). *Leucocytozoon* had the highest number of OTUs

found in Morocco (N = 9 OTUs). Why these countries have such high numbers of avian malaria OTUs could be a result of the manner of the research conducted (Outlaw et al., 2017). In these regions, looking at a broad number of hosts increasing the chances of identifying a greater variety of parasites as seen from research conducted by Drovetski *et al.* (2014) and Lutz *et al.* (2015). While in contrast, the countries and regions that had the lowest number of OTUs recovered were often the countries that had been studied least frequently. Although there are many contributing factors that can determine parasite diversity in different regions across Africa, such as avian host diversity and population sizes (Doussang et al., 2021; Marzal et al., 2011; Neto et al., 2015).

This research also aimed to identify any correlation between the host-specificity of the OTUs and their geographic distribution. Trends found across the different avian malaria OTUs was that the more generalist OTUs, infecting a broad range of hosts were almost always recorded in all five African geographic regions supporting the niche breadth hypothesis (Rivero and Gandon, 2018). However, the OTU H3, which had a large host range was not identified in the Middle African region. Considering that location of this region and that all its surrounding regions had recorded H3's presence, it is highly likely that it would be present there. This highlights the importance of conducting studies in this region. When looking at the host specific OTUs, none of these OTUs were distributed across all five African geographic regions. This means that their host-specificity is limiting their dispersal and likely causing geographic isolation. However, with the limited information available for the vectors of these OTUs, it would be highly beneficial to increase focus on studying the vectors as well to determine how big a role the vectors have on limiting the dispersal of these OTUs. The most host-specific OTU, H4, which was found in only one host species, was also only found in one geographic region (Northern Africa).

The geographic distribution and diversity of avian malaria OTUs seem to also correlate to the distribution and intensity of studies conducted in Africa. Further research in understudied regions, especially those with a high diversity of birds could reveal further diversity of malaria parasites. This could also aid in bettering our understanding of the host ranges of these parasites by increasing the number of host species examined.

4.5 Conclusion

Although the OTUs for each of the three malaria genera vary in degrees of host-specificity, *Plasmodium* parasites tend to be more generalist, while *Haemoproteus* parasites tend to be more host-specific. Another trait identified through this research was that OTUs that showed generalistic characteristics also were distributed throughout Africa, while host-specific OTUs were not. Going forward, research should look into the relationship between the distribution of the avian hosts and vectors of these OTUs to determine to what extent they play on malaria distributions. My research also highlights the need to focus research on *Leucocytozoon* which had too little data to draw true conclusions on the host ranges of these OTUs. My research highlights the need for research outside of Africa's research hotspots.

CHAPTER 5

INCONGRUENT AVIAN MALARIA PATTERNS IN FOUR SPECIES OF SYMPATRIC FOREST BIRDS

Abstract

Climate-driven habitat fragmentation and loss can alter the distribution of species and cause declines in populations, which in turn can lead to lowered genetic variation, population fitness, and evolutionary potential. South Africa's forests are naturally fragmented but are becoming less connected due to human activity. Here I test for avian malaria (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) prevalence in four forest bird species (*Camaroptera brachyura*, *Cossypha dichroa*, *Phylloscopus ruficapilla* and *Pogonocichla stellata*) collected from forest patches along the south-eastern coast of South Africa. A total of 460 birds were screened by PCR methods using genus-specific primers that amplified the mitochondrial cytochrome *b* gene region of malarial parasites. The prevalence of avian malaria among sampling sites ranged from 4-40 %, with sites further inland exhibiting higher levels of avian malaria than those closer to the coast. Three of the host/bird species had similar levels of avian malaria parasite infection (10-12%). However, in the fourth host/bird species, *C. dichroa*, the prevalence of these parasites was substantially higher (56 %), especially for *Haemoproteus* (47 %). My research indicates that although geography does play a role in parasite prevalence, unique life history characteristics of either host, vector or parasite may also play an important role in parasite prevalence.

Keywords: *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, host-specificity, distribution

5.1 Introduction

Human driven climate change and habitat loss are among the main threats to biodiversity (Thomas et al., 2004). Urbanization or land use change can often result in a reduction in habitat size, an increase in habitat fragmentation, a decrease in the size of these habitat fragments, or an increased isolation of these fragments, or any combination of these mentioned processes (de Lima Filho et al., 2021; Fahrig, 2003).

How biodiversity responds to this fragmentation, varies greatly. In general, habitat fragmentation has negative consequences for biodiversity, with fragmented habitats supporting less species richness which can affect the functionality of ecosystems (Haddad et al., 2015; Hill and Caswell, 1999; Thompson et al., 2017). In contrast, fragmentation may have no effect or may actually promote biodiversity, especially with lower levels of habitat fragmentation (Chisholm et al., 2018; Fletcher et al., 2018; Rösch et al., 2015). The life history characteristics of species could determine the impact fragmentation has on them. It has been found that generalist species usually thrive while specialist species suffer under fragmentation (Pfeifer et al., 2017). The effect that habitat fragmentation has on local species has shown to be complex and difficult to predict (Fletcher et al., 2018). This makes it difficult to predict impacts of fragmentation on complex interactions such as host-parasite interactions (Bonneaud et al., 2009; Mostowy and Engelstädter, 2011). For parasites that make use of a vector, this interaction becomes increasingly complex as the habitat alteration may affect host and vector differently (Loiseau et al., 2012).

Three genera of haemosporidian parasites infect birds globally: *Plasmodium*, *Haemoproteus* and *Leucocytozoon*. These haemosporidian parasites make use of arthropod vectors to transmit to their

avian hosts. The most common vectors for *Plasmodium* are members of the Culicidae family, Ceratopogonidae and Hippoboscidae for *Haemoproteus*, and Simuliidae for *Leucocytozoon* (Rivero and Gandon, 2018; Roberts et al., 2013; Valkiūnas, 2005; Valkiunas and Iezhova, 2018). Parasite species belonging to these three malarial genera fall on a spectrum of host specificity, with some members being highly host specific and others being more generalist. In general, *Haemoproteus* species tend to be more host specific towards their avian hosts, while *Plasmodium* species tend to be more generalist (Beadell et al., 2009; Doussang et al., 2021; Rivero and Gandon, 2018). Avian malaria's parasitic lifestyle, its spectrum of host specificity and complex heteroxenous lifestyle make it difficult to determine how these parasites will respond to habitat fragmentation (Cumming et al., 2013; Wood et al., 2007). The prevalence and diversity of avian malaria has been found to decrease in areas that have undergone habitat alteration (Bonneaud et al., 2009; Chasar et al., 2009; Neto et al., 2015). The magnitude of decrease depends on the extent of this habitat alteration, for example prevalence will still be higher in landscapes altered for agricultural purposes in comparison to urban development (Loiseau et al., 2010; Okanga et al., 2013b). A decrease in avian malaria prevalence has been directedly related to a decrease in vector abundance (Neto et al., 2015; Tchoumbou et al., 2020). Avian hosts in suboptimal habitat conditions may be under more stress and are more susceptible to infection (Chasar et al., 2009).

