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Phylogeny and Prevalence of Haemosporidian Parasites of Free-ranging Domestic Birds in Northwestern Uganda

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Abstract

Infection of avian malaria parasites is a general problem for both wild and domestic birds. Most infected birds exhibit suppressed immunity, poor productivity and high mortality. Despite this concern, there are limited studies on haemosporidian parasites in free-ranging domestic birds in Africa and its subregions. The aim of this study is to investigate the distribution, prevalence and diversity of haemosporidian parasites among free-ranging domestic birds in Uganda. Blood samples were collected from free-ranging chickens (n=304), ducks (n=70), turkeys (n=14), and guinea fowl (n=19) and screened for haemosporidian parasites. Microscopy and PCR approaches were used to detect and identify parasites based on morphological characteristics and a 600-900 bp amplified fragment of Cytochrome b (cyt b). We detected haemoproteus (17.25%, n=69), plasmodium (22%, n=88) and leucocytozoon (1.75%, n=7) in the sampled birds. The sequences from these genera were nested within their respective clades in a phylogenetic tree constructed using sequences from the MalAvi database. Our study showed that free-ranging domestic birds habour haemosporidian parasites in Uganda thus providing insight for more conscious management practice in poultry systems to prevent widespread infection of the parasites.

Key words: Avian Malaria, Birds, Ecosystem, Haemosporidia, Uganda

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Introduction

Over the years, high human population growth has increased urban migration and demand for food. Consequently, this has created pressure on land and food production such that farming systems which maximize yield per unit area (e.g., poultry production) have become priority. Poultry production systems are an integral part of mixed farming that involves chicken production, as well as turkeys, guinea fowls, ducks, pigeons, geese, and ostriches. Based on number, biosecurity, and management of birds, poultry practices can be categorized as commercial and free-range systems (Byarugaba, 2007). In commercial systems birds are reared in large number in an enclosure, and intensively managed; whereas in a free-range system birds are independent, poorly managed and less secured.

In Uganda, poultry farming is an important source of livelihood for the expanding population. Nevertheless, this sector currently faces daunting challenges, including weak veterinary and extension services, inoperative laws, and failed policies (Bhat *et al.*, 2014). These have led to disease outbreaks, transmission and infection, poor productivity, and high mortality of poultry birds (Soulsby, 1982; Conroy *et al.*, 2005). The role of parasites in disease induction and economic loss cannot be overemphasized. In-depth studies of the adverse effects of parasites on some avian species have been documented, and haemosporidian parasites such as *Plasmodium*, *Haemoproteus* and *Leucocytozoon* have received significant attention (Martinsen *et al.*, 2007).

Avian malaria is a parasitic disease of birds caused by protozoan parasites belonging to the genus *Plasmodium* (Lefèvre *et al.*, 2008) with close linkage to the genera *Leucocytozoon* and *Haemoproteus*. There are currently more than 50 *Plasmodium* species of birds (Valkiūnas, 2005; Valkiūnas *et al.*, 2014; Walther *et al.*, 2014) transmitted by diverse species of mosquitoes in the Culicinae and Anophelinae subfamilies (Valkiūnas, 2005; Njabo *et al.*, 2011). In contrast to human malaria which is transmitted solely by the Anophelinae subfamily of mosquitoes, avian *Plasmodium* species (spp.) is transmitted by mosquitoes in the Culicidae. *Haemoproteus* spp. is transmitted by biting midges (Ceratopogonidae) or louse flies (Hippoboscidae) (Valkiūnas, 2005) and *Leucocytozoon* spp. are transmitted by black flies (Diptera: Simuliidae). Generally, transmission of haemosporidian parasites occurs across the globe.

Haemosporidian parasites have negative impact on wild bird populations, cumulating in reduced fitness, mortality, and extinction of the host (Lefèvre *et al.*, 2008; Dinhopl *et al.*, 2015). Recently, *Coquillettidia* species have been shown to be a potential vector of avian malaria transmission among African birds (Njabo *et al.*, 2011), suggesting that our understanding of avian malaria vectors is incomplete in the tropical region.

