

**PREVALENCE AND DIVERSITY OF AVIAN MALARIA PARASITES AT
MIDDLE AND HIGH ELEVATIONS IN MONTANE PAPUA NEW GUINEAN
BIRDS**

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Wildlife Ecology and Conservation with Distinction


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ABSTRACT

Since its discovery, avian malaria parasites have served as an important model system for understanding the ecological and evolutionary forces behind host-parasite relationships. However, these parasites remain poorly characterized in many under-researched areas. Samples were collected from Mount Karimui and the YUS conservation area to better understand the avian malaria parasites in Papua New Guinea (PNG). We used molecular methods to detect and identify avian malaria parasites (genera: *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) from DNA in avian blood samples. For infected samples, we sequenced the parasite cytochrome *b* (*cyt b*) gene to identify parasite genetic lineages. We tested for avian malaria parasites in 75 blood samples from 29 bird species. Over half the samples (42 samples; 56%) were infected with at least one avian malaria parasite. Samples at the middle elevation site (1,350 masl - 1,575 masl) in Mount Karimui had a higher prevalence (37 samples; 67%) than samples from the high elevation (2,550 masl) YUS conservation area (5 samples; 25%). Infections from *Haemoproteus* parasites made up a majority of the overall infections (35 samples; 83%), while *Plasmodium* (21 samples; 50%) and *Leucocytozoon* (4 samples; 1%) were less common. About 40% of infected birds ($n = 17$) were infected by parasites from more than one genus; the majority were mixed *Haemoproteus* and *Plasmodium* infections ($n = 16$) while one sample was infected with all three genera. We identified 21 genetic parasite lineages, 12 of which have not been previously reported. This study is an important first step in characterizing the prevalence and diversity of avian malaria parasites in the montane birds of PNG.

Chapter 1

INTRODUCTION

Malaria parasites belong to an order of blood parasites (*Haemosporida*) that are transmitted between vertebrate hosts by dipteran vectors. While several species of malaria parasites in the genus *Plasmodium* infect humans (Pérez-Tris et al., 2005), malaria parasites represent a diverse clade of wildlife parasites that infect a great variety of hosts and vectors including birds, bats, deer, primates, and other vertebrates (Galen et al., 2018). Birds are primarily infected by malaria parasites of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (Valkiūnas 2005).

The avian malaria parasite genera are transmitted by different vectors. *Plasmodium* is transmitted by mosquitoes (family: *Cuclidae*). Parasites in the *Haemoproteus* genus are transmitted by two different families of dipteran vector (Valkiūnas, 2005). Parasites in the *Haemoproteus* subgenus are transmitted by louse flies (family: *Hippoboscidae*) and also differ in host preference. Parasites in this subgenus are only known to infect doves, pigeons, and some seabirds (Valkiūnas, 2005). Meanwhile, the *Parahaemoproteus* subgenus infects a more diverse array of bird species (songbirds, raptors, and more) and is transmitted by biting midges (family: *Ceratopogonidae*; Valkiūnas, 2005). *Leucocytozoon* is transmitted by black flies in the *Simuliidae* family. These avian malaria parasite species were first identified through the use of microscopy though this method has seen less use due to its difficulty. Instead, most identification of avian malaria parasites today is conducted

with polymerase chain reaction (PCR) and sequencing of a barcoding region of the parasite's mitochondrial cytochrome *b* gene (*cyt b*; Hellgren et al. 2004). Genetic lineages of avian malaria parasites are defined by unique sequences of *cyt b*. In many cases, avian malaria genetic lineages represent unique species (Bensch et al. 2004), but other times they do not (Hellgren et al. 2015), and in most cases it is not known.

Historically, the avian malaria parasite *Plasmodium relictum* was used as a model to demonstrate mosquitoes as competent biological vectors of malaria parasites in the late 1890s (Ishtiaq, 2021). Avian malaria has also served as a model for understanding host-parasite relationships as well as the impact of infectious disease on species conservation (Ishtiaq, 2021). One of the most notable examples of infectious disease impacting native species populations is the introduction of avian malaria to the islands of Hawaii. *P. relictum* was first detected in the blood of birds in Hawaii in the 1930s and was likely introduced with caged birds (Van Riper et al. 1986; Atkinson & LaPointe, 2009). The mosquito vector of *P. relictum*, *Culex quinquefasciatus*, was introduced to Hawaii about 100 years earlier, probably through boat ballast water (Joyce, 1961). Endemic Hawaiian birds are highly susceptible to *P. relictum* and experience high mortality from infection, likely because they evolved in the absence of avian malaria. The introduction of avian malaria to Hawaii led to the restriction of endemic songbirds like the 'I'iwi (*Drepanis coccinea*) to upper elevations in mountains where mosquitoes are less likely to transmit the malaria parasite (Van Riper et al. 1986). The 'I'iwi were once ubiquitous in Hawaii and made frequent visits to all elevational ranges in all of the major Hawaiian Islands as part of their ecological niche