In South Africa only about 0.4% of total landmass is naturally forested and the distribution of natural forests is strongly linked to prevailing climate (Eeley et al., 1999; South African Government, 2014). Since the Pliocene, the fragmentation of the forest biome in southern Africa has been driven by periods of climate change-driven aridity and increased fire frequency (Geldenhuys, 1989; Scott and Lesch, 1997). Fragmentation has been further accentuated by human

activity, such as logging, and landscape alteration, with over 80% of the natural forest landscape lost (Kotze and Lawes, 2007; Olivier et al., 2013). Habitat fragmentation has shown to affect the genetic structure of forest adapted birds (Adams and Burg, 2015; Coetzer et al., 2019; Hermes et al., 2016; Mulvaney et al., 2021). Although research conducted within South Africa has shown that avian malaria can be affected by land use and climate (Okanga et al., 2013a, 2013b), limited research has been conducted on avian malaria within fragmented habitats in South Africa. It has been suggested that in South Africa's low lying forests, there is a high diversity of avian malaria parasites and a large number of specialist parasites in comparison to other, more climatically variable habitats in South Africa such as the fynbos (Loiseau et al., 2012).

In this study I examine the genetic structuring of avian malaria (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) in four forest bird species (*Camaroptera brachyura*, *Cossypha dichroa*, *Phylloscopus ruficapilla* and *Pogonocichla stellata*) collected from forest patches along the south-eastern coast of South Africa. I aim to identify if the fragmentation of these forests limits the distribution of avian malaria parasites in sympatric bird species.

5.2 Materials and methods

5.2.1 Sampling

Sampling was conducted at 12 sampling sites, across six major forest types in south-eastern South Africa between January 2017 and April 2019 (Figure 5.1). This sampling was conducted by Jake Mulvaney for his PhD. These forest types are the Albany Forest (Alexandria and The Island), Amatole Mistbelt Forest (Fort Fordyce, Kubusi and Pirie), Eastern Scarp Forest (Manubi), Transkei Mistbelt Forest (Baziya, Gomo and Nqadu), Pondoland Scarp Forest (Mbotyi and Oribi

Gorge) and Eastern Mistbelt Forest (Ngele). At each sampling site four sympatric bird species (green-backed camaroptera *Camaroptera brachyura*; chorister robin-chat *Cossypha dichroa*; yellow-throated woodland warbler *Phylloscopus ruficapilla*; and white-starred robin *Pogonocichla stellata*) were caught using mist nets. Blood was sampled venously with ethical clearance from Stellenbosch University (SU-ACUD16-00195). The blood was stored in ethanol. A total of 460 birds were screened for the three genera of avian malaria: 127 green-backed camaropteras, 90 chorister robin-chats, 77 yellow-throated woodland warblers and 166 white-starred robins.

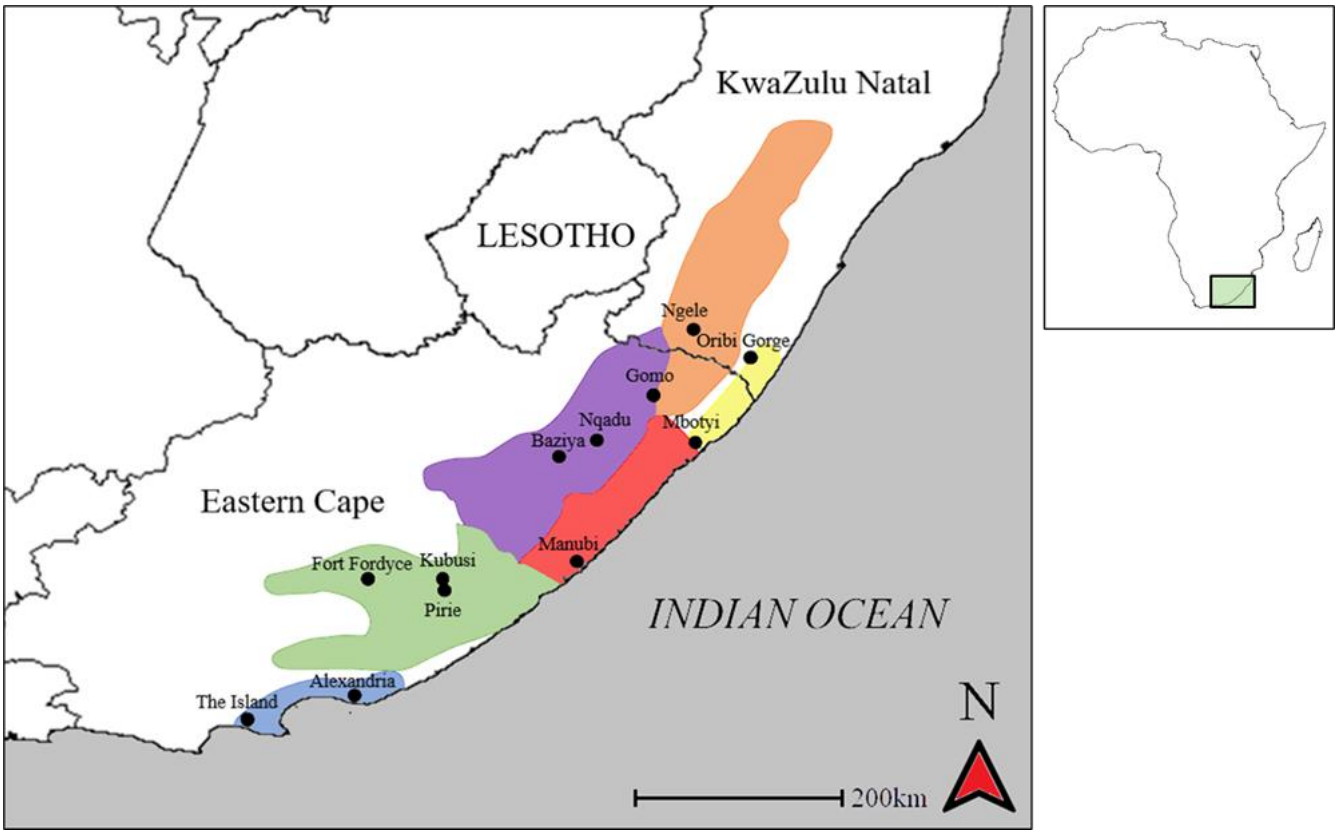


Figure 5.1. Map of eastern South Africa, indicating the sampling sites and main forest types sampled. Blue = Albany Forest, Green = Amatole Mistbelt Forest, Red = Eastern Scarp Forest, Purple = Transkei Mistbelt Forest, Yellow = Pondoland Scarp Forest, Orange = Eastern Mistbelt Forest (distribution of forest types taken from von Maltitz *et al.*, 2003).