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For example, avian haemosporidian diversity and prevalence are poorly understood in free-ranging birds in tropical Africa, especially in some regions of Uganda and East Africa. Free-ranging birds exhibit independent foraging and roosting habits, a behavioral pattern that keeps them highly vulnerable and predisposed to diseases and parasites coexisting in the ecosystem (Malatji *et al.,* 2016). Moreover, free-ranging birds serve as link between poultry and wild bird populations. Uganda is a major migratory route and stopover site on migration pathways for most Palearctic migrants; therefore, coalescence between migratory, resident, free-ranging, and poultry populations of birds hold potential for shared transmission, exchange, and introduction of new pathogens across the avifauna communities within the Ugandan ecosystem (Kirby *et al*., 2008).

Studies have shown that the distribution and heterogeneity of haemosporidian parasites depends on host-parasite compatibility (Ricklefs, 2010; Knowles *et al.*, 2011), vector-parasite compatibility (Carlson *et al.,* 2015) and mosquito feeding behavior (Medeiros *et al.,* 2015). Available data shows that the majority of malaria parasite species have a broad range of avian hosts (Medeiros *et al.*, 2013); therefore, detecting and screening parasites across this host continuum requires accurate techniques. With the recent development in molecular diagnostics for haemosporidian parasites, limitations inherent in microscopy-based approaches for identifying haemosporidian parasites have been addressed. Molecular approach has advantages in the effective characterization and detection of parasitemia (Bensch *et al.,* 2000a; Waldenström *et al.*, 2004). However, some studies of avian malaria in wild birds have suggested the parallel application of both microscopy- and molecular-based techniques (Valkiūnas *et al.,* 2008; Garamszegi, 2010). Molecular-based identification of avian haemosporidia often uses mitochondrial genes (mtDNA), and more genomic markers have recently been explored in the phylogeny of avian malaria parasites and related haemosporidia (Braga *et al.,* 2011). The results showed high genetic diversity among malaria parasites and haemosporidia lineages (Martinsen *et al.*, 2008). Currently, there are several DNA sequences for haemosporidian parasites deposited in GenBank or related databases that can facilitate studies aimed at the phylogeographical comparison of the diversity and prevalence of these parasites. Herein, we aimed to investigate the distribution, prevalence and diversity of haemosporidian parasites in free-ranging domestic birds in Uganda, East Africa.

Materials and Methods

Compliance with Ethical Standards

Sampling of the birds was done with verbal consent from the flock owners who were informed about the purpose of the project. Clearance was obtained from the Science committee of National Livestock Resources Research Institute (NaLIRRI) Uganda.

 DOI 10.5455/ijlr.20191104070146

Blood Sample Collection

The present study was carried out in Northwestern Uganda across four districts: Adjumani (03° 37′ N, 31° 78′ E), Moyo (03° 65′ N, 31° 72′ E), Yumbe (03° 46′ N, 31° 24′ E), and Koboko (03° 41′ N, 30° 95′ E). Blood samples were collected from free-ranging chickens (*Gallus domesticus*), ducks (*Anas domesticus*), turkeys (*Meleagris gallopavo*) and guinea fowl (*Numida meleagris*) via the brachial vein (venipuncture). Approximately 10–50 μL of blood was collected and preserved in EDTA-containing vacutainers and stored in a cool box prior to analysis. Blood smears were prepared on microscopic slides and fixed in 10% formalin. Slides were carefully stored in slide boxes and taken to the parasitology laboratory at the College of Veterinary Medicine, Animal Resources, and Biosecurity, Makerere University (Kampala, Uganda), where they were later Giemsa stained. Microscopic detection of avian haemosporidian parasites was completed thereafter.

Polymerase Chain Reaction (PCR)