(Samuel et al., 2015). Now these birds have been extirpated entirely from the warmer lower elevations of their former range, especially in habitats with wetter conditions that are more suitable to mosquito vectors (Van Riper et al., 1986; Samuel et al., 2015). Indeed, the impact of avian malaria on the tropical montane birds of Hawaii continues to unfold and many believe it may worsen from climate change. Smaller islands like O'ahu offer less disease refugia at even their highest ranges and species like the 'I'iwi have become completely extirpated from O'ahu as a result (Neddermeyer et al., 2023).

While the avian malaria parasites of Hawaii and many other places (Bensch et al., 2009) have been closely monitored and studied, some parts of the world remain understudied in terms of their avian malaria parasite prevalence and diversity. Lying just north of the Torres Strait near Australia is the tropical island of New Guinea. New Guinea is politically divided along the 141° E longitudinal with the western half forming the Indonesian province of West Papua and the eastern half forming the independent country of Papua New Guinea (PNG). PNG is renowned as a biodiversity hotspot and a haven for many endemic species. Despite making up less than 1% of earth's landmass, PNG hosts over 5% of the earth's biodiversity (Daith et al., 2001). Furthermore, as a result of tectonic collisions that shaped the landmass, the island has massive northern and central mountain ranges that define much of the surrounding landscape (Gressitt, 1982). Despite being a tropical area that is potentially conducive to avian malaria, avian malaria parasite prevalence and diversity has had only limited investigation.

Initial studies of avian malaria in PNG revealed that the prevalence of infection has been closely related to avian host phylogeny rather than abiotic factors like elevation and temperature (Bodawatta et al., 2020, Lau et al., 2023). PNG lies just east of the Wallace line and shares much of its native flora and fauna with Australia. This presents PNG with a greater chance of parasite dispersal between landmasses than other more remote tropical montane places like Hawaii. However, more comparisons of avian malaria parasites between PNG and Australia are needed (Beadell et al., 2004). Recent studies have reported high overall parasite prevalence in PNG as high as 45.5%; especially for *Haemoproteus* parasites (Lau et al., 2023). Parasite prevalence has also been known to peak at mid-elevational points rather than low-elevational points (Bodawatta et al., 2020, Lau et al., 2023). This is in spite of the seemingly better environmental conditions present at lower elevations for dipteran parasite vectors. In part of an ongoing effort to better understand the prevalence and diversity of PNG parasites and their respective patterns along the elevational gradient, we used molecular methods to screen for and identify avian malaria parasites. Specifically, we sought to explore the prevalence and diversity of cytochrome *b* genetic lineages of the three main parasite genera (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) in blood samples collected from wild birds in PNG.

Chapter 2

METHODS

2.1 Sample collection

Avian blood samples were collected at two sites in PNG by Dr. Benjamin Freeman and collaborators in 2012 under permit number 99902084881 from the National Research Institute of Papua New Guinea. The first site was at the YUS Conservation area (YUS being an acronym for the Yopno, Urawa, and Som rivers) in the northern Saruwaged mountain range in the Morobe province. The second site was at Mount Karimui in the central mountain range of the Chimbu province. Wild birds were captured with mist nets and blood samples were collected from the bird's brachial vein. Captured birds were then released. Blood samples were stored in a lysis buffer, imported to the US by Dr. Freeman, and stored at Cornell University. The samples were then shipped to the Ellis Lab at the University of Delaware under APHIS permit #639-23-159-98012. Blood samples from the YUS site were all taken at 2,550 masl and collected in May of 2012. In total, the YUS site had 21 samples from eight species. Blood samples from Mount Karimui were collected from June to July of 2012 and covered the entire elevational gradient of the site. The elevational gradient of Mount Karimui spanned from the lowlands at 1,150 masl to the much higher elevations nearing the summit at 2,425 masl. Samples were collected at 11 intervals that ranged in size from 50 – 150 m. The sample set from the Karimui site was far

larger with 764 samples from 85 bird species.

