ABSTRACT

Title of Dissertation: EMERGING INFECTIOUS DISEASE: HOST AND PARASITE PERSPECTIVES

Jon S. Beadell, Doctor of Philosophy, 2007

Directed By: Dr. Robert C. Fleischer, Head, Center for Conservation and Evolutionary Genetics, National Zoological Park

Dr. Gerald S. Wilkinson, Professor
Behavior, Ecology, Evolution & Systematics

Avian malaria and related haematozoa are nearly ubiquitous parasites that can impose fitness costs of variable severity and may, in some cases, cause substantial mortality in their host populations. One example of the latter, the emergence of avian malaria in the endemic avifauna of Hawaii, has become a model for understanding the consequences of human-mediated disease introduction. The drastic declines of native Hawaiian birds due to avian malaria provided the impetus for examining more closely several aspects of host-parasite interactions in this system. Host-specificity is an important character determining the extent to which a parasite may emerge. Traditional parasite classification, however, has used host information as a character in taxonomical identification, potentially obscuring the true host range of many parasites. To improve upon previous methods, I first developed molecular tools to identify parasites infecting a particular host. I then used these molecular techniques to

characterize host-specificity of parasites in the genera *Plasmodium* and *Haemoproteus*. I show that parasites in the genus *Plasmodium* exhibit low specificity and are therefore most likely to emerge in new hosts in the future. Subsequently, I characterized the global distribution of the single lineage of *P. relictum* that has emerged in Hawaii. I demonstrate that this parasite has a broad host distribution worldwide, that it is likely of Old World origin and that it has been introduced to numerous islands around the world, where it may have been overlooked as a cause of decline in native birds. I also demonstrate that morphological classification of *P. relictum* does not capture differences among groups of parasites that appear to be reproductively isolated based on molecular evidence. Finally, I examined whether reduced immunological capacity, which has been proposed to explain the susceptibility of Hawaiian endemics, is a general feature of an "island syndrome" in isolated avifauna of the remote Pacific. I show that, over multiple time scales, changes in immune response are not uniform and that observed changes probably reflect differences in genetic diversity, parasite exposure and life history that are unique to each species.

EMERGING INFECTIOUS DISEASE: HOST AND PARASITE PERSPECTIVES

By

Jon Sumner Beadell

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2007

Advisory Committee:
Professor Gerald S. Wilkinson, Chair
Professor Robert C. Fleischer, Co-Advisor
Professor Dina M. Fonseca
Professor Matthew P. Hare
Professor David J. Hawthorne
Professor Sarah A. Tishkoff

Preface

This dissertation is comprised of an introduction and four chapters. Chapters I (*Journal of Parasitology*. 2005. 91, 683-685), II (*Molecular Ecology*. 2004. 13, 3829-3844) and III (*Proceedings of the Royal Society London B*. 2006. 273, 2935-2944) are presented in the formats in which they were originally published. Chapter IV is formatted for submission to *The American Naturalist*. A single bibliography provides information on references cited throughout the dissertation.

Dedication

In memory of Far Far and Grandpa Chuck.

Acknowledgements

I am grateful to my advisor Dr. Robert Fleischer for opening the door to evolutionary genetics, for his generosity, for making available numerous opportunities to expand my horizons, and for his unfailingly positive view towards my work. Thanks, too, to my co-advisor Dr. Gerald Wilkinson and to Lois Reid for guiding an absentee student through the mechanics of graduate life. And for making 5 years of life at the zoo a fun and productive time, I am especially indebted to my pipette-wielding labmates at the Genetics Program.

Thank you to Colm Atkins, Farah Ishtiaq, Carl McIntosh and Jen Reed for their help in the lab and especially to Erin Cashion and Michelle Jonker for their valiant efforts in the field. Drs. Estelle Russek-Cohen and Larry Douglass provided invaluable statistical advice. And many thanks to the long list of people in Hawaii, Australia, French Polynesia, Christmas Island and even further afield who helped me coordinate fieldwork, provided tissues or data, or consulted on methodologies.

My work was funded by the University of Maryland Biology Department (block grant), National Science Foundation (graduate fellowship, doctoral dissertation improvement grant, and grant to R. Fleischer), National Institutes of Health (grant to R. Flesicher), Smithsonian Institution (predoctoral fellowship and Abbott Fund grant), Cosmos Club Foundation and the American Ornithologists' Union.

Table of Contents

List of Tables

List of Figures

Figure 3. Relationships among hematozoan parasites based on maximum likelihood using the model GTR + I + G. Lineage number and host species (color-coded for family) are indicated at right. Red branches indicate bootstrap support greater than 70% (stepwise addition, 100 replicates).

Figure 4. Logistic regression curves relating the predicted probability of host relatedness to genetic differentiation of parasites in the genus *Plasmodium* (P) and *Haemoproteus* clade A (HA) and clade B (HB). Dotted lines indicate the genetic distance at which parasite pairs from clades A and B were equally likely to be found in hosts of the same or different families.

CHAPTER III

Figure 1. Phylogenetic relationships among morphologically-identified species of *Plasmodium* estimated using ML, MP and ME with cytochrome *b* sequences. Numbers above branches indicate bootstrap support based on 1000 replicates. Numbers before species names correspond to mitochondrial lineage numbers in figure 3. Sequences were obtained directly from Genbank (accession number in italics) or from extracts of blood smears obtained from the International Reference Collection for Avian Haematozoa (IRCAH) and M. Peirce.

Figure 2. Map depicting the global distribution of the single mitochondrial lineage of malaria parasite (GRW4) found in resident Hawaiian passerines. Pie charts indicate

the proportion of all sequenced *Plasmodium* infections in a given region that were identical to GRW4 (red). Details concerning locations and sampling effort are in table 1. Red dots indicate additional locations in which GRW4 has been reported previously (Ishtiaq *et al.*; Mendes e*t al.* 2005; Schrenzel *et al.* 2003; M. Kimura pers. comm.) or in which GRW4 was recovered from a relatively small group of samples (Kazakhstan).

Figure 3. Phylogenetic trees of parasite mitochondrial lineages (cyt *b*; left; numbered) and associated nuclear haplotypes (DHFR-TS; right; lettered), constructed using maximum likelihood (GTR+I+G for both markers). The distribution of mitochondrial lineages across global regions is indicated with squares, color-coded to help identify the associated DHFR-TS sequence (when available, otherwise black). Background shading reflects the limits of two clades with good support in analysis of combined data (figure 4). Mitochondrial lineages which derived from at least one parasite identified as *P. relictum* by morphology are indicated in bold. Bootstrap support values (>60) are indicated above branches.