Total DNA was extracted from bird blood samples using a DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol. Extracted DNA was subjected to nested PCR amplification of a 600-900 bp cytochrome b (*cyt b*) gene region of Mitochondrial DNA (mtDNA). Primary amplification used two primers, DW2-Forward (5'-TAATGCCTAGACGTATTCCTGATTATCCAG-3') and DW4- Reverse (5'-TGTTTGCTTGGGAGCTGTAATCATAATGTG-3'), which were designed as previously described by Perkins and Schall (2002). Secondary primers previously designed in conserved regions flanking *cyt b* based on recent studies of avian *Leucocytozoon* spp., *Plasmodium* spp., and *Haemoproteus* spp. Primers were denoted as LCytb-Forward (5'-CAAATTCTTACTGGTGTATTATTAGC-3') and LCytb-Reverse (5'-ATAATAGATAATGAATAATCTCTTGG-3') (Sato *et al*., 2009), APF-Forward (5'CTTATGGAATTATGGATTTCTTTTAGG-3') and APRN-Reverse (5'-ATAATAAAGCATAGAATGAACATATAAACC-3') (Ejiri *et al*., 2009), and L15183-Forward (5'-GTGCAACYGTTATTACTAATTTATA-3') and H15730-Reverse (5'-CATCCAATCCATAATAAAGCAT-3') (Ishak *et al*., 2008) for *Leucocytozoon*, *Plasmodium* and *Haemoproteus* respectively. PCRs were run in 25 μL total volumes containing 12.5 μL of Taq PCR master mix, 1 μL (10 mM) each of forward and reverse primers, 8.5 μL of distilled water, and 2 μL of extracted DNA. For each PCR run, one negative control was routinely used to detect false amplification since nested PCR is highly sensitive. The cycling conditions for amplification started with an initial denaturation for 10 min at 95 °C followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s, and 72 °C for 5 min of final extension. Amplification products were detected by gel electrophoresis using 5 μL of the final PCR products on a 2.0% agarose gel followed by visualization of the ethidium bromide-stained DNA under ultraviolet light.

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DNA Sequencing and Phylogenetic Analysis

All successfully amplified samples were prepared for sequencing. These samples were selected randomly basing on positive microscopy results. The PCR products were purified using a QiAquick PCR Purification kit (Qiagen), sequenced directly from the primed 5'-end using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), and loaded into an ABI Prism 3100 automated sequencer (Applied Biosystems). All sequences were proofread and trimmed in CodonCode Aligner version 7. The new sequences were submitted to GenBank and assigned accession numbers (MF547704–MF547715).

To distinguish among the three investigated parasite genera, sequences from the present study were combined with those downloaded from the MalAvi database (Bensch *et al*., 2009) for species of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*. The detailed information of downloaded sequences is available in the MalAvi database website (http://mbio-serv2.mbioekol.lu.se/Malavi/). Sequences were aligned with ClustalW using MEGA version 7.0.26 (Tamura *et al.*, 2007; Kumar *et al.*, 2016). The phylogenetic relationships among parasite lineages were estimated using Bayesian inference and maximum likelihood methods. Phylogenetic trees were constructed and rooted using a mammalian *Plasmodium falciparum* as the out group. We applied the GTR+G+I best fitting model of DNA substitution based on Akaike Information Criteria corrected for small sample size in jModelTest (Posada, 2008). Bayesian inference was performed using MrBayes version 3.1.2. Two independent Markov Chain Monte Carlo were run for five million generations sampling every 100 generations. Convergence diagnostics were assessed, and 25% of generations were removed as burnin by visual examination of the plots and estimates of effective sample size (ESS>420) using Tracer version 1.5 (Drummond and Rambaut, 2007). Tree Annotator was used to summarize remaining evenly sampled trees as a posterior distribution from which a maximum clade credibility tree using median node height was generated.

To identify the lineages of parasite sequences, the placement of our sequences was determined relative to those downloaded from the MalAvi database. Congruence and support for placement and sister taxon cluster were assessed using the posterior probability of the Bayesian inference tree. We also compared the bootstrap support values of the maximum likelihood tree constructed in MEGA7 (Kumar *et al.*, 2016) at 1000 iterative replicates using the same model of DNA substitution from jModelTest (Palinauskas *et al*., 2011). Maximum likelihood obtained an initial tree for the heuristic search by applying BioNJ and Neighbor-Joining algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and selecting the appropriate topology based on log likelihood values. The support values are shown for the maximum clade credibility trees obtained from both methods.