2.2: Molecular testing and DNA sequencing

From these sample sets, we selected a subset of 80 samples from 29 species. This included 59 samples from the Mount Karimui sample set and all 21 samples from the YUS sample set. The selected Karimui samples were collected at mid-elevational habitats at two sampling intervals; 45 samples were collected at 1,350 masl – 1,450 masl and the remaining 14 samples were collected at 1,450 masl – 1,575 masl. All 21 YUS samples were collected at 2,550 masl. To extract DNA from the samples, two protocols were used. We first used the Qiagen DNeasy Blood & Tissue kit, following the manufacturer's instructions. This method was used to extract DNA from the first 16 samples in the YUS sample set and produced DNA concentrations consistently below the ideal 25 ng/ μ l DNA concentration recommended for avian malaria PCR testing. Nonetheless, these samples were still tested. The remaining 64 samples from the YUS and Karimui datasets were extracted with a standard ethanol-based protocol. Ethanol DNA extractions were conducted by using Proteinase K to first digest structural proteins in the samples of lysed bird blood. Ammonium acetate (NH_4Ac) and ethanol (EtOH) were then used to purify and precipitate DNA. DNA precipitate was eluted with ultrapure water. This method was generally more effective at obtaining higher DNA concentrations. We quantified DNA concentration using 3 μ l of the extracted DNA sample with a NanoDrop One spectrophotometer (ThermoFisher Scientific).

However, very low concentrations of extracted DNA were still found in five of

the ethanol extractions. These samples were excluded from avian malaria testing (in the future we will try concentrating these samples and subsequently testing them). The remaining 75 samples comprised 20 samples from the YUS sample set and 55 samples from the Kairmui sample set and included the same 29 species. Four of the excluded samples belonged to the Karimui 1,350 masl – 1,450 masl sampling interval thereby reducing the subset to 41 samples at the 1,350 masl – 1,450 masl. Samples that had DNA concentrations exceeding the desired 25 ng/ μ l DNA concentration were subsequently diluted to 25 ng/ μ l of DNA with ultrapure water and stored in a separate 1.5 ml microcentrifuge tube.

We began screening these samples for avian malaria parasites using a multiplex PCR assay (Ciloglu et al., 2019). This PCR produces specifically sized fragments of mitochondrial DNA (mtDNA) that correspond to the three main genera (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*). This allowed us to not only test a sample for avian malaria infection, but also simultaneously determine the genera of parasites in the sample. The multiplex PCR is conducted using 0.2 μ l of genus-specific forward and reverse primers (HMF, HMR, PMF, PMR, LMF, LMR); each primer is at an initial concentration of 10 μ M (Ciloglu et al., 2019). Additionally, 5 μ l of Qiagen Multiplex PCR Master Mix 2X and 1.8 μ l of ultrapure water are used for each sample. To begin the multiplex PCR, the 8 μ l of the aforementioned components and 2 μ l of DNA extract were loaded into wells of a 96-well PCR reaction plate. Negative controls of 2 μ l ultrapure water were used throughout the well plates to ensure contamination was not present. Positive controls were artificially created by mixing

samples from previously tested birds to ensure the PCR reaction successfully amplified fragments from all three genera (i.e., a positive control included DNA from all three parasite genera). These well plates were then sealed and placed in an Applied BioSystems MiniAmp Thermal Cycler for the PCR reaction, with cycling times as described in Ciloglu et al. (2019).

After the multiplex PCR reaction, amplified DNA was visualized using gel electrophoresis to determine the presence or absence of avian malaria parasites. We mixed 5 μ l of PCR product with 1 μ l of 6X TriTrack loading dye (ThermoFisher Scientific) and loaded the mixture into 2 % agarose gels (made with TAE) stained with GelRed (Biotium) in a 1X TAE buffer along with 100 bp (base pair) ladders at 90V for *ca.* 40 min. We imaged the gels using a Bio-Rad GelDoc Go Imaging System. Samples testing positive for *Haemoproteus*, *Plasmodium*, and/or *Leucocytozoon* would have distinct PCR product bands: *Haemoproteus* bands are *ca.* 533 bp, *Plasmodium ca.* 378 bp, and *Leucocytozoon ca.* 218 bp (Figure 1; Ciloglu et al. 2019).

Samples that tested positive for any of the parasite genera in the multiplex PCR were then run in a nested PCR assay to amplify the barcoding region of the *cyt b* gene (Hellgren et al., 2004). Samples from doves were run regardless of their multiplex result to ensure the separate *Haemoproteus* subgenera commonly found in these birds (and not captured by the multiplex PCR) did not avoid detection. The nested PCR included two separate PCR reactions. The first PCR reaction, or outer PCR reaction, included samples infected with any of the three parasite genera. This first PCR reaction amplifies a large 617 bp fragment of *cyt b*. For every sample, a master mix of