5.2.2 DNA extraction and avian malaria screening

Genomic DNA was extracted from blood using the E.Z.N.A. Tissue DNA Kit following standard tissue protocol (Omega Bio-Tek Inc, Georgia, USA). Extracted DNA was stored at -20° C. All samples were sexed by amplifying a portion of the CHD gene using the primers CHD1F and CHD1R (Lee et al., 2010). A portion of the cytochrome *b* gene region was used to detect avian malaria. Screening followed a nested PCR method (Hellgren et al., 2004). An initial PCR was conducted using the primers HaemNFI (5'-AGACATGAAATATTATGGITAAG - 3') and HaemNR3 (5' - GAAATAAGATAA GAAATACCATTC-3'). PCR products from this initial step were used as template in the next round of PCRs. For *Plasmodium* and *Haemoproteus*, the primer pair used was HaemF (5' – ATGGTGCTTTCGATATATGCATG – 3') and HaemR2 (5' – GCATTATCTGGATGTGATAATGGT – 3'), while for *Leucocytozoon* the primer pair used was HaemFL (5' – ATGGTGTTTTAGATACTTACATT – 3') and HaemR2L (5' – CATTATCTGGATGAGATAATGGIGC – 3'). PCR reactions included 0.2 µL forward primer, 0.2 µL reverse primer, 2 µL extracted DNA/PCR product, 5 µL of OneTaq (New England BioLabs Inc), 2.4 µL H_2O and 0.2 µL Bovine serum albumin. Thermal cycler conditions for all primer sets were as follows: 2 minute denaturation at 94° C, followed by 30 cycles of denaturation for 30 seconds at 94° C, annealing for 30 seconds at 50° C and extension for 1 minute at 68° C, final 5-minute extension step at 68° C. Negative controls were included with all PCR reactions. Positive controls were included with all PCR reactions and were used to identify differences in *Plasmodium* and *Haemoproteus* infections or identify coinfections on a 2% agarose gel. Positive PCR amplicons were sent to the Central Analytical Facility at Stellenbosch University, South Africa for

Sanger sequencing using the BigDye Terminator V3.1 sequencing kit and ABI3730xl machine (Applied Biosystems).

5.2.3 Phylogenetic analyses

Sequence electropherograms were manually checked in BioEdit (Hall, 1999). Poor quality ends and primer binding sites were trimmed before sequences were BLASTed against NCBI GenBank and MalAvi to assign sequences to MalAvi lineages (Altschul et al., 1990; Bensch et al., 2009). There is evidence that these MalAvi lineages may represent individual parasite species, although some intraspecific variation may also be incorporated (Bensch et al., 2009). As a compliment to this taxonomic assignment, I also assigned sequences to operational taxonomic unit (OTUs) using the method outlined in Chapter 3. Data for each parasite genus were aligned separately using Clustal W (Thompson et al., 1994) in BioEdit. Alignments were optimized manually to ensure homology. Phylogenetic trees were constructed using the maximum likelihood and Bayesian inference optimality criteria. The best-fit substitution model was determined to be GTR+I+G for each alignment using the AIC criteria in jModelTest2 on XSEDE (Darriba et al., 2012). Maximum likelihood analyses were conducted using GARLI 2.10 on XSEDE (Zwickl, 2006). Nodal support was assessed using 1000 bootstrap replicates. MrBayes version 3.2.7a on XSEDE (Ronquist et al., 2012) was used to conduct the Bayesian inference. Two Bayesian runs were simultaneously conducted, each consisting of four Markov chains run for 25 million generations. Trees were sampled every 1000th generation. Convergence was determined in Tracer 1.7 (Rambaut et al., 2018) when Effective Sampling Size (ESS) values ≥200 for all parameters (Drummond et al., 2006). The first 20% of trees were removed as burn-in and 50% majority rule consensus trees were created in the CONSENSE module of PHYLIP 3.698 (Felsenstein, 2005). All trees were midpoint

rooted in Figtree 1.4.4 (Rambaut, 2018). Posterior probability values and bootstrap values were annotated onto the most likely trees for each parasite genus.

5.3 Results

5.3.1 Prevalence of avian malaria

In total, 460 birds were successfully sexed and screened for the three genera of avian malaria. Of these 460 birds, 20% were infected with at least one of the three avian malaria genera (Table 5.1). Overall prevalence for *Plasmodium* and *Leucocytozoon* were fairly similar with a total 4.13% and 4.35% of birds found to be infected with these parasites respectively. The prevalence for *Haemoproteus* was higher with 14.57% of birds sampled in this study were infected by this parasite genus. While 3.04% of birds sampled were infected by two genera of avian malaria parasites. The prevalence of avian malaria in male and female birds were similar with 20.24% of female birds found to be infected and 19.87% male birds infected.

Table 5.1. Number of host birds sampled per species as well as the number of males and females sampled per species. The table also shows the number of sampled birds found to be infected with at least one of the three avian malaria genera, as well as the number of samples infected with *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*.

Host species	**N**	**M/F**	**Infected**	***Plasmodium***	***Haemoproteus***	***Leucocytozoon***
Chorister robin-chat *Cossypha dichroa*	90	57/33	50	10	42	10
Green-backed camaroptera *Camaroptera brachyura*	127	80/47	14	6	9	0
White-starred robin *Pogonocichla stellata*	166	113/53	20	1	12	7
Yellow-throated woodland warbler *Phylloscopus ruficapilla*	77	47/30	8	2	4	3
Total	**460**		**92**	**19**	**67**	**20**

Of the four bird species, the chorister robin-chat had the highest infection rate with 55.56% of the birds screened being infected with avian malaria. It also had the highest prevalence for each of the avian malaria genera found in a single host species. Of the three avian malaria genera, *Haemoproteus* had the highest prevalence in the chorister robin-chats with 46.67% of birds infected. While *Plasmodium* and *Leucocytozoon* infections were recorded at much lower frequencies in this species, with an equal 11.11% of birds infected for both genera.

The white-starred robin recorded an avian malaria prevalence of 12.05%. Of the three avian malaria genera, *Haemoproteus* was the most prevalent, found in 7.23% of the robins screened. *Leucocytozoon* was the second most prevalent avian malaria genus (4.22%) while only one white-starred robin (0.60%) infected with *Plasmodium*. The green-backed camaropteras were similar to the white-starred robins in terms of overall avian malaria infection and *Haemoproteus* prevalence (11.02% and 7.09% respectively). In the green-backed camaropteras, *Plasmodium* was the second most prevalent avian malaria infections (4.72%) while *Leucocytozoon* was not detected in any of the green-backed camaropteras tested.

The yellow-throated woodland warbler recorded the lowest avian malaria prevalence with only 10.39% of birds tested being positive for one of the three parasite genera. Again, *Haemoproteus* was the most prevalent infection (5.19%). Only three yellow-throated warblers (3.90%) were infected with *Leucocytozoon* and only two (2.60%) infected with *Plasmodium*.

5.3.2 Geographic distribution of avian malaria infections

Prevalence of avian malaria was highest in the Amatole Mistbelt Forest type (28.3%), while the other forest types at similar latitudes had lower incidents of avian malaria (Figure 5.2a): Eastern Scarp Forests (18.09%) and Transkei Mistbelt Forests (17.28%), as well as the Pondoland Scarp Forests (11.76%) and Eastern Mistbelt Forests (12.12%).

The sampling site at Nqadu had the highest prevalence with 40% of the birds sampled here infected, while Gomo Forest had the lowest malaria prevalence with less than 5% of the birds sampled here infected (Figure 5.2b). The prevalence of avian malaria was not evenly distributed amongst the sites, including sites within the same forest types. For example, in the Transkei Mistbelt Forest type the Nqadu and Baziya Forest sites have very different levels of avian malaria infection (40.00% and 28.57% respectively) while Gomo Forest site had the lowest (4.65%) number of avian malaria infections. However, differences in numbers of samples collected at each site may influence the results obtained.