Figure 4. Majority rule consensus tree of avian malaria parasite lineages generated by Bayesian analysis of combined mitochondrial (cytb *b*) and nuclear (DHFR-TS) sequence. Parasite genotypes are identified by their respective cytb *b* lineage (number) and DHFR-TS haplotype (letter), which are depicted separately in figure 3. Clade credibility values are indicated above branches. Background shading identifies two well-supported clades (A and B) referenced in the text. Dots within Clade A

indicate nodes for which New World (open circle) or non-New World (black) origin could be confidently assigned based on ancestral trait reconstruction performed with Mesquite.

Figure 5. Statistical parsimony network of *Plasmodium* mitochondrial lineages related to the Hawaiian strain (lineage 15). Sampled haplotypes are numbered as in figure 3 and inferred haplotypes are indicated by black dots. Shading indicates whether the lineage was detected in the Old World (white), New World (black) or in both regions (grey). Lineages 9, 19, and 36 were not included due to missing sequence. Lineages 7, 8, and 29 were joined at the 90% connection limit (13 substitutions).

CHAPTER IV

Figure 1. Prevalence of the haematozoan parasites *Plasmodium* spp. (black), *Haemoproteus* spp. (light grey) and *Leucocytozoon* spp. (dark grey) across mainland and island populations of *Z. lateralis*, *N. temporalis*, and *Acrocephalus* spp. Asterisks indicate significantly lower parasite prevalences in recently introduced island populations (*Haemoproteus* spp. in *Z. lateralis*) or island endemic species (*Plasmodium* spp. in *A. aequinoctialis*, above, and *A. rimitarae*, below) relative to their mainland counterparts.

Figure 2. Cell-mediated (PHA-induced patagial swelling) and innate (agglutination and lysis of rabbit red blood cells) measures of immune response in endemic and

recently-introduced island taxa (circles) relative to a mainland control (centered at zero). Numbers indicate sample size and standard error is depicted by vertical bars, or by grey shading in the case of the mainland control. Filled circles indicate a significant difference from the mainland control.

INTRODUCTION

"The role of disease in wildlife conservation has probably been radically underestimated" – Aldo Leopold, 1933

Infectious disease, long recognized as a threat to human health and an economic problem in domestic animals, has increasingly been acknowledged as a major factor driving ecological and evolutionary processes in wild animal populations (Deem et al. 2001). In the decades immediately following Leopold's observation, the role of disease in wildlife population dynamics remained somewhat obscure due in part to the absence of proper tools to detect and identify pathogens and skepticism on the part of some researchers that disease could be important in regulating wild populations (reviewed in Price 1991, Toft 1991). The advent of molecular biology, however, has allowed for agents of disease to be identified and traced on an increasingly fine scale. In addition, theoretical models have increasingly revealed the role parasites can have in the regulation of host populations (May and Anderson 1983). And over the past several decades, globalization of travel and trade, increasing encroachment of a burgeoning human populace into previously isolated habitats, monotypic agricultural and animal husbandry practices, and changes in climate have precipitated the emergence of numerous infectious diseases with undeniably catastrophic consequences for both humans and wildlife (Daszak et al. 2000, Woolhouse and Gowtage-Sequeria 2005, Pearce-Duvet 2006).

Emerging disease may be broadly defined as any infectious agent that has been recently discovered, is newly evolved, or has recently increased in incidence, geographical distribution or host distribution (Cleaveland et al. 2001). Factors leading to emergence may be inherent to the host (e.g., immunosuppression due to increased exposure to pollution or stress) or the parasite (e.g., mutation, selection for higher virulence in the presence of a competing parasite) as well as extrinsic elements such as changes in the distribution, behavior or competency of vectors or alternative hosts. Although infectious disease may be extremely detrimental to wild populations, empirical data and theory both suggest that disease by itself is generally not capable of driving populations to extinction. Smith et al. (2006) reported that disease was a contributing factor in fewer than 4% of all extinctions known to have occurred in the last 500 years and that fewer than 8% of critically endangered taxa are threatened by disease. And in fact, while hypothesized to be involved in several extinctions (de Castro and Bolker 2005), infectious disease has been demonstrated to be the ultimate cause of extinction only in the case of a captive remnant population of Polynesian tree snail (due to a microsporidian parasite; Cunningham and Daszak 1998) and one wild frog species (due to fungal infection; Schloegel et al. 2006). Theoretical models predict that host-specific diseases that cause high mortality and exhibit density-dependent transmission are likely to die out as the host population falls below some threshold density (Anderson and May 1992). Therefore, infectious disease may be more important in driving populations to small sizes at which point stochastic fluctuations in demographics or environment, allee effects, or genetic complications of inbreeding provide the ultimate force behind extinction.

Examples of emerging diseases that have detrimentally impacted wildlife include chytridiomycosis (fungi) in amphibians, rinderpest in African ruminants, phocine distemper in North Sea seals, rabies in African wild dogs and Ethiopian wolves, and myxomatosis in European rabbits (summaries in Cleaveland et al. 2001, Dobson and Foufopoulos 2001, Daszak et al. 2003). In birds, several diseases have come to public awareness not only because of their impact on their hosts, but also their potential to spillover into humans (e.g. HPAI H5N1, SARS, West Nile Virus). Other notable disease outbreaks (reviewed in Cooper 1993, Friend et al. 2001) have driven the decline of house finches on the eastern seaboard of the U.S. (mycoplasmosis; Hochachka and Dhondt 2000), pelicans in the Salton Sea (botulism), and the honeycreepers of Hawaii (avian malaria; Warner 1968, van Riper et al. 1986). This last example, the human-mediated introduction of avian malaria into the native avifauna of Hawaii, represents one of the best known examples of emerging disease and provides the motivation for the work described in this dissertation.

Avian malaria (family Plasmodidae) and related haematozoan parasites in the families Haemoproteidae and Leucocytozoidae have been detected on every continent except Antarctica. Excluding a few host taxa restricted to extreme arctic environments (Bennett et al. 1992) and perhaps several island taxa, the vast majority of bird species are host to avian haematozoa with estimated prevalences based on microscopy averaging about 10% (Janovy 1997). Recent application of sensitive molecular techniques to the detection of haematozoa have indicated that actual prevalences are probably substantially higher (Jarvi et al. 2003, Kimura et al. 2006) though these values can vary dramatically with host age class (Mendes et al. 2005),

season of sampling (Fallon et al. 2004) and geographical location (Bensch and Åkesson 2003). Molecular techniques have also revealed a previously unrecognized diversity of parasite lineages (Perkins 2000, Ricklefs and Fallon 2002), calling into question previous species limits and raising the possibility that haematozoan species diversity is on the order of avian species diversity (Bensch et al. 2004). Supporting this hypothesis are surveys of single host species that have recovered between 5 and 26 distinct parasite mitochondrial lineages (Bensch et al. 2007, Durrant et al. unpublished, Fallon et al. 2006, Ishtiaq et al. 2006, Kimura et al. 2006) and initial indications that mitochondrial lineages may represent good species (Bensch et al. 2000).