12.5 µl DreamTaq Master Mix 2X (ThermoFisher Scientific), 1 µl of 10 µM HaemNFI (forward primer), 1 µl of 10 µM HaemNR3 (reverse primer), and 9.5 µl ultrapure water was created. This master mix was combined individually with 1 µl of extracted DNA; ultrapure water was used as negative controls and positive controls were also included in a 96-well PCR reaction plate for a 25 µl reaction in each well. The plate was then sealed and placed in an Applied BioSystems MiniAmp Thermal Cycler following the thermal profile in Hellgren et al. (2004). After the first reaction, an inner PCR was performed using 2 µl of the PCR product from the outer PCR. Separate inner PCR reactions were performed based on two sets of primers for different genera; PCR products with *Plasmodium* and/or *Haemoproteus* infections were amplified in an inner nested PCR separate from the inner PCR with *Leucocytozoon*. For all reactions, samples were loaded from the outer PCR plate into a new inner PCR plate with 12.5 µl DreamTaq Master Mix 2X and 8.5 µl of ultrapure water; 1 µl each of HaemF and Haem R2 primers was used in the *Plasmodium* and *Haemoproteus* inner reaction and 1 µl each from HaemFL and HaemR2L primers was used in *Leucocytozoon* reactions as described in Hellgren et al. (2004). To ensure contamination did not occur during the inner PCR, every eighth well in the inner nested 96-well PCR plate was loaded with a negative control of ultrapure water.

PCR product from the nested PCR reactions was visualized with gel electrophoresis as described for the multiplex PCR except that they were conducted at 80V for *ca.* 45 min. Samples in both inner PCRs that successfully amplified the *cyt b* gene of the parasites would display a band of *ca.* 479 bp (the size of the amplified

barcoding fragment of *cyt b*). All samples that returned a positive result in the nested PCRs were sent to Azenta Life Sciences for Sanger sequencing in both directions.

2.3: *Sequence analysis*

We analyzed Sanger sequences with the software program Geneious Prime 2023.1.2 (<https://www.geneious.com>). We manually screened each sequence to ensure we had high quality sequences with strong peaks. We also checked for mixed nucleotide base pair calls that could be indicative of mixed lineage infections. Once sequences were manually screened and trimmed for low quality base calls (typically at the beginning and end), the forward and reverse reads were assembled into a consensus contig using the DeNovo Assembly feature in Geneious. These consensus sequences were then identified to lineage name using the basic local alignment tool (BLAST) on the MalAvi database website (<http://130.235.244.92/Malavi/>).

2.4: *Statistical analysis*

We calculated the prevalence and diversity of the parasites using pivot tables in Google Sheets (Table 1 – 3). We plotted the prevalence of species that had sample sizes greater than three individuals (Figure 2) using R v.4.2.1 (R Core Team, 2022) with the ggplot2 and ggtext R packages (Wickham, 2016; Wilke et.al 2022).

Chapter 3

RESULTS

Of the 75 samples that were screened in multiplex PCRs from both sites, 42 samples (56%) from 19 avian host species tested positive for one or more parasite genus (Table 1). Five of the 20 samples (25%) from the YUS site tested positive for one genus; four samples (20%) were infected with *Haemoproteus*, while one (5%) was infected with *Leucocytozoon*. Of the 55 Karimui birds that were tested, 37 samples (67%) tested positive for one or more avian malaria parasite genus; 31 samples (56%) were infected with *Haemoproteus* and 21 samples (38%) were infected with *Plasmodium*; *Leucocytozoon* infections were scarcer with four samples (7%) testing positive. Many of these positive screening results were found with mixed-genus infections (i.e., a bird infected by more than one parasite genus): 16 samples (29%) from two avian host species tested positive for both *Haemoproteus* and *Plasmodium* from the multiplex screening and one sample (2%) from a White-Eyed Robin (*Pachycephalopsis poliosoma*) tested positive for all three avian parasite genera. Among bird species with more than 3 individuals sampled ($n = 7$ species), prevalence varied for *Plasmodium* (range: 20 – 94 % infected), *Haemoproteus* (14 – 100 % infected), and *Leucocytozoon* (13 – 33 % infected; Figure 2).

We were able to successfully amplify the *cyt b* gene of the parasites in 38 samples using the nested PCR assay. Nine of the 38 infections returned unusable (low

quality) sequences. As a result, all genetic lineages present in these nine infections are currently unresolved. As for the remaining 29 infections, we were able to successfully sequence the entire 479 bp barcoding region from the *cyt b* gene of 32 individual avian malaria parasites (more parasites than infections because of mixed parasite infections). These 32 individual avian malaria parasites represented 21 unique genetic lineages from 12 avian host species (Table 2 & 3). Of these 21 genetic lineages, 12 were previously undescribed (Table 3). These 12 novel lineages include: seven *Haemoproteus* lineages, two *Plasmodium* lineages, and three *Leucocytozoon* lineages. The remaining nine genetic lineages have been previously described in the MalAvi database (Table 2). All nine of these genetic lineages belong to the *Haemoproteus* genus. Eight of these genetic lineages have only been found in PNG while one genetic lineage, SERCIT02, has been found in both Australia and PNG.