As seen in Figure 5.3, of the three malaria genera, *Haemoproteus* was the most common parasite infection and had the highest prevalence in ten of the twelve sampling sites (including Gomo which had an equal prevalence for *Haemoproteus* and *Leucocytozoon*). In contrast in birds sampled from Oribi Gorge, were not infected by *Haemoproteus*. While the only other sampling site which did not have *Haemoproteus* as the most abundant avian malaria genus was The Island in the Albany Forest type, which had a higher prevalence of *Plasmodium* (18.18%) and a lower prevalence for *Haemoproteus* and *Leucocytozoon* (9.09%).

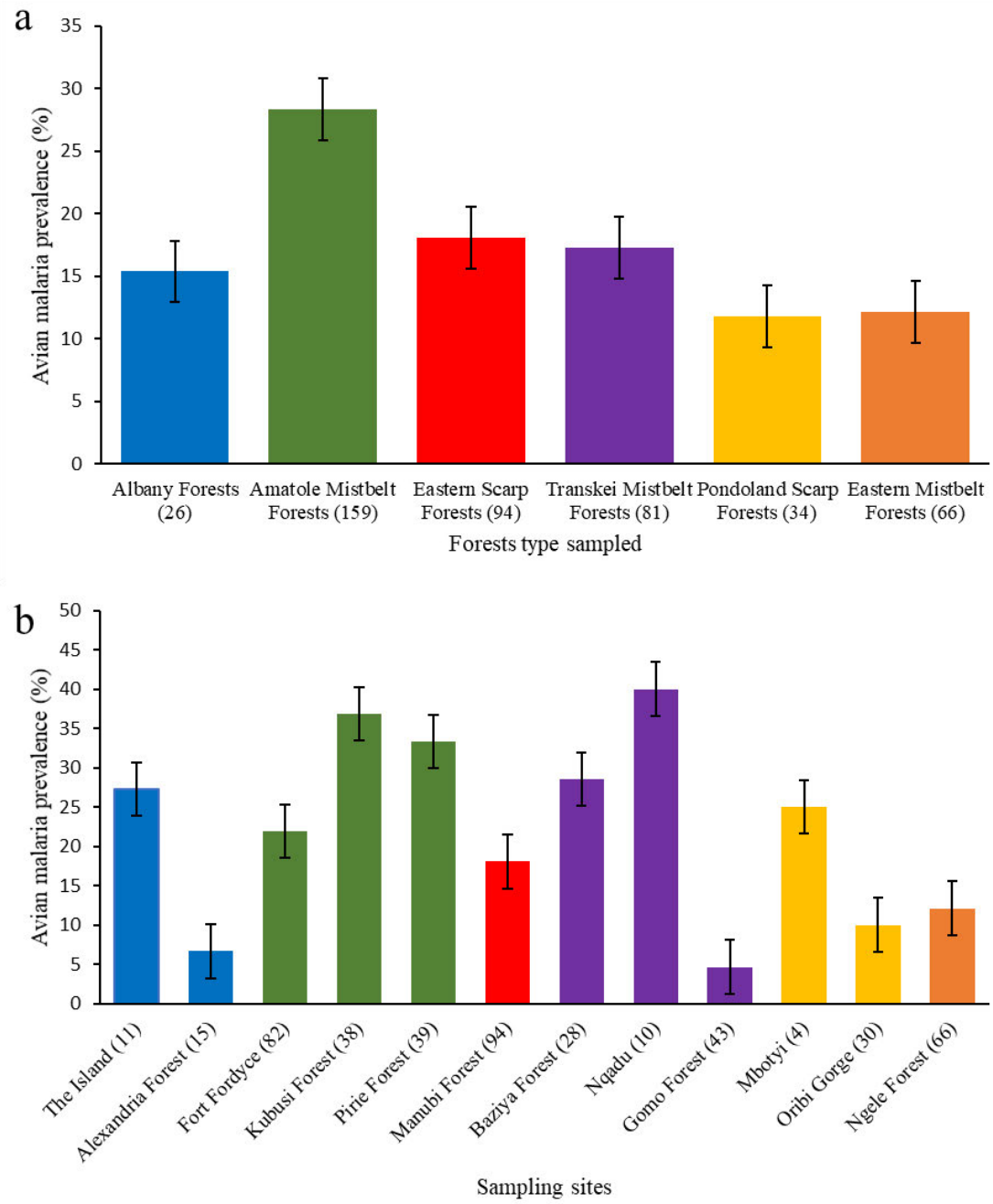


Figure 5.2. a) Prevalence of avian malaria (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon* combined) for each forest type where sampling took place, with the total number of samples collected indicated in brackets. b) Prevalence of avian malaria (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon* combined) for each sampling site, with the total number of samples collected indicated in brackets.