Direct measures of haematozoon infection intensity (Bensch et al. 2007), as well as the discrepancies typically encountered when detecting parasites by microscopy and PCR (Bentz et al. 2006), suggest that most infected birds carry low level chronic infections. While it is hypothesized that these infections are unlikely to have serious consequences for host fitness (Valkiunas 2005), quantifying non-lethal effects of infection in wild populations is notoriously difficult and therefore, the true impact of haematozoans is likely to be blurred. At least several studies have demonstrated negative consequences of haematozoan infection for survival, clutch size, incubation period, fledging success, motor activity and fat accumulation (Bennett et al. 1993, Gustafsson et al. 1994, Nordling et al. 1998, Merino 2000, Valkiunas 2005) though numerous other studies have yielded more equivocal results. Bennett et al. (1993) suggested that severe mortality attributable to haematozoan parasites appears to be limited to cases involving domesticated birds or species

introduced to exotic ranges. If haematozoa are important in limiting the range of most bird species, as hypothesized by Bennett et al. (1993), then changes to the distribution of local haematozoan faunas could have disastrous consequences for bird populations worldwide. High mortality observed upon translocation of presumably naïve species to foreign zoos (penguins, reviewed in Valkiunas 2005; New Zealand avifauna, Bennett et al. 1993, Tompkins and Gleeson 2006) and upon introduction of malaria to Hawaii provide experimental support for this possibility.

Unlike most continental species, the native birds of Hawaii were likely not exposed to malaria until some time in the early 19th century, when mosquitoes were first introduced to the Hawaiian Islands (see Fonseca et al. 2006). Once established, the presence of a suitable vector (*Culex quinquefasciatus*) allowed for colonization of the islands by the parasites themselves, which may have arrived via migratory ducks and shorebirds or via the importation of domestic fowl, gamebirds and caged song birds (Warner 1968). Preliminary screening of historical specimens suggests that avian malaria only arrived as late as the middle of the twentieth century (Fleischer et al. unpublished data). Many native bird species, apparently unusually susceptible to disease and already decimated by the introduction of mosquito-vectored pox virus, experienced further declines and possible extinction as a result of exposure to malaria. Disease-driven extinction in this case may have been made possible by the presence of competent alternative hosts (non-native birds) which are largely unaffected by malaria (van Riper et al. 1986, Jarvi et al. 2003) yet provide a large reservoir of parasites which may then be transmitted to native hosts. The presence of alternative host reservoirs provides an important exception to models which predict

the density-dependent decline of parasite impact along with the host (Smith et al. 2006). The extreme declines observed in Hawaii raise the following questions: To what extent can avian malaria exploit various hosts and thus, to what extent does avian malaria present an emerging threat to bird populations elsewhere across the globe? What is the identity of the parasite introduced to Hawaii? What is the geographical distribution of the Hawaiian parasite, what is its prevalence, and can we use this information to infer anything about its virulence outside of Hawaii? And finally, are other remote avifaunas of the Pacific likely to exhibit susceptibility similar to that of the Hawaiian honeycreepers? These questions are addressed in the chapters that follow.

In Chapter I, I describe the development of a PCR assay that allows for the rapid and sensitive detection of haematozoan DNA within a sample extracted from bird tissue or blood. This method is an improvement upon previous assays based on microscopy, which can drastically underestimate the prevalence of chronic infections (Jarvi et al. 2003) and other PCR methods, which are inefficient because they employ nested amplifications (Valkiunas et al. 2006) or target relatively large fragments. The assay described in this chapter targets a small conserved fragment of mitochondrial DNA identified by sequencing full mitochondrial genomes from five avian haematozoan parasites. The assay also employs restriction fragment length polymorphism to provide a genetic fingerprint that is diagnostic of the genus of parasite present in an infected sample.

In Chapter II, I investigate the degree to which haematozoan parasites are constrained to a particular host species or family. Most emerging diseases infect a

broad range of hosts (Taylor et al. 2001, Woolhouse and Gowtage-Sequeria 2005), and therefore, host-specificity is likely a key factor determining both the extent to which a parasite may "emerge" from its typical environment and also, whether density-dependent effects associated with the decline of a primary host are likely to extinguish the pathogen threat. An initial application of molecular techniques to this question may have been misleading due to spartan sampling and the fact that the authors neglected to distinguish between haematozoan genera (Ricklefs and Fallon 2002). Here, I present the results of a parasitological survey of closely related hosts from several avian families found in a single geographic region. I show that host-specificity of *Haemoproteus* spp. is high relative to *Plasmodium* spp. at multiple depths within the parasite phylogeny. This work highlights the potential for *Plasmodium* spp. to emerge as a problematic parasite in novel hosts. In addition, I show that host-specificity is not uniform even across lineages of *Haemoproteus*, suggesting that parasite differentiation has been the result of periodic host-switching events, followed by vicariance.

In Chapter III, I use mitochondrial and nuclear markers to track the origins and spread of *Plasmodium relictum*, a species of avian malaria that emerged in the naïve avifauna of Hawaii. First I demonstrate that the Hawaiian parasites exhibit a uniform genetic signature and therefore, may be defined much more narrowly than the collection of parasites encompassed by the morphological species *P. relictum*. Using this genetic signature, I show that Hawaiian-like parasites are distributed across the globe, and currently occur on several other remote islands which could potentially share a similar history of avian demise. Finally, I use phylogenetic reconstructions of

additional parasite lineages recovered from a survey of over 13,000 specimens to show that the Hawaiian parasite likely originated from an Old World source. This, in turn, may influence conclusions regarding the evolution of virulence.

Finally, in Chapter IV, I approach the topic of emerging disease from the perspective of a putatively naïve island avifauna. Here I ask the questions a) Is loss of immune function a component of an "island syndrome"?, and b) On what time scale do changes in immunity occur? To answer these questions, I characterized parasite exposure, genetic diversity and immune response in recently-introduced and endemic island taxa and compared the results to related mainland counterparts. As expected, I found that parasite exposure on the islands was generally lower than on the mainland and indices of genetic diversity also tended to be lower on the islands, particularly for long-term endemic residents. Interestingly, immune response in the island taxa was not necessarily lower than mainland taxa and the response was not uniform even among island taxa with similar residence times.