Table 1: Multiplex PCR results for all infected samples

Avian host species	Sample size (n)	Site Elevation (masl)	<i>Haemoproteus</i>	<i>Plasmodium</i>	<i>Leucocytozoon</i>	<i>Haemoproteus & Plasmodium</i>	<i>Haemoproteus, Plasmodium, & Leucocytozoon</i>	Grand Total
Ashy Robin (<i>Heteromyias albispecularis</i>)	8	YUS 2,550	-	-	1	-	-	1
Black Berrypecker (<i>Melanocharis nigra</i>)	2	Karimui 1,350 - 1,450	2	-	-	-	-	2
Black Fantail (<i>Rhipidura atra</i>)	7	Karimui 1,350 - 1,450	1	-	-	-	-	1
Chestnut-Bellied Fantail (<i>Rhipidura hyperythra</i>)	1	Karimui 1,350 - 1,450	-	1	-	-	-	1
Dimorphic Fantail (<i>Rhipidura brachyrhyncha</i>)	1	YUS 2,550	1	-	-	-	-	1
Dwarf Honeyeater (<i>Oedistoma iliolophus</i>)	1	Karimui 1,350 - 1,450	1	-	-	-	-	1
Fan-tailed Berrypecker (<i>Melanocharis versteri</i>)	1	Karimui 1,350 - 1,450	1	-	-	-	-	1
Large Scrubwren (<i>Sericornis nouhuysi</i>)	1	Karimui 1,450 - 1,575	1	-	-	-	-	1
Lesser Melampitta (<i>Melampitta lugubris</i>)	1	YUS 2,550	1	-	-	-	-	1
Little Shrike-thrush (<i>Colluricincla megarrhyncha</i>)	3	Karimui 1,350 - 1,450	1	-	-	-	-	1
Loria's Satinbird (<i>Cnemophilus loriae</i>)	1	Karimui 1,350 - 1,450	1	-	-	-	-	1
Mottled Whistler (<i>Rhagologus leucostigma</i>)	2	Karimui 1,350 - 1,450	1	-	-	-	-	1
Northern Scrub Robin (<i>Drymodes superciliaris</i>)	1	Karimui 1,350 - 1,450	-	-	1	-	-	1
Rusty Mouse-Warbler (<i>Crateroscelis murina</i>)	3	Karimui 1,350 - 1,450	-	-	1	-	-	1
Slaty-Chinned Longbill (<i>Toxorhamphus poliopterus</i>)	5	Karimui 1,350 - 1,450	4	-	-	1	-	5
Spangled Honeyeater (<i>Melipotes ater</i>)	1	YUS 2,550	1	-	-	-	-	1
White-Eyed Robin (<i>Pachycephalopsis poliosoma</i>)	1	Karimui 1,350 - 1,450	-	-	-	-	1	1
	3	Karimui 1,450 - 1,575	-	3	-	-	-	3
White-Faced Robin (<i>Tregellasia leucops</i>)	10	Karimui 1,350 - 1,450	1	-	-	9	-	10
	6	Karimui 1,450 - 1,575	-	-	-	6	-	6
White-Winged Robin (<i>Peneothello sigillata</i>)	1	YUS 2,550	1	-	-	-	-	1
Grand Total			18	4	3	16	1	42

Table 1: The table above shows the number of infections in each host species separated by parasite genus infections and sample site/elevation; sample size of birds tested is also shown. These results are from the multiplex PCR.

Table 2: Known cytochrome *b* lineages table

Cyt <i>b</i> Lineage	Host species	Site elevation (masl)	Lineage in MalAvi	Host species in MalAvi	Locations in MalAvi
H_PENBIM01	White-Faced Robin (6/11)	Karimui: 1,350 - 1,450		White-Faced Robin; White-Rumped Robin	Papua New Guinea
	White-Faced Robin (5/11)	Karimui: 1,450 - 1,575	Yes		
H_SERPER01	Black Fantail (1/1)	Karimui: 1,350 - 1,450	Yes	Buff-Faced Scrubwren	Papua New Guinea
H_MELMON01	Slaty-Chinned Longbill (3/5)	Karimui: 1,350 - 1,450	Yes	Slaty-Chinned Longbill; Forest Honeyeater	Papua New Guinea
H_SYMAXI01	Slaty-Chinned Longbill (1/5)	Karimui: 1,350 - 1,450	Yes	Black Monarch; Yellow-Bellied Longbill; Slaty-Chinned Longbill	Papua New Guinea
H_MELNIG02	Black Berrypecker (1/2)	Karimui: 1,350 - 1,450	Yes	Black Berrypecker	Papua New Guinea
H_COLMEG05	Little-Shrike Thrush (1/3)	Karimui: 1,350 - 1,450	Yes	Little-Shrike Thrush	Papua New Guinea
H_OEDILI01	Dwarf Honeyeater (1/1)	Karimui: 1,350 - 1,450	Yes	Rusty Mouse-Warbler; Spectacled Longbill; Canary Flyrobin	Papua New Guinea
H_MELVER01	Fan-Tailed Berrypecker (1/1)	Karimui: 1,350 - 1,450	Yes	Black Berrypecker; Streaked Berrypecker; Fan-Tailed Berrypecker; Slaty Robin; Buff-Faced Scrubwren; Slaty-Chinned Longbill	Papua New Guinea
H_SERCIT02	Large Scrubwren (1/1)	Karimui: 1,450 - 1,575	Yes	Yellow-Throated Scrubwren; Large-Billed Scrubwren; Large Scrubwren	Australia, Papua New Guinea