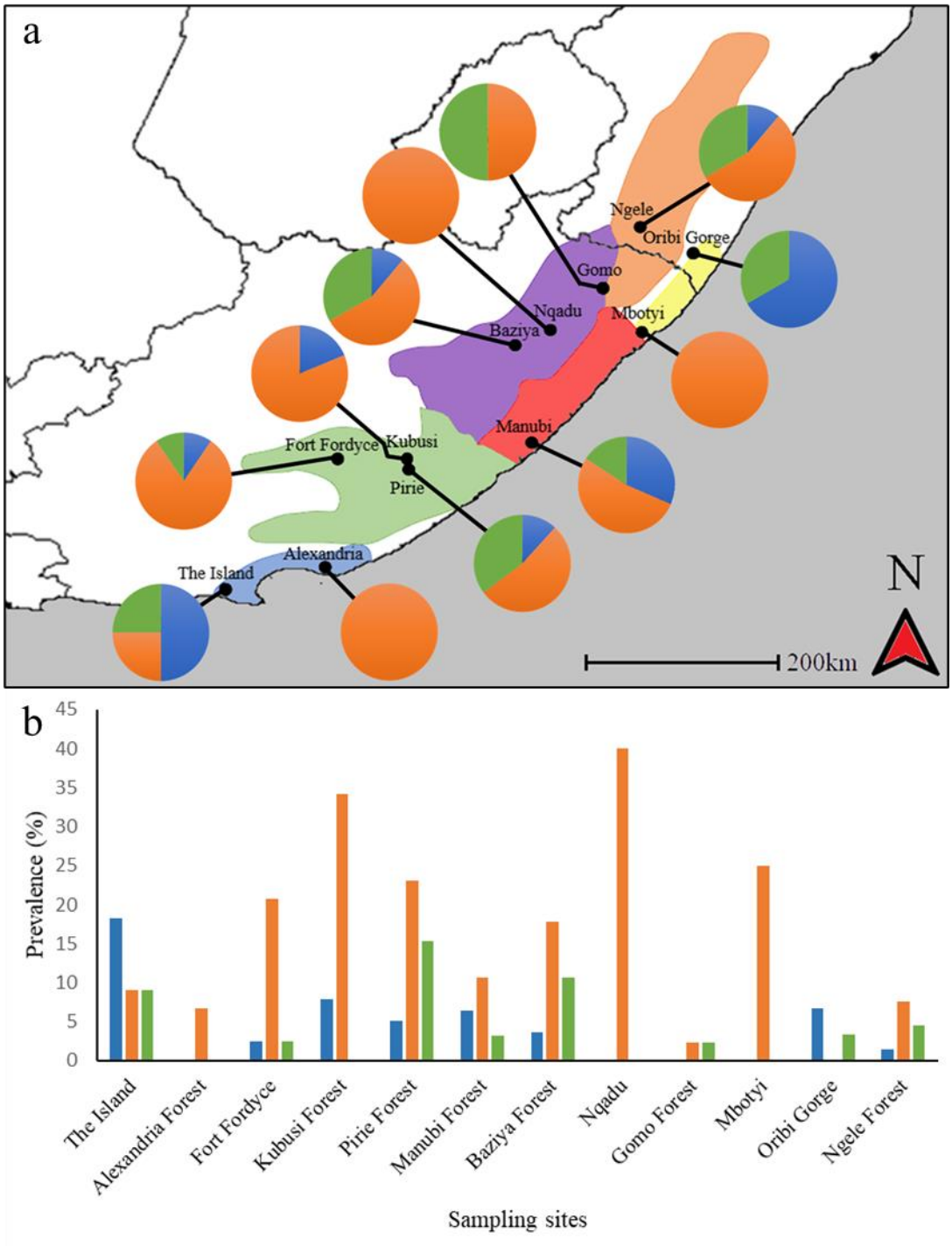


Figure 5.3. a) Map of the sampling sites and pie charts showing the proportion of each avian malaria genus recorded at each site. b) Bar graph showing the prevalence of each avian malaria genus at each sampling site. Blue = *Plasmodium*, Orange = *Haemoproteus*, Green = *Leucocytozoon*.

5.3.3 Phylogenetic analyses of avian malaria

Of the 19 *Plasmodium* infections, nine were successfully sequenced, two OTUs were identified (PI and PII; Figure 5.4). Within these two OTUs, six MalAvi lineages were identified (Figure 5.4). These lineages revealed some host specificity with CINCHA01 found exclusively within yellow-throated woodland warblers and GRW04 and GRW06 found exclusively within the green-backed camaropteras. Lineage GRW04 was the only *Plasmodium* lineage only found in a single forest type (Albany Forest), while CINCHA01 and GRW06 were found in forest types that were adjacent to one another (CINCHA01 being found in Amatole Mistbelt Forest and Eastern Scarp Forest types, GRW06 found in Eastern Scarp Forest and Transkei Mistebelt Forest types) showing some geographic correlation in the distribution of lineages.

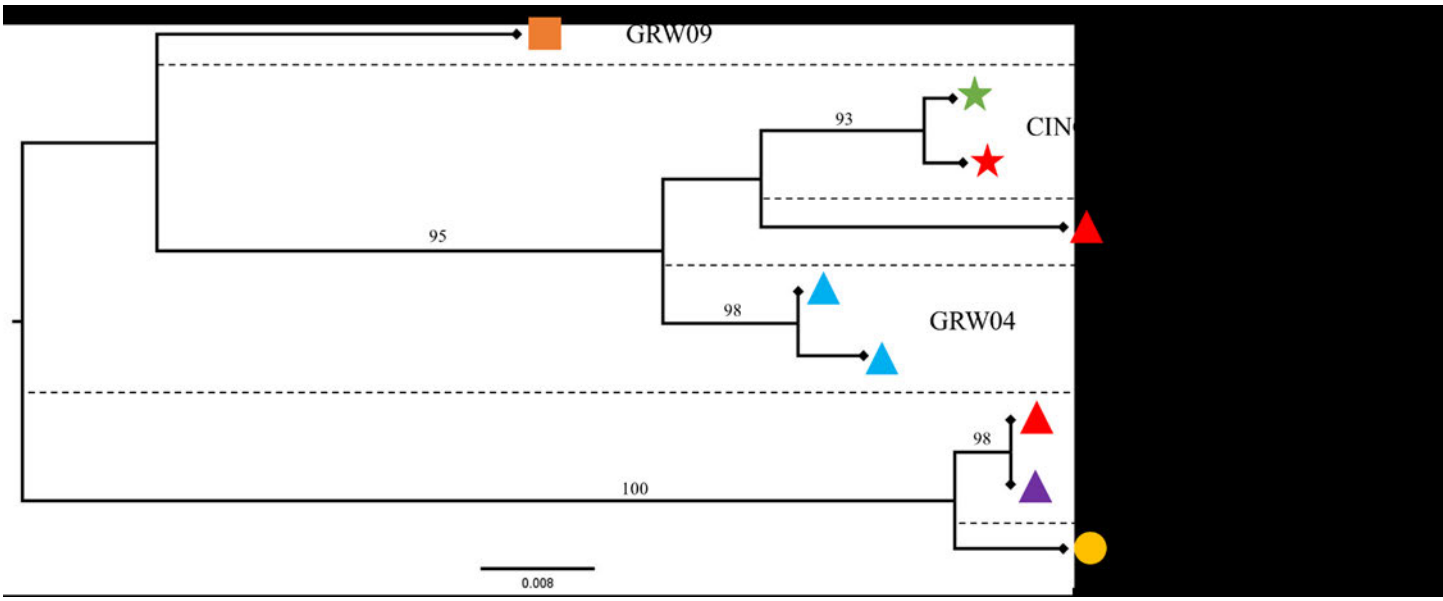


Figure 5.4. Maximum likelihood phylogeny of *Plasmodium* isolated from four sympatric forest bird species (*Camaroptera brachyura*; *Cossypha dichroa*; *Phylloscopus ruficapilla*; and *Pogonocichla stellata*). The dotted line differentiates MalAvi lineages. OTUs are labelled PI and PII. The symbols represent the different host species; Circle = chorister robin-chat, Triangle = green-backed camaroptera, Square = white-starred robin, Star = yellow-throated woodland warbler. Symbols are coloured by forest type; Blue = Albany Forest, Green = Amatole Mistbelt Forest, Red

= Eastern Scarp Forest, Purple = Transkei Mistbelt Forest, Yellow = Pondoland Scarp Forest, Orange = Eastern Mistbelt Forest. Sequence length was 503 bp.

Of the 67 *Haemoproteus* infections detected, 57 were successfully sequenced and were used to construct phylogenetic trees (Figure 5.5). Two *Haemoproteus* OTUs were identified (HI and HII) that corresponded to two MalAvi lineages (ZOSMAD01 and SFC10). The majority of the *Haemoproteus* sequences used to construct the phylogeny (Figure 5.5) were from chorister robin-chats and this host species was the most common host for both OTUs. The OTU HI occurs primarily in the chorister robin-chats but was also found within white-starred robins. Both chorister robin-chats and white-starred robins belong to the same avian family (Muscicapidae), and this could be an example of host specificity at a family level. The OTU HII was again predominantly found in chorister robin-chats and white-starred robins. However, HII was also found in green-backed camaropteras and yellow-throated woodland warblers, making this the only OTU to be found within all four host species. Geographically, the OTU HI was found in three forest types (Amatole Mistbelt Forest, Eastern Scarp Forest and Transkei Mistbelt Forest) that were in close proximity to each other suggesting some geographic signal. In contrast, HII was the only avian malaria OTU that was found across all six forest types.