CHAPTER I

A restriction enzyme-based assay to distinguish between avian hemosporidians

ABSTRACT

We describe a reliable and relatively inexpensive method for detecting and differentiating between the commonly studied avian blood parasite genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*. The assay takes advantage of a *Haemoproteus*-specific restriction site identified by sequencing full mitochondrial genomes from two *Haemoproteus* and three *Plasmodium* lineages and an adjacent genus-specific restriction site identified in *Leucocytozoon* spp. The assay was sensitive to parasitemias of about 8×10^{-6} and was 100% accurate in differentiating between parasite genera isolated from a broad geographical and taxonomic sampling of infected hosts.

MAIN TEXT

Avian hemosporidia in the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* are widespread blood parasites occurring in diverse host species. The 3 genera are closely related evolutionarily (Perkins and Schall, 2002), but are characterized by considerable differences in ecology and life history. *Plasmodium* spp. are transmitted by culicine mosquitoes, whereas *Haemoproteus* spp. are transmitted by hippoboscid

and ceratopogonid flies, and *Leucocytozoon* spp. are vectored by simuliid flies. Species of *Plasmodium* generally exhibit broader host specificity (Atkinson and van Riper III, 1991) while both *Plasmodium* spp. and *Leucocytozoon* spp. are considered to be more pathogenic (Bennett, Peirce, and Ashford, 1993) than *Haemoproteus* spp. All 3 genera undergo alternating cycles of sexual and asexual reproduction, but only species of *Plasmodium* undergo schizogony in circulating erythrocytes (Atkinson and van Riper III, 1991), which leads to the symptoms of malaria (Garnham, 1966).

Recently, the polymerase chain reaction (PCR) has become a valuable tool for detecting these parasites and sequencing has uncovered a wealth of genetic diversity (Bensch et al., 2000; Ricklefs and Fallon, 2002; Schrenzel et al., 2003). PCR screens offer increased sensitivity over traditional analysis of blood smears (Feldman et al., 1995; Cann et al., 1996; Richard et al., 2002); however, the parasites (most often *Haemoproteus* spp. and *Plasmodium* spp.) are typically amplified indiscriminately, and identification to genus requires costly sequencing. Lineage-specific information may be useful when monitoring an epidemic or measuring migratory connectivity (Webster et al., 2002), but often, ecologists, wildlife managers, and zookeepers may benefit from genus-level knowledge of parasites present in an avian community. Here, we report the development of a relatively inexpensive restriction enzyme-based diagnostic for detecting and identifying *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp. We have confirmed the utility of the test by correctly identifying the genus of genetically diverse parasite lineages from a broad taxonomic and geographic sampling of hosts.

We initially searched for a method to distinguish *Haemoproteus* spp. and *Plasmodium* spp., the two most common genera, and the two most commonly co-amplified by existing PCR methods. To ensure that candidate nucleotide polymorphisms separating parasite genera fell in relatively conserved regions of DNA, we first sequenced the 6-kilobase mitochondrial genome of 5 avian parasite lineages. These included 2 lineages of *Haemoproteus* and 3 lineages of parasite putatively identified as *P. relictum* by morphology (Table I), but known to be genetically divergent (McConkey et al., 1996; J. Beadell unpubl. obs.). We extracted DNA using Qiaquick Dneasy kits (Qiagen, Valencia, California). We designed primers (available upon request) based on the sequences of relatively conserved regions of previously published mitochondrial genomes from mammalian malaria parasites. Primer pairs typically amplified between 500 and 600 base pairs (bp) and were spaced so that approximately 30 to 50 bp (excluding primed sequence) from one segment overlapped with the adjacent segment. Amplified DNA was sequenced bi-directionally on an ABI 377 (Applied Biosystems, Inc., Foster City, California). Sequences were assembled, aligned and edited using the program SEQUENCHER version 4.1. Further alignment was performed by eye when necessary. Sequences of the five mitochondrial genomes have been deposited in GenBank (Accession numbers AY733086 to AY733090, Table I).

Comparison of the frequency of insertions/deletions and nucleotide substitutions across the 5 genomes with the location of mitochondrial gene sequences (COI, COIII, CYb) identified in *P. falciparum* (AY283008, Joy et al., 2003) suggested that these regions were least conserved across hematozoan genera (Fig. 1).

Average uncorrected pairwise divergence among the 5 genomes was 2.5% outside gene-coding regions versus 7.7% within. Therefore, we targeted our search for polymorphisms to non-gene-coding regions where polymorphisms between genera were most likely to be fixed. We describe the resulting diagnostic below.

We designed primers 213F (5'- GAG CTA TGA CGC TAT CGA -3') and 372R (5'- GGA ATG AGA GTT CAC CGT TA -3') to amplify a 160 bp fragment of DNA encoding an Xmn I restriction site unique to *Haemoproteus*. Both primers shared 100% identity with corresponding sequence of mitochondrial DNA from the parasites listed in Table I, and the final fragment size was minimized so as to improve the likelihood of recovering PCR product from old and potentially degraded samples. We employed a PCR scheme typical of "ancient DNA" amplifications: initial denaturing step at 94 C for 8 min, 45 cycles of 92 C for 30 sec, 52 C for 30 sec, 72 C for 30 sec, and a final extension at 72 C for 7 min. We amplified 1.8 μl of DNA in a reaction volume of 25 μl, using final concentrations of 1X PCR Gold Buffer (Applied Biosystems, Inc.), 0.8 mM dNTPs, 2.0 mM $MgCl_2$, 0.8 mg/ml BSA, 0.6 μM each primer and 0.5 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Inc.). We digested 5.0 μl of the PCR product in a total volume of 10 μl for 3 hr at 37 C, using 1 U of restriction enzyme Xmn I, 0.1 mg/ml BSA and 1X NEBuffer 2 (New England Biolabs, Beverly, MA). We electrophoresed the digested product at 80 volts on a 4% 3:1 agarose gel (Amresco, Solon, OH) with 0.2X Gel Star (Cambrex Bio Science Rockland, Inc., Rockland, ME). PCR products derived from *Plasmodium* spp. remained undigested while PCR products derived from *Haemoproteus* spp. were

cleaved into fragments of 121 and 39 base pairs (Fig. 2). The smallest band was typically faint or indistinguishable from primer artifacts.