Results and Discussion

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Microscopic Detection of Avian Haemosporidian Parasites

A total of 407 bird samples comprised of 304 chickens, 70 ducks, 14 turkeys, and 19 guinea fowl were screened for haemosporidian parasites. Of these, we detected 69 *Haemoproteus* spp., 7 *Leucocytozoon* spp., and 88 *Plasmodium* spp. across the sampled bird species based on direct microscopy of Giemsa-stained blood smears (Fig. 1; Table 1). Domestic chicken were found infected with all three avian haemosporidian parasites, 3.3% *Haemoproteus* spp., 2.3% *Leucocytozoon* spp., and 21.7% *Plasmodium* spp.. Duck, turkey, and guinea fowl were only found infected with *Haemoproteus* spp. and *Plasmodium* spp.; duck samples were 62.9% *Haemoproteus* spp. and 25.7% *Plasmodium* spp., turkey samples were 50% *Haemoproteus* spp. and 21% *Plasmodium* spp., and guinea fowl samples were 42% *Haemoproteus* spp. and 5.3% *Plasmodium* spp. In general, avian haemosporidian parasite infection rate for chicken, duck, turkey, and guinea fowl were 27%, 61%, 71%, and 47%, respectively (Table 1). Mixed infections of *Haemoproteus* spp. and *Plasmodium* spp. was detected in chicken (*n*=81), duck (*n*=43), turkey (*n*=10) and guinea fowl (*n*=9). There were seven positive cases of single *Leucocytozoon* infection in chicken.

Table 1: Microscopic detection of haemosporidian parasites in birds and percentage infection of birds from Northwestern Uganda

Birds	***n***	***Haemoproteus***	***Leucocytozoon***	***Plasmodium***	**Total (%)**
Chicken	304	10	7	66	26.6
Duck	70	44	0	18	61.4
Turkey	14	7	0	3	71.4
Guinea fowl	19	8	0	1	47.4
Total	497	69	7	88	

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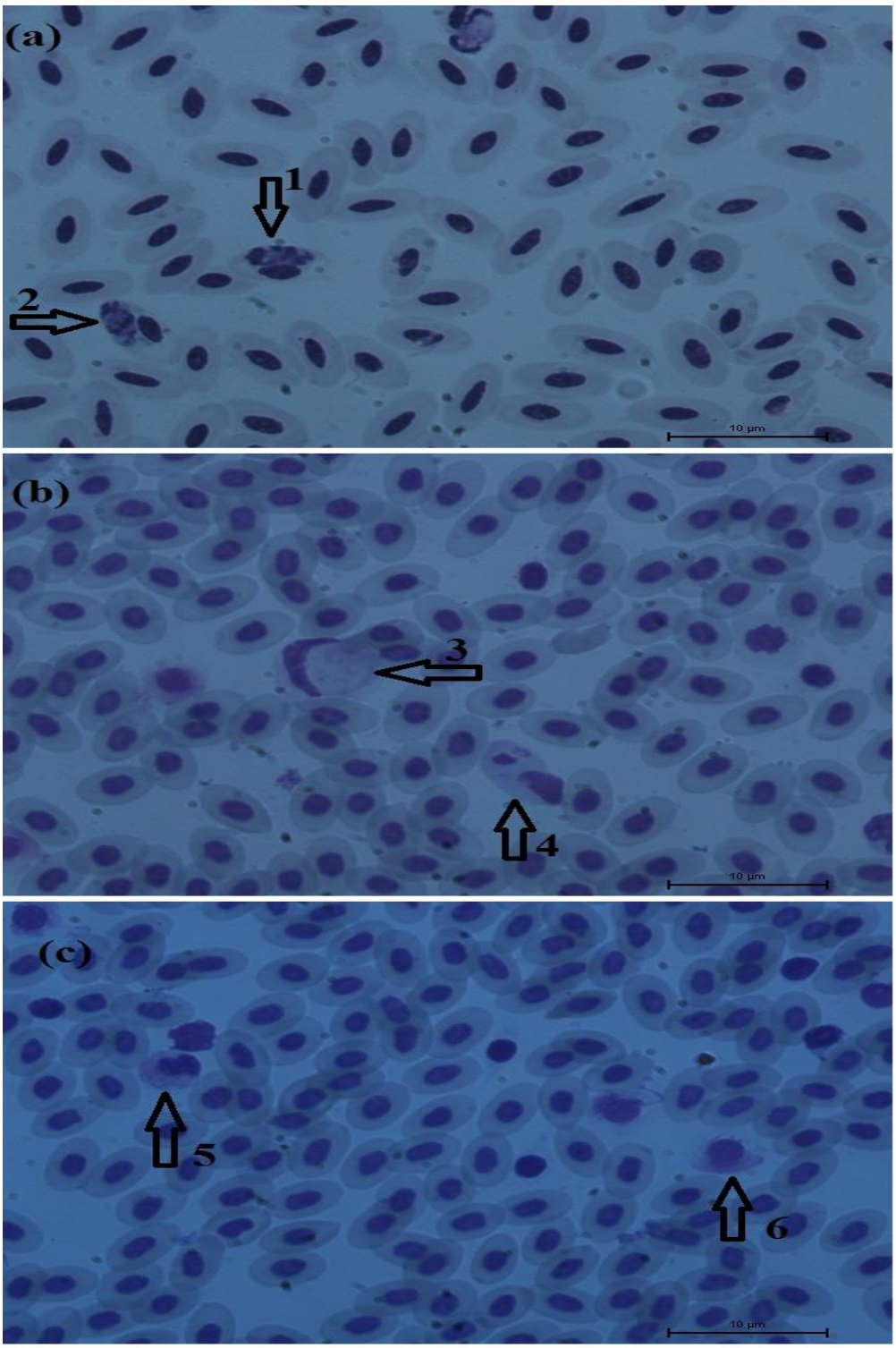