Table 2: Shown above are the 9 lineages discovered in the YUS and Karimui samples that have been previously described in the MalAvi database. The genus of the lineage is denoted with the first letter ('H' for *Haemoproteus*). Lineage names are based on the first three letters in the Latin binomial from the avian host species it was first discovered in. All 9 known lineages belonged to the *Haemoproteus* genus.

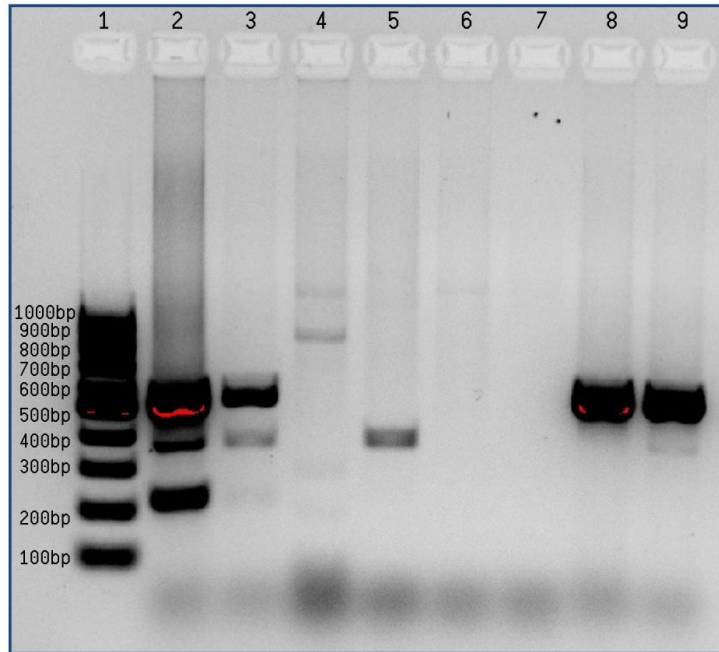
Table 3: Novel cytochrome *b* lineages table

Cyt <i>b</i> Lineage	Host species	Site elevation (masl)
H_Unique_Lineage01 (H_UL01)	Spangled Honeyeater (1/1)	YUS: 2,550
H_Unique_Lineage02 (H_UL02)	Slaty-Chinned Longbill (1/5)	Karimui: 1,350 - 1,450
H_Unique_Lineage03 (H_UL03)	Slaty-Chinned Longbill (1/5)	Karimui: 1,350 - 1,450
H_Unique_Lineage04 (H_UL04)	Black Berrypecker (1/2)	Karimui: 1,350 - 1,450
H_Unique_Lineage05 (H_UL05)	Black Berrypecker (1/2)	Karimui: 1,350 - 1,450
H_Unique_Lineage06 (H_UL06)	White-Eyed Robin (1/4)	Karimui: 1,350 - 1,450
H_Unique_Lineage07 (H_UL07)	Slaty-Chinned Longbill (2/5)	Karimui: 1,350 - 1,450
P_Unique_Lineage01 (P_UL01)	White-Eyed Robin (1/4)	Karimui: 1,350 - 1,450
P_Unique_Lineage02 (P_UL02)	White-Eyed Robin (1/4)	Karimui: 1,450 - 1,575
L_Unique_Lineage01 (L_UL01)	Ashy Robin (1/8)	YUS: 2,550
L_Unique_Lineage02 (L_UL02)	Northern Scrub Robin (1/1)	Karimui: 1,350 - 1,450
L_Unique_Lineage03 (L_UL03)	White-Eyed Robin (1/4)	Karimui: 1,350 - 1,450

Table 3: Shown above are the 12 lineages discovered in the YUS and Karimui samples that have been previously undescribed. The letter at the start of each lineage name denotes the genus it belongs to (“H” for *Haemoproteus*, “P” for *Plasmodium*, and “L” for *Leucocytozoon*). The number refers to the order in which they were found in our sample set for each genus. All lineage names are tentative.