We tested the method described above on DNA extracted from 33 infected birds representing 21 families from 3 continents (Appendix I). The genus of the infecting parasite had been determined by prior sequencing and phylogenetic analysis of a 295 base pair segment of cytochrome b. Uncorrected pairwise divergences ranged from 1.4 to 8.5% (average = 5.7%) among *Plasmodium* lineages tested (excluding samples from *Schoeniophylax phyganophila* and *Gnorimopsar chopi* for which the full 295 bp was not available) and from 0 to 9.5% (average = 5.2%) among *Haemoproteus* lineages. The diagnostic successfully resolved the genus of infection in all cases (17 *Plasmodium* sp. and 16 *Haemoproteus* sp.). Because the diversity of lineages tested within each genus resembled that recovered in other surveys of hematozoa (Bensch et al., 2000; Ricklefs and Fallon, 2002; Schrenzel et al., 2003), the test should be applicable on a broad scale. The test may also detect double infections by both genera when template DNA for each parasite occurs in sufficient concentration. We tested 4 samples for which prior sequencing and cloning had revealed infection with both *Haemoproteus* and *Plasmodium*. In each case, 2 bands were evident on the agarose gel (Fig. 2). Underdigestion of PCR products could also result in the appearance of a multi-genus infection, however, we did not observe double bands among the 33 samples tested above. The use of excess restriction enzyme and long incubation times should minimize these artifacts.

We subsequently used primers 213F/372R to amplify DNA from diverse lineages of *Leucocytozoon* spp. extracted from blood smears (Appendix 2).

Sequencing revealed an absence of the Xmn I site, and digestion with Xmn I confirmed that the PCR products were indistinguishable from the products obtained from *Plasmodium* spp. However, comparison of the sequences with those from the lineages of *Haemoproteus* and *Plasmodium* tested above revealed an Xba I site unique to lineages of *Leucocytozoon*. Digestion of PCR products from each of the *Leucocytozoon* spp. with Xba I under conditions identical to those described above produced 2 fragments of about 109 and 54 bp (small size differences arose from single base indels at two sites within the larger fragment), while digestion of the products obtained from *Haemoproteus* spp. and *Plasmodium* spp. resulted in no cleavage. The use of Xmn I and Xba I together, therefore, generated a diagnostic molecular fingerprint for the three hemosporidian genera (fragments of approx. 160 bp for *Plasmodium* spp., 121 bp for *Haemoproteus* spp., 109 and 54 bp for *Leucocytozoon* spp.) when PCR products were electrophoresed on a 4% agarose gel (Fig. 2).

To determine the utility of these primers in detecting low-level infection, we tested the primers on extractions of serial dilutions of duck blood containing the Hawaiian lineage of *P. relictum*. We estimated the parasitemia of the stock blood to be 3.23% by scanning 30 fields at 400x magnification. We consistently amplified extractions of stock blood diluted as much as 4,000-fold; however, amplification of more dilute DNA was sporadic or impossible. This level of sensitivity is similar to that for a protocol developed by Fallon et al. (2003), which outperformed previously published methods. Serological assays may be even more sensitive (Jarvi et al., 2002); however, any single serological assay is specific for a limited set of lineages

and may detect both current and previous infections. PCR-based methods of detection may be imperfect (Freed and Cann, 2003), but using multiple primer sets can help reduce false negatives. We suggest that the assay described here will complement previous methods by providing a relatively sensitive method for detecting current hematozoan infection and a robust and cost-effective means for identifying *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp.

We also note here that divergences (based on mean character differences) between mitochondrial lineages from the three parasites identified by morphology as *P. relictum* ranged from 3.4 to 3.9%. By comparison, *P. reichenowi* (AJ251941, Conway et al., 2000) and *P. falciparum* (AY283008, Joy et al., 2003), parasite species whose primary hosts are chimpanzees and humans, respectively, exhibited only 2.3% sequence divergence across their entire mitochondria. These data lend further support to authors who have questioned the phylogenetic significance of previous parasite classifications based on morphology (Manwell, 1936; Bensch et al., 2000; Perkins and Schall, 2002). If mitochondrial lineages are indicative of species-level differentiation, then extreme care should be taken in assessing evolutionary relationships or tracking the epidemiology of hematozoan parasites based solely on morphological and ecological phenotypes. This is particularly true for *P. relictum*, which has been implicated in the decline of Hawaii's native avifauna (van Riper et al., 1986; Atkinson et al., 1995), but has also been reported in blood smears from a broad distribution of avian hosts around the world (Bennett et al., 1993).

TABLE

Table I. Host, geographical origin, and sources of parasite lineages for which full mitochondrial genomes were sequenced.

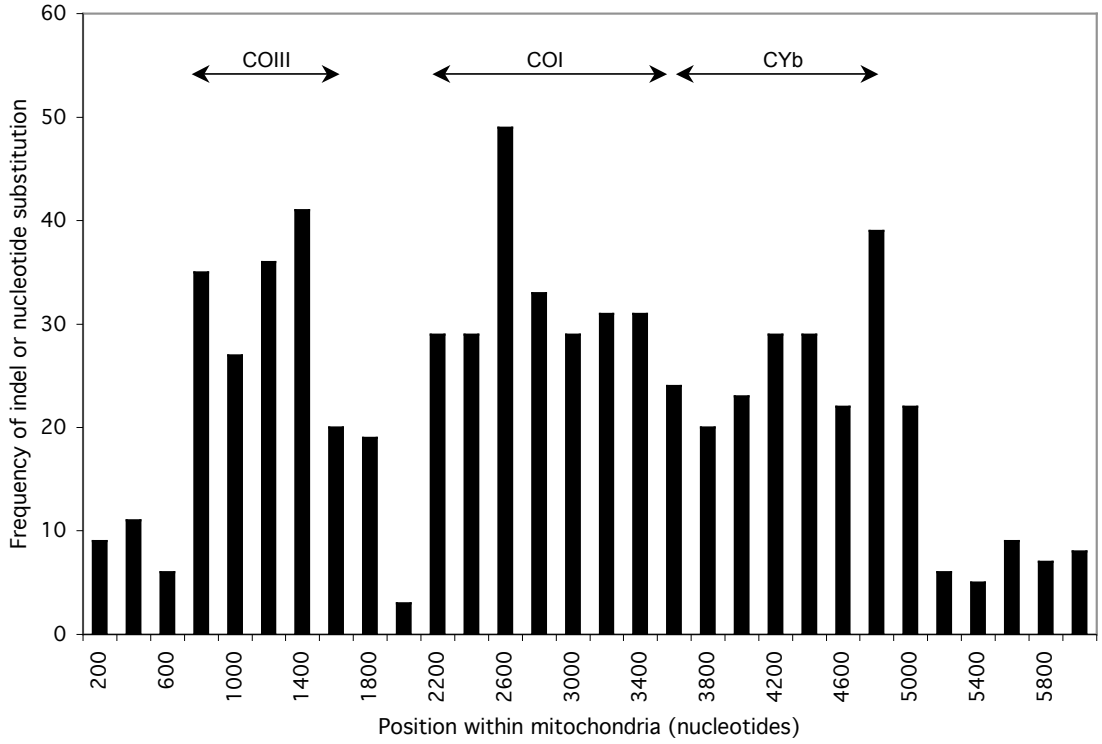
Lineage	Parasite name	Host name		Geographical origin	Collector	GenBank accession no.
		Scientific	Common			
1	*Haemoproteus sp.*	*Meliphaga lewinii*	Lewin's honeyeater	Queensland, Australia	J. Austin	AY733086
2	*Haemoproteus* sp.	*Lichenostomus frenatus*	Bridled honeyeater	Queensland, Australia	J. Austin	AY733087
3	*Plasmodium relictum*	*Spheniscus demersus*	African black-footed penguin	Baltimore Zoo, Maryland, U.S.A.	M. Cranfield	AY733088
4	*Plasmodium relictum*	*Zenaida macroura*	Mourning dove	Nebraska, U.S.A.	ATCC #30141	AY733089
5	*Plasmodium relictum*	*Hemignathus virens*	Amakihi	Hawaii, U.S.A.	R. C. F.	AY733090