Fig. 1: Microscopy detection of haemosporidian parasites for *Haemoproteus* spp. (a1-2); *Leucocytozoon* spp. (b3-4), and *Plasmodium spp.* (c5-6). Arrows point to *Haemoproteus* sp. gamonts (1000x); *Leucocytozoon* sp. gamonts in an erythrocyte (1000x), the nucleus of the host cell is peripheral; *Plasmodium* sp. schizont and macrogametocyte (1000x) from Giemsa-stained thin blood films. The scale bars in a, b and c is of length 10 µm

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Phylogenetic Analysis and Position of Parasites

We used 54 sequences (12 from this study, 41 from MalAvi, and outgroup from Genbank; and constructed phylogenetic trees using Bayesian inference (Fig. 2) and maximum likelihood (Fig. supplementary 1). The Bayesian inference tree showed the placement of sequences from the three avian Haemosporidia genera (*Haemoproteus, Plasmodium, and Leucocytozoon*) from the current study (Fig. 2) and were nested near their sister taxon from the MalAvi database. The nodes showed reasonable support for this sister taxon cluster, posterior probability values were greater than 50. The eight *Haemoproteus* spp. sequences formed a cluster near *Haemoproteus majoris*. The *Plasmodium* sequences were nested within the *Plasmodium* lineages from MalAvi, however, two of the three sequences formed a sister cluster with *Plasmodium gallinaceum*, while the other sequence was placed at the base of the clade comprised of *P. multivauolaris*, *P. homopolare*, *P. vaughani*, *P. juxtanucleare*, *P. heteronuclear* and *P. rouxi*. The only sequence from *Leucocytozoon* spp. (MF547715) was placed at the basal position of the *Haemoproteus*, between *Haemoproteus* and *Leucocytozoon* genera. The maximum likelihood tree showed a topology similar to the Bayesian inference tree, but some clades were poorly supported at some nodes compared to the Bayesian inference tree.