Figure 1: Example of a multiplex screening with PNG samples

The Gel “ladder” in well 1 shows the position of DNA at different base pair sizes.



The positive control in well 2 shows where bands will show up for all three parasite genera.

Well 7 contains a negative control that replaces a bird sample with ultrapure water to ensure contamination is not present.

Figure 1: Above is an example of one of our multiplex screenings. The wells are labeled above in numerical order. Well 2 represents a positive control for all three genera with a *Haemoproteus* band on top, a *Plasmodium* band in the middle, and a *Leucocytozoon* band on the bottom. Well 3 contained a natural triple infection from a White-Eyed Robin (*Pachycephalopsis poliosoma*) while well 5 contained a single *Plasmodium* band from a Chestnut-Bellied Fantail (*Rhipidura hyperythra*). Wells 8 & 9 contained PCR products from Slaty-Chinned Longbills (*Toxorhamphus poliopterus*). Well 8 contained a saturated *Haemoproteus* band. Well 9 also had a saturated *Haemoproteus* band with a faint *Plasmodium* band underneath. The samples in wells 4 and 6 contained non-specific bands and therefore did not test positive for any avian malaria parasites. Well 7 contained a negative control of ultrapure water and therefore contained no PCR amplicon.

Figure 2: Multiplex Screening results of Papua New Guinean birds (n ≥ 3)