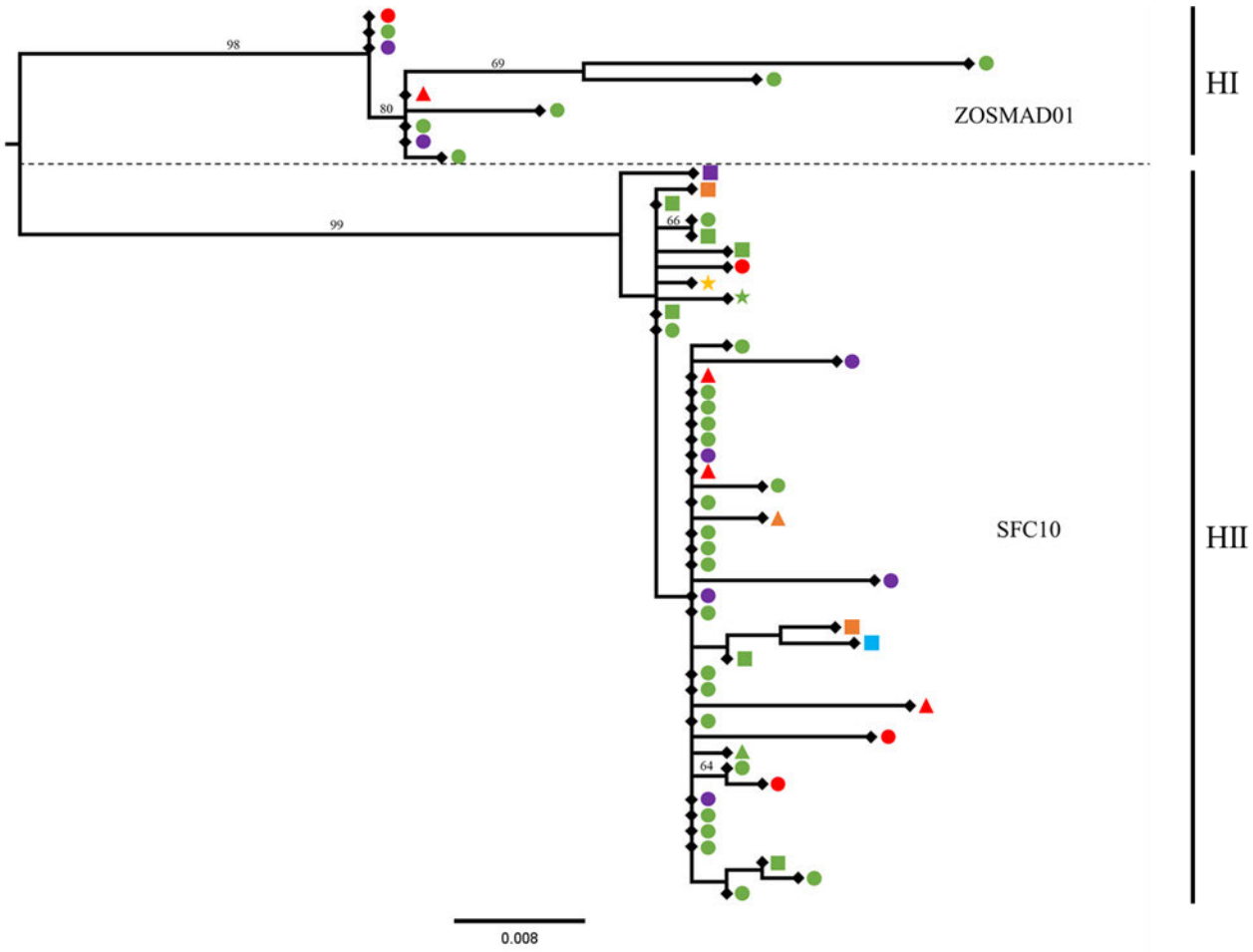


Figure 5.5. Maximum likelihood phylogeny of *Haemoproteus* from four sympatric forest bird species (*Camaroptera brachyura*; *Cossypha dichroa*; *Phylloscopus ruficapilla*; and *Pogonocichla stellata*). The dotted line differentiates MalAvi lineages. OTUs are labelled HI and HII. The symbols represent the different host species; Circle = chorister robin-chat, Triangle = green-backed camaroptera, Square = white-starred robin, Star = yellow-throated woodland warbler. Symbols are coloured by forest type; Blue = Albany Forest, Green = Amatole Mistbelt Forest, Red = Eastern Scarp Forest, Purple = Transkei Mistbelt Forest, Yellow = Pondoland Scarp Forest, Orange = Eastern Mistbelt Forest. Sequence length was 523 bp.

Of the 20 *Leucocytozoon* infections reported, 11 were successfully sequenced and were used to construct the phylogenetic tree shown in Figure 5.6. A total of four *Leucocytozoon* OTUs were identified (LI – LIV), while six MalAvi lineages were identified (CYAOLI16, SYBOR08, RS4,

SYBOR23, ANLAT16 and WW6). The OTU LIII (MalAvi lineage ANLAT16) was only found in chorister robin-chats, and CYAOLI16 was found exclusively in yellow-throated woodland warblers. OTU LIV (MalAvi lineage WW6) and RS4 were found in hosts belonging to the family Muscicapidae.

There was geographic pattern present within these parasites, with LIV and lineage RS4 (in LI) found only in a single forest type and LIII and lineage CYAOLI16 (in LI) shared between adjacent forests. Of the four *Leucocytozoon* OTUs, LI was the most abundant, found across four of the six forest types and found in three of the four host species screened.