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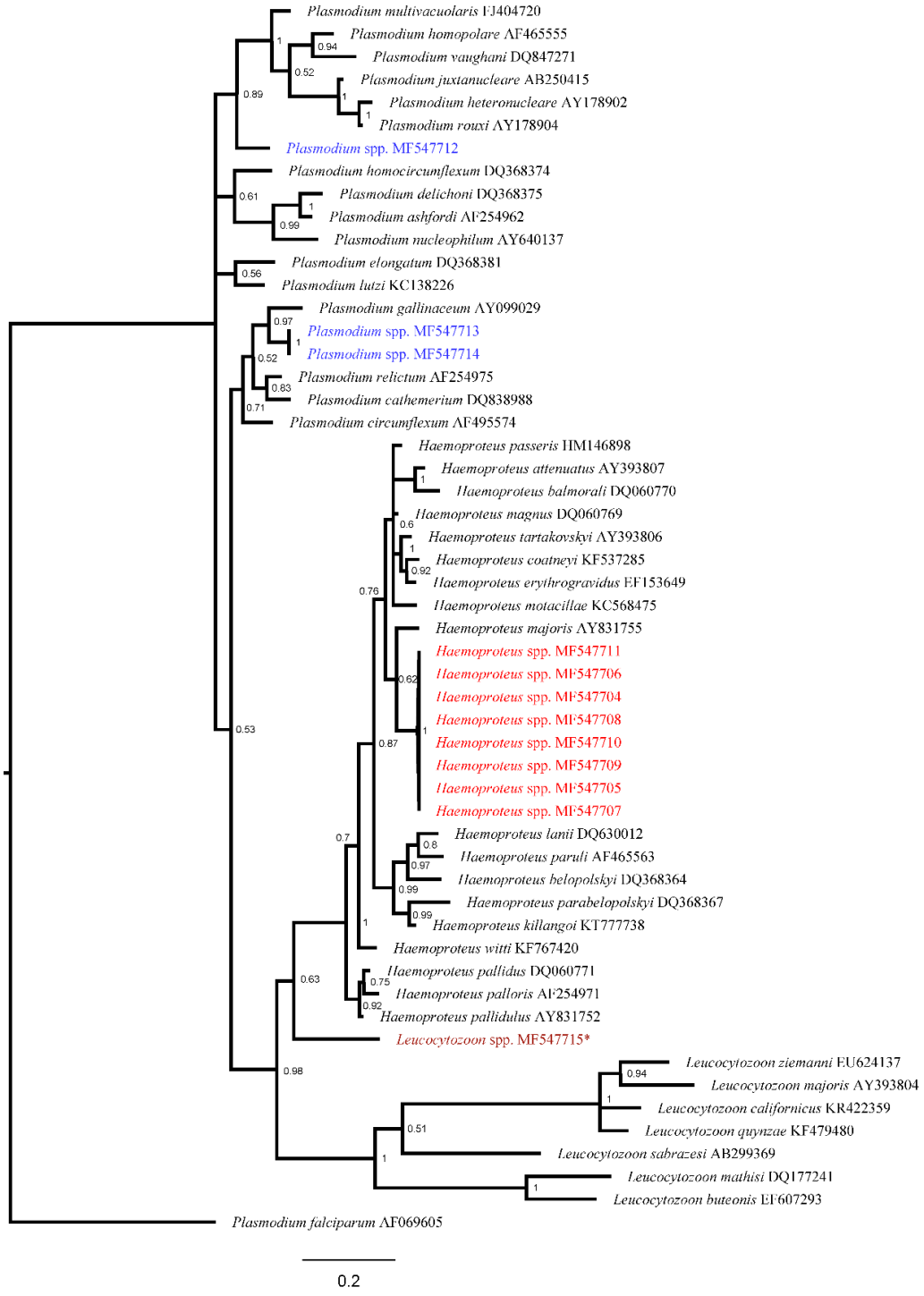

Fig. 2: Bayesian inference tree of three avian haemosporidian parasite genera (*Haemoproteus* spp., *Plasmodium* spp., and *Leucocytozoon* spp.) based on 54 mtDNA sequences. Sequences from the present study are colored to differentiate those obtained from the MalAvi database. Posterior probabilities are shown above or below branches. *The position of *Leucocytozoon* spp. was not clear on the trees.