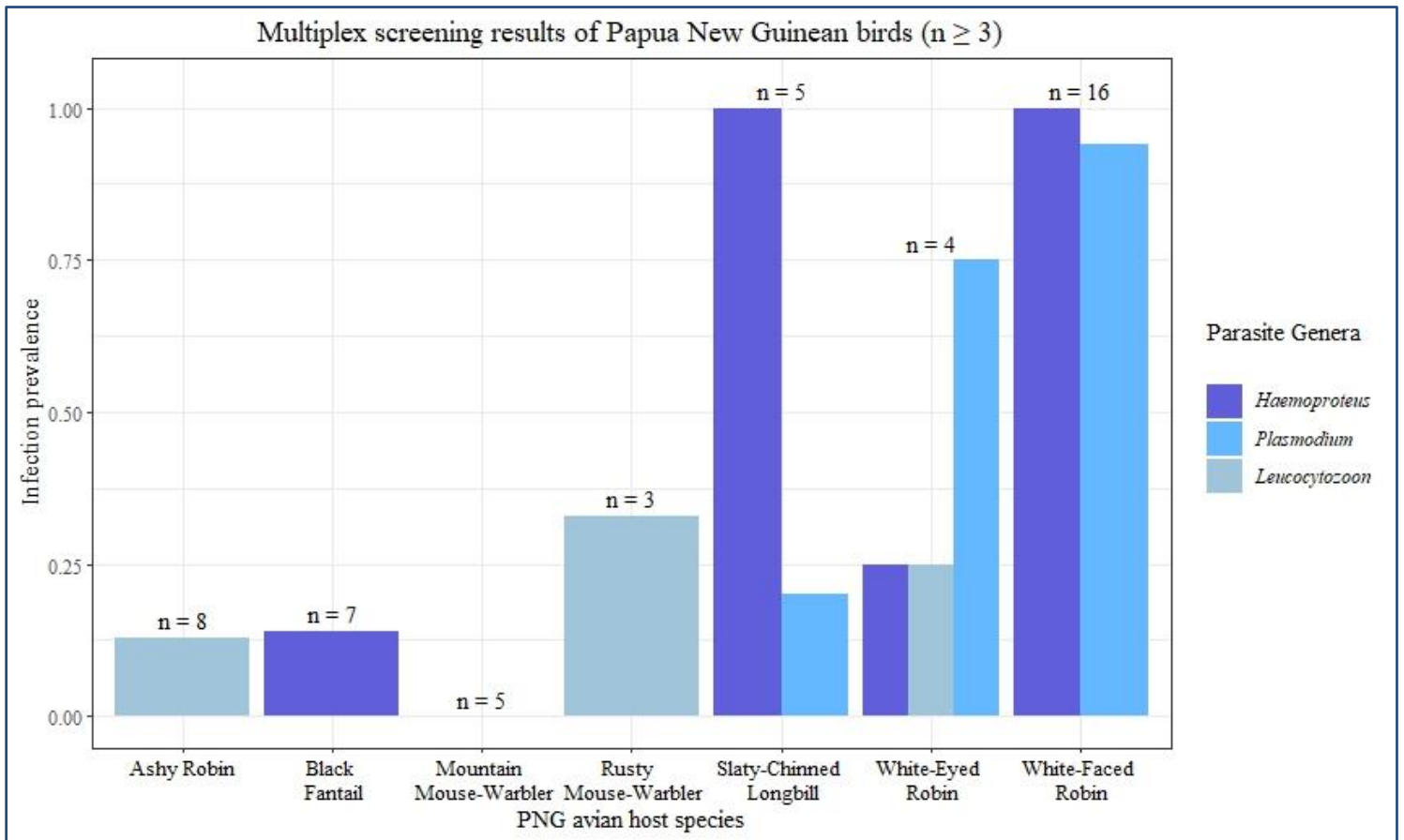


Figure 2: The prevalence of avian malaria parasites in bird species with sample sizes greater than or equal to 3 (sample sizes are presented above the prevalence bars). These prevalence data are derived from multiplex screening results at both PNG sites.

Chapter 4

DISCUSSION

We observed a high prevalence and diversity of avian malaria parasites at both PNG study sites (Table 1 - 3, Figure 2). Of the three genera, *Haemoproteus* appears to be the most common (20% of infections at YUS and 56% of infections at Karimui; Table 1). Previous studies have also found a similar trend of avian malaria parasite communities being dominated by *Haemoproteus* in PNG (Bodawatta et al., 2020, Lau et al., 2023). Moreover, our high level of infection (67%) at the 1,350 masl – 1,450 masl and 1,450 masl – 1,575 masl mid-elevation sampling intervals at Mount Karimui is also consistent with trends seen in previous studies (Bodawatta et al., 2020, Lau et al., 2023).

One of the frequently sampled species was the White-Faced Robin (*Tregellasia leucops*). Of all the species tested, this bird had the highest prevalence for both *Haemoproteus* (100% of individuals tested were infected; Figure 2) and *Plasmodium* (94%; Figure 2). As shown in Table 2, all of the 11 White-Faced Robin samples that were successfully sequenced had the PENBIM01 *Haemoproteus* lineage. It appears that PENBIM01 may be specialized on members of the Australasian robin family (Family: Petroicidae) based on MalAvi records (Table 2). Tables 2 and 3 also show how other species, like the Slaty-Chinned Longbill (*Toxorhamphus poliopterus*), host a diverse array of parasite lineages. In only five samples of the Slaty-Chinned Longbill, we were able to identify five genetic lineages, three of which were

previously undescribed. Table 2 shows that three Slaty-Chinned Longbill samples were infected with the *Haemoproteus* lineage MELMON01. These samples infected with MELMON01 also all contained a second *Haemoproteus* co-infection. One sample was co-infected with the *Haemoproteus* lineage SYMAXI01 while the other two were infected with UL07 (Table 2 & 3). Importantly, mixed lineage infections of the same genus (i.e., two or more *Haemoproteus* lineages infecting the same bird) can be difficult to resolve with Sanger sequencing and require additional methods (Illumina sequencing) that we did not use in this study. We hope to incorporate these additional methods in the future for currently unresolved lineage infections.

Unlike the White-Faced Robin's more narrow elevational range (*ca.* 1,250 masl - 1,700 masl), the Slaty-Chinned Longbill was more common across the elevational gradient and was found from 1,150 masl to 2,000 masl in Mount Karimui. Much like the 'I'iwi, the Slaty-Chinned Longbill is mostly nectarivorous; though its exact foraging habits are not completely understood (Gregory, 2020). A nectivorous life history strategy combined with frequent movement along the elevational gradient could in turn potentially expose Slaty-Chinned Longbills to more avian malaria parasites than species like the White-Faced Robin. Future studies using the complete dataset should investigate the importance of life history strategies and elevational range of avian hosts on parasite prevalence, diversity, and host specificity.

These findings offer a preview of the discoveries that await in the entire Karimui dataset ($n = 764$) that remains to be analyzed. Our findings have supplied new data for host specificity and presence along the PNG elevational gradient in nine

previously described parasite lineages (Table 2). We also describe 12 new parasite lineages. This includes: seven *Haemoproteus* lineages, two *Plasmodium* lineages, and three *Leucocytozoon* lineages (Table 3). Our findings also offer clues as to the distribution and presence of insect vectors along the elevational gradient. Some species like the Ashy Robin (*Heteromyias albispecularis*) at the YUS conservation area were infected with *Leucocytozoon* (Table 1). These birds typically inhabit only high montane elevations ($\geq 1,700$ masl) in PNG (Boles & Christie, 2023). This suggests that black flies may exist as competent vectors at high elevations in PNG. These observations and patterns can in turn be put through more rigorous statistical testing with a more robust sample size in the future. Future work on PNG samples and the integration of new parasites into the overall malaria phylogeny will likely shed additional light on the biogeography and evolution of these parasites and their relationships with their avian hosts.

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