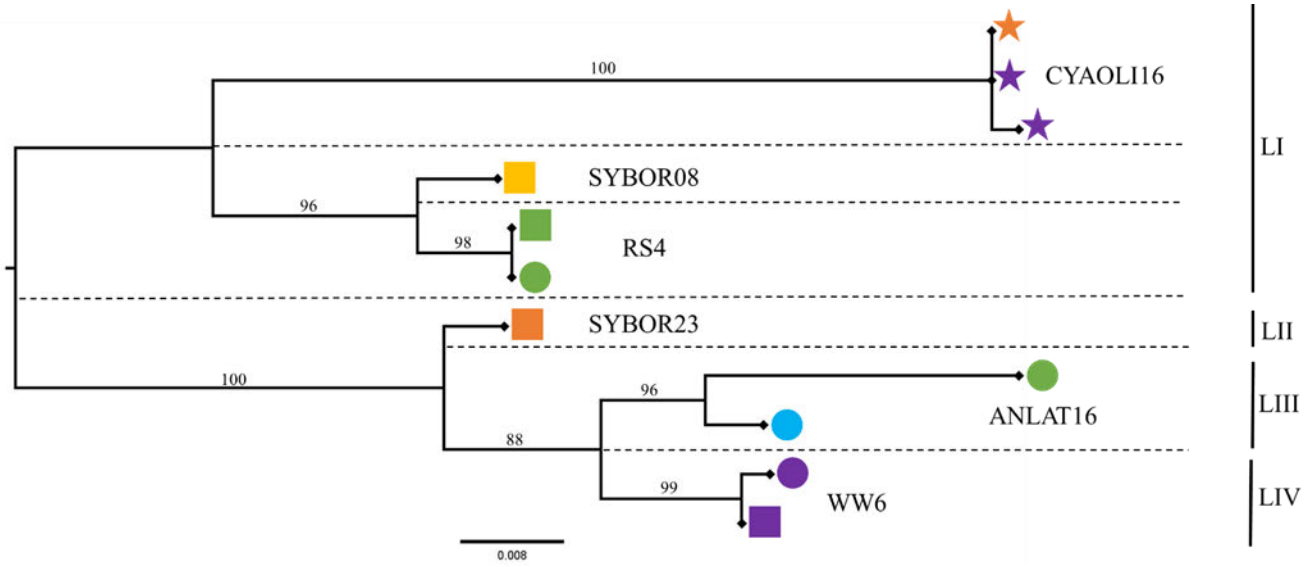


Figure 5.6. Maximum likelihood phylogeny of *Leucocytozoon* from four sympatric forest bird species (*Camaroptera brachyura*; *Cossypha dichroa*; *Phylloscopus ruficapilla*; and *Pogonocichla stellata*). The dotted line differentiates MalAvi lineages. OTUs are labelled LI – LIV. The symbols represent the different host species; Circle = chorister robin-chat, Triangle = green-backed camaroptera, Square = white-starred robin, Star = yellow-throated woodland warbler. Symbols are coloured by forest type; Blue = Albany Forest, Green = Amatole Mistbelt Forest, Red = Eastern Scarp Forest, Purple = Transkei Mistbelt Forest, Yellow = Pondoland Scarp Forest, Orange = Eastern Mistbelt Forest. Sequence length was 571 bp.

5.4 Discussion

Although previous studies (Jones et al., 2018; Nebel et al., 2020; Okanga et al., 2013a, 2013b; Schultz and Whittington, 2005) have provided information on the prevalence of avian malaria in South Africa, limited data is available for birds distributed across South Africa's low-lying forests. Studies suggest that the forest biome supports a high diversity of avian malaria parasites and a large number of specialist parasites in comparison to other, more climatically variable habitats (Loiseau et al., 2012). This study examines the prevalence of avian malaria in four forest dependant bird species. All four of the bird species occur sympatrically and were collected from the same localities and have similar life history traits (diurnal, resident insectivores). Although these species showed some differences in dispersal and dependency across these forests, it was assumed that they were all exposed to similar insect vectors (Mulvaney et al., 2021). Despite this I found differences in the prevalence of avian malaria in the four species. In particular the chorister robin-chats recovered a very high infection rate. This bird species made up more than half of the recorded infections and also had the highest prevalence recorded for each of the three avian malaria genera of any host. This could be related to a pronounced decline in effective population size these birds have experienced (Mulvaney et al., 2021). Even without the chorister robin-chat data, the genus *Haemoproteus* remained the most prevalent of the three parasite genera.

Unequal infection of sympatric bird species in fragmented habitats has been recorded previously (Chasar et al., 2009). Various factors may be responsible for the differences in infection rate seen across these four species of sympatric birds. Host species may be able to avoid infection through behavioural traits, making it less likely to be fed on by invertebrate vectors, while other hosts may have a higher ability to fight off avian malaria infections (Samuel et al., 2015; Scordato and

Kardish, 2014). Migration patterns and high altitudes can also play a role in the prevalence of these parasites (Samuel et al., 2015; Sorensen et al., 2019). Migration to high altitude habitats tends to result in lower prevalence of parasites in avian hosts (Samuel et al., 2015; Sorensen et al., 2019). The results of our study do not support this as the chorister robin-chats was the most frequently infected and does migrate to local high elevation areas over winter. However, research conducted by Mulvaney et al. (2021) showed that these birds may not be as mobile in recent years. Co-infections may also result in higher prevalence of these parasites, however only 12 of the 50 infected chorister robin-chats had co-infections (Dimitrov et al., 2015). For further clarity on the results obtained in this study, focus should also be placed the vectors themselves, to reveal if there is any host preference shown by the vectors or variations in presence at different sampling sites (Scordato and Kardish, 2014).

Each genus of avian malaria had a single OTU that was the most abundant, (in this study PI, HII and LI). These three OTUs were all somewhat generalist, infecting three (PI and LI) or all four host species (HII). The OTUs PI and LI, correspond to the OTUs P1 and L1 from Chapter 3, and were shown to be generalists in Chapter 4. While OTU HII, which corresponds to H7, showed to be somewhat host-specific towards members of the Muscicapidae family in Chapter 4 and was only reported in five African host species prior to this study. While the MalAvi lineage SFC10 had previously only been reported in *Muscicapa striata*, a member of the family Muscicapidae (Drovetski et al., 2014; Mata et al., 2015). The four bird species screened in this chapter are all novel African host species for OTU H7 and MalAvi lineage SFC10, as well as the first report of its presence in southern Africa. Although HII was more frequently found in Muscicapidae hosts

(chorister robin-chats and white-starred robins) it does provide new information on the host-specificity and geographic distribution of one of Africa's *Haemoproteus* OTUs.

OTUs PI, HII and LI were also found across the most forest types. Other OTUs showed a degree of host specificity and were somewhat geographically isolated which is to be expected (Ishtiaq et al., 2010). When looking at the MalAvi lineages of these parasites, almost all the lineages were found to be somewhat host specific and somewhat geographically isolated, except for SFC10 (HII). This MalAvi lineage was the most abundant lineage across all three genera and was found in all four host species and all six forest types. I suggest that each parasites species life characteristics may play a role in the abundance and distribution of parasites. The more generalist OTUs and MalAvi lineages were always the most abundant and most geographically widespread. While the observed decline in habitat range in these fragmented forests may be the cause of geographic isolation (Mulvaney et al., 2021). Research on other, less forest dependent bird species should be conducted to identify if other avian host species facilitate the transmission of generalist avian malaria OTUs if the host species studied here are isolated (Fecchio et al., 2018).

For *Leucocytozoon,* it is very clear that studies on this genus of avian malaria are lacking. The OTU LI was the most frequently reported *Leucocytozoon* OTU in Africa (L1) and still there were two new host species identified in this research for this OTU. These species were chorister robin-chat and white-starred robin. The *Leucocytozoon* OTUs LII (L7), LIII (L15), and LIV (L17) had never been reported in southern Africa before, making these the first reported cases for this region for these OTUs. The host species these OTUs were found in were also novel host species and for L15 the Muscicapidae family was a novel host family. Of the MalAvi lineages identified from

OTUs LII, LIII and LIV, SYBOR23 had only been reported in one other species, ANLAT16 had been reported in two species and WW6 had been recorded in three species (Drovetski et al., 2014; Hellgren et al., 2007b; Lutz et al., 2015; Valkiunas et al., 2009). These results clearly shown for *Leucocytozoon* as well as *Haemoproteus* and *Plasmodium* that there is still much we do not know about these parasites in Africa in terms of their host range and distribution. However, WW6 has been reported in the genus *Phylloscopus* previously (Hellgren et al., 2007b) but in this study was not reported in the yellow-throated woodland warbler. Avian malaria lineages have been found to be host specific towards avian families, so it is peculiar as to why this lineage was not found in a host species from a genus already reported to be a host (Valkiūnas, 2005).