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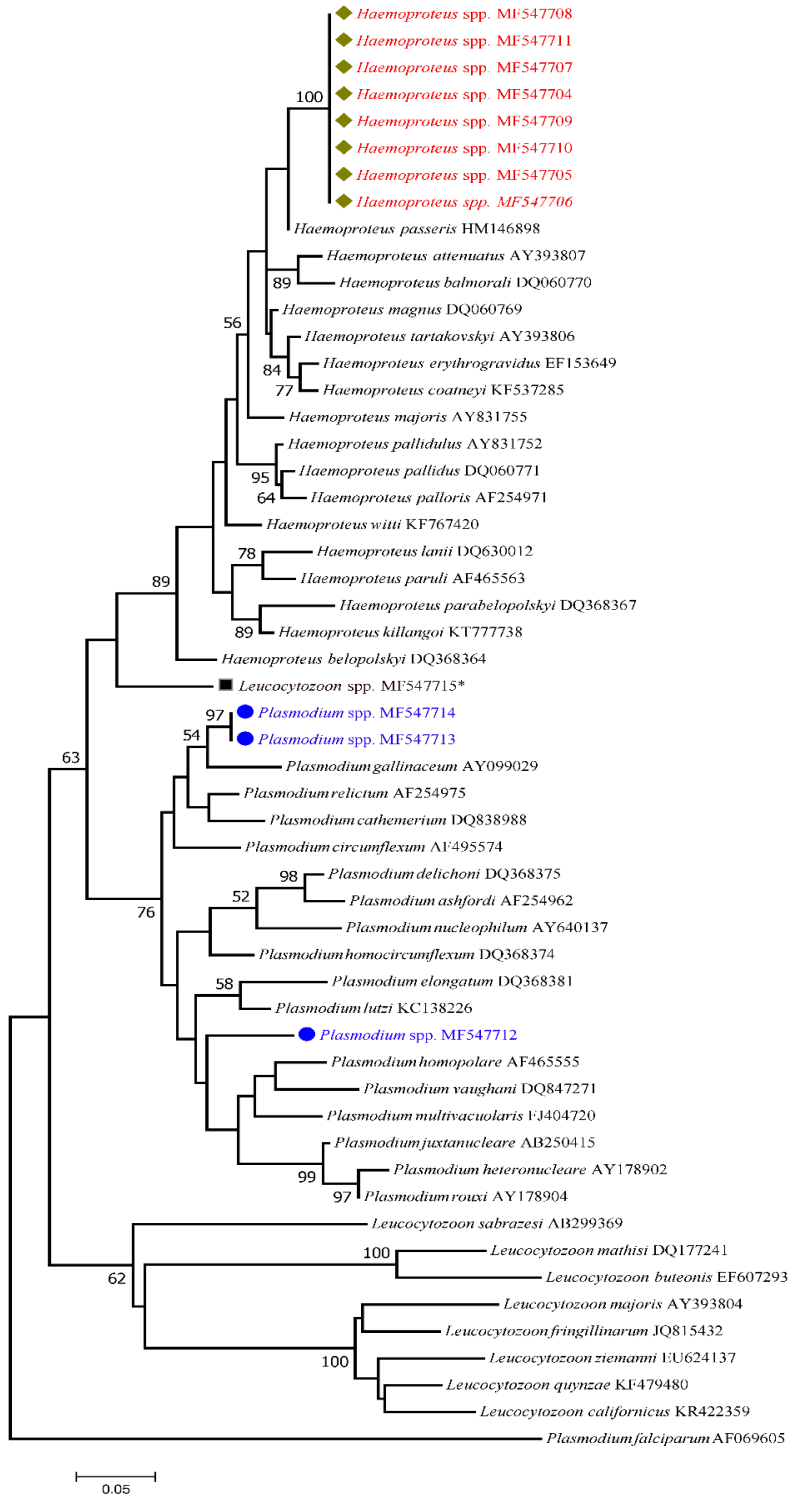

Fig. Supplementary 1: Maximum likelihood tree of three avian haemosporidian parasite genera (*Haemoproteus* spp., *Plasmodium* spp., and *Leucocytozoon* spp.) based on 54 mtDNA sequences. Sequences from the present study are colored to differentiate those obtained from the MalAvi database. Bootstrap values >50 are shown at nodes. *The position of *Leucocytozoon* spp. was not clear on the trees.

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Only a few studies have investigated haemosporidian parasites of birds in Uganda (Howard *et al*., 2018); however, there were studies of these parasites in wild birds within surrounding African islands including Cape Verde (Hille *et al*., 2007) and Madagascar (Barraclough *et al*., 2008; Ishtiaq *et al*., 2012; Schmid *et al*., 2017). Although Uganda has a diversity of habitat suitable for vector development, this does not translate into more diversified blood parasite transmission within the area. Low prevalence of haemoparasites has been found in wild bird populations due to resistance of most avian species to infections of the parasites (Martínez-abraín *et al*., 2004). Also, implementation of control measures for the development of arthropod vectors can mitigate the transmission of blood parasites (Piersma, 1997). The present study showed that host range varied for the three haemosporidian parasites. *Haemoproteus* spp. and *Plasmodium* spp. exhibited a wider host range of infection; for example, the four free-ranging bird species investigated were found infected with parasites from these haemosporidian genera. In contrast, *Leucocytozoon* spp. seemed to exhibit a narrowed host range. Our study only detected infection in one host, the domestic chicken. There was one case (i.e. MF547715) we were unable to decide whether *Haemoproteus* spp or *Leucocytozoon* spp due to the position on the tree.