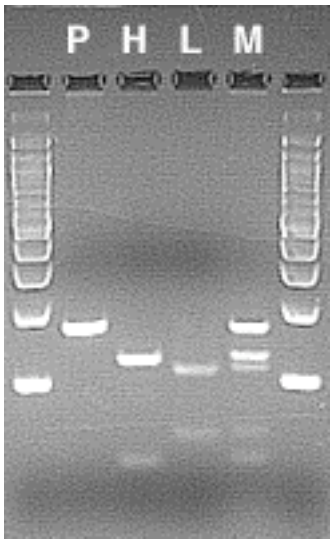
FIGURE LEGENDS

Figure 1. Frequency of insertions/deletions and nucleotide substitutions across five mitochondrial genomes from *Plasmodium* spp. and *Haemoproteus* spp. Upper limits of 200-nucleotide intervals are numbered with respect to positions in *P. falciparum* (AY283008, Joy et al., 2003). The locations of cytochrome *c* oxidase subunits 1 (CO1) and III (COIII) and cytochrome *b* (CYb) are indicated at the top.

Figure 2. Electrophoretic banding patterns indicative of infection with *Plasmodium* sp. (P), *Haemoproteus* sp. (H) or *Leucocytozoon* sp. (L) relative to a 100 base pair ladder. Multi-genus infections (M) may be resolved by electrophoresis of PCR products for 1.3 hours on a 4% agarose gel.

FIGURES


COIII
COI
CYb
Frequency of indel or nucleotide substitution
0
10
20
30
40
50
60
200
600
1000
1400
1800
2200
2600
3000
3400
3800
4200
4600
5000
5400
5800
Position within mitochondria (nucleotides)


P H L M

APPENDICES

Appendix I. Geographical origin and family designation of avian host species with known infections used to test ability of assay to distinguish between *Plasmodium* sp. (P) and *Haemoproteus* sp. (H).

Host	Host family	Parasite
Australia		
Acanthiza katherina	Acanthizidae	P
Sericornis citreogularis	Acanthizidae	H
Sericornis citreogularis	Acanthizidae	H
Meliphaga notata	Meliphagidae	P
Xanthotis macleayana	Meliphagidae	H
Monarcha trivirgatus	Monarchidae	P
Colluricincla megarhyncha	Pachycephalidae	H
Eopsaltria australis	Petroicidae	H
Eopsaltria australis	Petroicidae	H
Pitta versicolor	Pittidae	P
Ailuroedus melanotis	Ptilonorhynchidae	P
Gabon		
Ipsidina lecontei	Alcedinidae	H
Merops breweri	Meropidae	H
Trochocercus nigromitratus	Monarchidae	H
Stiphrornis erythrothorax	Muscicapidae	H

Zoothera cameronensis	Muscicapidae	P
Zoothera cameronensis	Muscicapidae	P
Nectarinia fuliginosa	Nectariniidae	H
Camaroptera brachyura	Sylviidae	P
Illadopsis cleaveri	Timaliidae	H
Illadopsis cleaveri	Timaliidae	P
Illadopsis rufipennis	Timaliidae	P
Uruguay		
Basileuterus culicivorus	Emberizidae	P
Gnorimopsar chopi	Emberizidae	P
Icterus cayanensis	Emberizidae	H
Molothrus badius	Emberizidae	H
Schoeniophylax phyganophila	Furnariidae	P
Mimus saturninus	Mimidae	P
Colaptes campestris	Picidae	H
Polioptila dumicola	Polioptilidae	P
Aramides ypecaha	Rallidae	P
Rallus sanguinolentus	Rallidae	H
Troglodytes aedon	Troglodytidae	P

Appendix II. Host, geographical origin, source and number of *Leucocytozoon* spp. tested in assay.

Parasite	#	Host	Geographical origin	Source
L. mardouxi	2	*Nesoenas mayeri*	Mauritius	International Reference Centre for Avian Haematozoa, Brisbane, Australia
L. toddi	2	*Milvago chimango*	Chiloe Island, Chile	U.S. National Parasite Collection, Maryland, U.S.A.
Leucocytozoon sp.	1	*Carduelis spinus*	Lithuania	G. Valkiunas
Leucocytozoon sp.	1	*Sylvia borin*	Lithuania	G. Valkiunas
Leucocytozoon spp.	6	*Junco hyemalis*	Alaska, U.S.A.	P. Deviche

CHAPTER II

Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region

ABSTRACT

The degree to which widespread avian blood parasites in the genera *Plasmodium* and *Haemoproteus* pose a threat to novel hosts depends in part on the degree to which they are constrained to a particular host or host family. We examined the host distribution and host-specificity of these parasites in birds from two relatively understudied and isolated locations: Australia and Papua New Guinea. Using PCR, we detected infection in 69 of 105 species, representing 44% of individuals surveyed (n = 428). Across host families, prevalence of *Haemoproteus* ranged from 13% (Acanthizidae) to 56% (Petroicidae) while prevalence of *Plasmodium* ranged from 3% (Petroicidae) to 47% (Ptilonorhynchidae). We recovered 78 unique mitochondrial lineages from 155 sequences. Related lineages of *Haemoproteus* were more likely to derive from the same host family than predicted by chance at shallow (avg. LogDet genetic distance = 0, n = 12, P = 0.001) and greater depths (avg. distance = 0.014, n = 11, P < 0.001) within the parasite phylogeny. Within two major *Haemoproteus* subclades identified in a maximum likelihood phylogeny, host-specificity was evident up to parasite genetic distances of 0.029 and 0.007 based on logistic regression. We found no significant host relationship among lineages of *Plasmodium* by any method of analysis. These results support previous evidence of strong host-family specificity

in *Haemoproteus* and suggest that lineages of *Plasmodium* are more likely to form evolutionarily-stable associations with novel hosts.