In this study I delimited species using both the gap method outlined in Chapter 3 and the method used by MalAvi. For most of the more specialist OTUs, there was a close match between OTU and MalAvi lineage with the exception of PII, which had two (GRW06 and PLACAS02). While MalAvi lineages such as CINCHA01 and GRW04 (within PI) and CYAOLI16 and RS4 (within LI) also showed host specificity and geographic isolation. But these lineages were found to be associated with more generalist and geographically dispersed OTUs. This could reveal that the fragmentation of these forests is limiting the dispersal of even the more generalist and easily dispersed OTUs. Mulvaney et al. (2021) showed how the dispersal of the host species studied is being limited, but these parasites may use other host species (Ellis et al., 2015). Research should be conducted on other avian host species as well as vectors to get a better understanding of the low dispersal observed in this study.

The prevalence of avian malaria differed among the different forest types and also among the different sampling sites within the same forest type. These results showed that avian malaria prevalence was highest in the Amatole Mistbelt Forest type (28.3%). There was no geographic pattern to avian malaria prevalence observed. Even within the same forest type, the number of reported infections was often quite different. For example, Nqadu had an avian malaria prevalence of 40.00% and Gomo Forest only recorded a prevalence of 4.65%. The reasons for these disparities are unclear, but may include different micro-climatic conditions, differences in vector abundance or loss of connectivity to other patches of the forest (Mulvaney et al., 2021; Okanga et al., 2013a, 2013b). Subtle climatic differences may be a factor attributed to differences in the relative prevalence of the parasites, as the sampling sites further away from the coast all shared a high prevalence of *Haemoproteus*.

Geography did seem to shape the distribution of many of the MalAvi lineages and parasite species identified. Many of the lineages within each parasite genera seemed to be isolated either to a single forest type or to adjacent forest types, with the only exception being *Haemoproteus* lineage SFC10 (HII) which found in all four host species and all six forest types. The phylogeographic patterns seen in this data may be responsible for variation of infection rates across the different sampling sites as the diversity of parasites present in hosts were not uniform across the different sites.

5.5 Conclusion

In this study avian malaria was shown to affect sympatric bird host species differently. The infection rates of the different parasites varied across sites and did not show a strong geographic signal, in contrast birds collected from the same forest patch or adjacent forest patches were often

infected by same species or lineage of parasite. I did find a few generalist OTUs/lineages that were abundant and geographically most widely distributed, but most of the parasite species I recorded in this study were geographically isolated. The role of geography does seem to play a role in the distribution of these parasites and additional research on vector abundance would be vital to fully unpack the patterns.

CHAPTER 6

CONCLUSIONS

6.1 Avian malaria in Africa

Plasmodium, *Haemoproteus* and *Leucocytozoon* have been reported and studied in birds across the world. Research on these parasites has traditionally been primarily based on microscopy, however the introduction of molecular-based means of detection of these parasites has allowed for an increase in research on avian malaria. This increase in research has led to the construction of MalAvi, a database for molecular avian malaria research across the globe. The centralization of this information allows for continental analyses. Although there has been an increase in research, less research is conducted in Africa in comparison to other continents. By reviewing all research conducted in Africa, we can obtain a better understanding of avian malaria in Africa, including diversity and distribution of these parasites, as well as the host species that are frequently infected. But reviewing all gathered information will also highlight key knowledge gaps that should be the focus of future avian malaria research in Africa.

6.2 Avian malaria studies in Africa

Compiling all published molecular research on avian malaria conducted in Africa reveals that since the development of molecular-based diagnostics, there has been an acceleration in avian malaria research. However, this research has not been evenly distributed across the continent. Most of the research is conducted in "research hotspots" such as the Gulf of Guinea and Southern Africa. This results in cross continental examination to be heavily skewed by the research conducted in these hotspots. With 20% of all African bird species reported to be infected with these parasites, this

could be higher if research was conducted in high avian diversity regions and under studied countries. Another key issue with research conducted in Africa is the lower levels of *Leucocytozoon* research. However, there is an increase in the tendency to study all three genera (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) in single studies, which may help address this issue.

6.3 Delimiting avian malaria species

Using the compiled research on avian malaria in Africa, I was able to identify a "gap" between inter- and intraspecific genetic distances which I used to delimit phylogenetic species of avian malaria. This did reveal that there could be 18 *Plasmodium* species, 19 *Haemoproteus* species and 19 *Leucocytozoon* species found in Africa, although these are likely underestimates of the true number of malaria species present. This study did highlight some knowledge gaps. First, there was very little information on *Leucocytozoon* parasites, with only one morphological *Leucocytozoon* species associated to a phylogenetic species. Other issues identified was that several morphological species for avian malaria were found in single phylogenetic species. The best way to address these issues is a combination of traditional morphology-based methods and new molecular based methods to identify possible misidentifications, synonymous morphological species and enhance our knowledge on the species delimitation threshold. Using a marriage of these two methods, future research should also focus on broader host ranges and be conducted in understudied geographic regions.

6.4 Geographic distribution and host range of avian malaria species

Using the phylogenetic species delimited in Chapter 3, and the additional information compiled from all avian malaria research conducted in Africa, I could identify the geographic spread and host range of phylogenetic parasite species. These identified species varied in degrees of host-specificity. In general, *Plasmodium* parasites tend to be more generalist, while *Haemoproteus* parasites tend to be more host-specific. The species that were found to be more generalist also tend to be distributed throughout Africa while host-specific species were geographically isolated. Once again there was very limited information available on *Leucocytozoon* parasites, indicating the need for more focus to be placed on the parasites from this genus. But there is also very limited information on the vectors of these parasites and future research should look into the role the vectors play on the host-specific patterns observed here.

6.5 Avian malaria in fragmented forests

Within the naturally fragmented forests of south-eastern South Africa, avian malaria was found to infect bird hosts differently, with the chorister robin-chats in particular showing a susceptibility to infection, while the remaining host species had lower malaria prevalence. There were also variations in prevalence at the different sampling sites, but in general the sampling sites further from the coast all had *Haemoproteus* as the most abundant malaria parasites, indicating that climate may also be a factor. There was a variety of host-specific malaria species that had small geographic distribution, while generalist species were abundant and was found across all forest types and all four bird hosts examined. To further understand some of the observations, research should also focus on the vectors transmitting these parasites and what role they may play in prevalence.

6.6 Future research

My research shows that although more and more research is being conducted, there are still key knowledge gaps that must be addressed. In particular there are many regions of Africa that lack avian malaria research. Another focus should be on the avian malaria genus *Leucocytozoon*, which has been severely understudied in comparison to *Plasmodium* and *Haemoproteus*. Although this is being addressed more and more in current research. However current research does predominately use a molecular approach, but my findings reveal that there is a need for both molecular based and microscopy-based research, especially when it comes to species delimitation. In future research, more work should also be done on the vectors of these parasites to better understand what factors of infection are due to the vectors.

In conclusion, molecular-based research in Africa has been increasing since its inception and has been providing vital information on avian malaria in African birds. However, understudied regions within Africa and the understudied *Leucocytozoon* parasites should take particular focus to obtain a better understanding of what the true malaria diversity and host ranges of these parasites are.

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