The phylogenetics of the mtDNA (*cyt* b) revealed low diversity of avian haemosporidian parasites in free-ranging birds in Uganda. A single *Haemoproteus* cluster was observed suggesting it may be the only lineage in circulation in free-ranging birds in Uganda. Of the three *Plasmodium* spp. sequences identified in the current study, two (MF547713 and MF547714) formed a cluster with *P. gallinaceum*, the other (MF547712) was placed at the base of the clade comprised of *P. multivauolaris*, *P. homopolare*, *P. vaughani*, *P. juxtanucleare*, *P. heteronucleare* and *P. rouxi*. The phylogeny of avian haemosporidian parasites has not been extensively studied in Uganda; therefore, further research is needed to identify avian reservoirs and mosquito vectors for haemosporidian parasites in this country.

Haemosporidian parasites can have adverse effects on the physiological processes of birds. For examples, Atkinson *et al.* (1995) detected declines in food consumption and body weight of native Hawaiian forest birds in an experimental study of pathogenicity of avian malaria. Death was mainly due to anemia following erythrocyte parasitemia. This makes it important to bleed the birds and measure their hematocrit. Presence of pale watery heart blood, high parasitemia with abundant immature erythrocytes, and diffuse areas of extramedullary erythropoiesis in the liver and kidney and lesions have been detected (Atkinson *et al*., 2000). Clinically, avian malaria produces a wide range of effects in avian hosts, from no apparent clinical signs to severe anaemia and death. Post-mortem findings reveal intense and severe anemia as main cause. Further, associated tissue hypoxia can produce tissue necrosis; hypertrophy of the spleen and the liver (Ishtiaq *et al*., 2012). *P. gallinaceum*, *P. juxtanucleare* and *P. durae* appear to be the most dangerous for poultry, producing up to 90% mortality. Some 65 *Plasmodium sp*. have been isolated from over 1,000 different species of birds. Few of the *Plasmodium* sp. which has been identified appears to be natural parasites of

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domestic poultry. Susceptibility occurs primarily in passerine birds rather than domestic fowl. The birds sampled in this study were apparently healthy and there was no existing knowledge of avian haemosporidia among the farmers and veterinary stakeholders.

Current lapses in the poultry industry in Uganda in terms of weak veterinary services and policies may increase disease transmission. Though intensive disease control and management systems are employed by some commercial poultry farmers, very little intervention has been directed to managing disease or infection in free-ranging domestic birds. This makes disease control unsustainable and difficult to monitor across managements in poultry production. Moreover, Uganda is a major destination for migratory birds from the northern hemisphere. With a total of 1061 bird species within its borders, Uganda is among bird watching destinations in Africa. The limited research on avian parasites has resulted in poor awareness and control measures of haemosporidian parasites; therefore, a threat to the avian diversity in Uganda and the region at large. Further, global climate change has affected the movement of both migratory and resident species of birds including response to changes in resources such as food availability, habitats and weather conditions, in the wintering grounds (Waldenström *et al*., 2004). Together, this can increase predisposition to infection or disease transmission, along flying routes. Avian malaria is one of the more likely diseases to be contracted at stopover points during migration following exposure to mosquito bites. Also, avian malaria and its mosquito vectors are believed to play a major role in the decline and extinction of native bird species (Walther *et al*., 2014). These parasites are frequently lethal in non-adaptive avian hosts. The pathogenicity of malaria parasites towards birds usually differs during the acute and chronic stages of infection (Cranfield *et al*., 1994; Graczyk *et al*., 1994; Atkinson *et al*., 1995; Valkiūnas, 2005; Palinauskas *et al*., 2011).

Conclusion

The present study used both molecular- and microscopy-based techniques to detect and identify the presence of haemosporidian parasites in free-ranging domestic birds in Northwestern Uganda. We detected *Haemoproteus* spp., *Leucocytozoon* spp. and *Plasmodium* spp. The Haemosporidian parasites exhibited limited diversity; the phylogeny revealed distinct clades with similar evolutionary relationships. The current level of research and awareness regarding these parasites is low in Uganda therefore requires intensified effort to understand the diversity, prevalence and distribution of haemosporidian parasites in the region.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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