INTRODUCTION

The application of molecular methods to the study of avian hematozoa has revealed surprising levels of genetic diversity. This diversity has been exploited to reveal phylogenetic relationships (Perkins and Schall 2002), assess disease linkage between breeding and wintering grounds (Waldenstroem et al. 2002), and investigate host-parasite fidelity (Bensch et al. 2000, Ricklefs and Fallon 2002, Fallon et al. 2003). This last issue is of particular importance as human activities alter the ranges of vectors and avian hosts, thereby increasing exposure of potential hosts to novel parasites. In Hawaii, the introduction of the malarial parasite *Plasmodium relictum* has been implicated in the decline of native honeycreepers (van Riper et al. 1986). The negative impact of hematozoa introduced to domesticated birds has also been well documented (reviewed in Bennett et al. 1993a); however, discerning the fitness consequences of infections in wild birds with long histories of parasite exposure has been more difficult (Hatchwell et al. 2001, Siikamaki et al. 1997). Predicting the consequences of introduced disease is difficult, but we can begin to assess the chances of an exotic parasite spreading to novel hosts by determining the extent to which that parasite is evolutionarily constrained to a particular host or host family.

Two of the most common and best-studied genera of avian blood parasites are *Plasmodium* and *Haemoproteus*. Earlier studies have suggested that *Haemoproteus* exhibits greater host-specificity than *Plasmodium* (Bennett and Peirce 1988, Bennett et al. 1993b). Traditional means of classifying parasites at the species level, however,

have often included host taxonomy as a character, thereby providing a biased estimate of host-parasite conservatism (Atkinson and van Riper 1991). In addition, reconstructions of parasite phylogenies based on DNA sequences have yielded evolutionary relationships that differ from those derived from traditional classification methods (Escalante et al. 1998).

A recent molecular study of *Haemoproteus* lineages in old world warblers and tits produced discordant host and parasite phylogenies, suggesting frequent host-switching (Bensch et al. 2000). A survey of parasites in African residents and European migrants revealed numerous cases of a single parasite lineage shared by multiple hosts; all *Haemoproteus* lineages were shared among hosts of the same family while at least one *Plasmodium* lineage occurred in multiple host families (Waldenström et al. 2002). On a global scale, Ricklefs and Fallon (2002) demonstrated relative conservatism of host-parasite evolution, but no distinction was made between the specificity of *Plasmodium* and *Haemoproteus*. Here, we attempt to merge the strengths of these studies by investigating host-parasite relationships at several evolutionary depths across multiple well-diversified host families within a single region.

As part of a global survey for the original host and geographical source of the Hawaiian parasite, we examined malarial parasites from a subset of bird species from tropical Australia and Papua New Guinea. To our knowledge, this is the first molecular exploration of host-parasite relationships in this fauna. The avifauna of this region is relatively isolated, both taxonomically and geographically, potentially reducing noise associated with transient introduction of foreign parasites. Prior

surveys for hematozoan parasites have identified *Haemoproteus* and *Plasmodium* in many of the hosts included here, but relatively few parasites have been morphologically identified beyond the genus level (Ewers 1967, Bennett and Campbell 1973, Jones 1985). Our goals were to 1) characterize the prevalence of hematozoa across varied bird families in this region and 2) determine the extent to which *Haemoproteus* and *Plasmodium* differ in host-specificity.

MATERIALS AND METHODS

Sample Collection and Preparation

JA collected blood samples from mist-netted birds in 2002 and 2003 at sites in the Wet Tropics of northeastern Queensland, Australia and at Eungella National Park, which encompasses an isolated fragment of rainforest to the south (Figure 1). Blood smears for 40 samples were fixed with methanol and then stained with Giemsa for 30 min. For each slide, we searched 100 fields at 400x magnification to determine infection status. High resolution digital images of representative parasites were used for final identification.

TP provided blood samples from birds captured in 2003 from the d'Entrecasteaux Islands, Papua New Guinea. JD provided blood and tissue samples of birds netted between 1991 and 2002 from forested sites across the main island of Papua New Guinea.

We extracted host and parasite DNA from blood and tissue samples using the relevant protocols accompanying Qiagen DNeasy kits. Each extraction included a negative control, which was screened for contamination.

Parasite Detection

In order to detect divergent and possibly degraded parasite DNA, we screened samples with two primer sets originally designed to successfully amplify *Haemoproteus* and *Plasmodium* DNA from dried blood smears up to 30 years old: 850F (5'-CTT CAA CTA TTC TTA TAA AGT ATG T-3') with 1024R (5'-AGG TGA GTG TTT TGC ATC ATT-3') and F2 (5'-AAG TGA CCC AAC CTT AAA AAG-3') with R2 (5'-GCT GTA TCA TAC CCT AAA GG-3'). Prior use of these primers in a wide array of avian hosts from varied geographical regions amplified no other hematozoa (e.g. *Leucocytozoon*, *Trypanosoma*, *Hepatozoon*). Primers 850F/1024R and F2/R2 amplify small fragments (167 and 132 base pairs) with homology to portions of mitochondrial cytochrome oxidase III and cytochrome *b* genes (Feagin 1992), respectively. We used annealing temperatures of 50 C and 52 C, respectively, and typical PCR reactions employed conditions developed for amplification of "ancient" DNA (Fleischer et al. 2000).

For those samples that were positive based on the tests above, we amplified a larger fragment of cytochrome *b* (533 bp + primers) for use in phylogenetic analyses using primers 3760F (5'-GAG TGG ATG GTG TTT TAG AT-3') and 4292Rw2 (5'-TGG AAC AAT ATG TAR AGG AGT-3'). If this fragment did not amplify, we attempted to amplify smaller fragments of either 433 bp or 295 bp (+ primers) using either F1 (5'-CAT ATT TAC CTT TAT CAT GGA T-3') or F3 (5'-CCA GGA CTT GTT TCA TGG AT-3') with 4292Rw2. The annealing temperature for these latter reactions was 51 C.

To ensure that DNA extractions were successful for those samples in which we did not detect infection, we amplified a small fragment (268 bp) of avian

cytochrome *b* DNA using primers cytb-2RC and cytb-wow following the methods described in Dumbacher et al. (2003). This amplification was successful in all cases.

Following purification of PCR products using Qiaquick kits (Qiagen), we bi-directionally sequenced the largest fragment available for a given sample on an ABI 3100 Sequencer (Applied Biosystems, Inc.). Sequences were assembled, aligned and edited using the program SEQUENCHER version 4.1. Phylogenies based on cytochrome *b* sequence have consistently recovered two discrete clusters of lineages corresponding to *Haemoproteus* and *Plasmodium* (Bensch et al. 2000, Perkins and Schall 2002). Therefore, we assigned mitochondrial sequences (lineages) to each genus based on their associations in a phylogenetic tree (see below). Inclusion of sequence data from prior studies and morphological assessments of parasites for which we had smears generally allowed easy delineation of the two genera. In cases where limited sequence data did not provide sufficient resolution, we used a restriction enzyme test (JSB and RCF unpubl. data) to assign parasite lineages to genera.

To assess whether prevalence of *Haemoproteus* and *Plasmodium* varied across host families, we performed an ANOVA (GLM in SAS v 8.2, SAS Institute, Inc., Cary, NC) on arcsine square-root transformed prevalences observed at the level of host species. We included only those species from families represented by greater than 10 individuals total (Table 1). We estimated the proportion of variance attributable to host family using the NESTED procedure in SAS.

Cloning

In several cases, we detected multiple infections based on the occurrence of multiple peaks throughout the chromatogram. In these cases, we repeated the PCR and cloned the fragment using a TOPO-TA cloning kit (Invitrogen) following manufacturer guidelines. We picked 6 to 24 blue/white-selected colonies for each fragment cloned, boiled the colonies for 10 minutes, and amplified 2 ul of the resulting lysate for 30 cycles with the relevant primer set. Fragments from successful amplifications were cleaned and sequenced as described above. Inspection of sequences obtained for a given clone, and comparison of those sequences with the original sequence, allowed for easy identification of PCR artifacts arising from polymerase error or *in vitro* recombination (Thompson et al. 2002).

Phylogenetic Analysis

We estimated parasite phylogenetic relationships using all samples for which we had at least 295 base pairs of cytochrome *b* sequence, though 533 bp were available for most samples (see Appendix A). Following the phylogeny developed by Perkins and Schall (2002), all trees were rooted with mammalian *Plasmodium* sequences (GenBank accession nos. AY069614, AF069624, AF055587, AY099051, AY283019, and AF069610). The program ModelTest v3.06 (Posada and Crandall 1998) indicated that the most likely model of base pair substitution was general time reversible (GTR), with the proportion of invariable sites = 0.3604 and gamma shape parameter = 0.5372. We used maximum likelihood (ML) to reconstruct a phylogeny using these parameters. We used 100 replicates and the "fast" heuristic in PAUP* (Swofford 1999) to estimate bootstrap support. We also performed a full heuristic search for the shortest tree using tree-bisection reconnection (TBR) on both GTR and LogDet

(Lockhart et al. 1994) distances. We compared the resulting minimum evolution tree to 1000 trees generated by bootstrap resampling with a TBR heuristic search. Nodes with greater than 50% support were retained.

Host-Specificity

We followed the binomial probability approach of Ricklefs and Fallon (2002) to assess the extent to which parasites of varying relatedness were likely to be found in host species from the same family. Host species were grouped into families as listed with the Handbook of the Birds of the World (2003), but we grouped all kingfishers in the Alcedinidae and included *Rhipidura* fantails within the Monarchidae (Sibley and Ahlquist 1985). First, we tested for a significant difference between the observed and expected probability that a shared parasite lineage (i.e., mitochondrial haplotypes indicated by light blue dots in Figure 2) derived from two host species of the same family. We calculated this separately for shared *Haemoproteus* and *Plasmodium* lineages. In cases where a single parasite lineage was found in more than two host species, we randomly paired hosts to represent that lineage. For example, if a lineage occurred in six different hosts, we randomly paired those hosts to form three observations.

Subsequently, we repeated the analysis using pairs of parasite lineages joined by 1st-step nodes with greater than 70% bootstrap support (dark blue dots in Figure 2). When a 1st-step node joined more than two host taxa, we randomly chose just a single independent pair. To quantify the phylogenetic depth being analyzed, we calculated average pairwise LogDet distances among parasites compared at each level. For all comparisons we used only lineages with greater than 470bp of sequence.

In order to extend the analysis beyond 1st-step nodes and to assess the parasite genetic distance at which host family conservatism was lost, we performed a logistic regression of host family (same or different) versus LogDet parasite distance (Ricklefs and Fallon 2002). We tested for a significant influence of region (same or different) on host family similarity before using the full data set for each application of the model. Logistic regression employs the model $\ln(P/(1-P)) = a + b*d$ where "P" is the probability that two parasites derive from hosts of the same family, "d" is genetic distance, and "a" and "b" are coefficients estimated by the model. We performed this regression on all pairwise comparisons of parasite lineages and their hosts at several levels of evolutionary organization. Because multiple pairwise distance comparisons violate assumptions of independence, we determined significance of the coefficients using a permutation of the original data. We randomly reassigned host families to the parasite phylogeny 999 times and performed logistic regression upon each iteration. Coefficients based on the original data were compared to those generated by randomization in order to estimate the probability of recovering the original estimates by chance alone.

RESULTS

Parasite Prevalence

We used PCR to screen 428 individuals in total. Of 209 individuals from Papua New Guinea, 64 (31%) tested positive for *Haemoproteus* and 20 (10%) tested positive for *Plasmodium*. Of 77 species tested, 46 were positive for one or both genera and we detected infection in all 12 species for which we tested 5 or more individuals. Of 219 individuals tested from Australia, 62 (28%) were positive for *Haemoproteus* and 30 (14%) were positive for *Plasmodium*. We recovered *Haemoproteus* or *Plasmodium* from 27 of 32 species tested, and we found infection in 17 of 19 species for which 5 or more individuals were screened. Chi-squared tests revealed no significant difference in prevalence of either parasite between regions.

Low PCR amplification, poor-quality sequence, or unresolved multiple infections reduced the number of samples for which we could identify parasites to genus, and therefore, estimates of prevalence (Table 1) were biased low. Prevalence of *Haemoproteus*, which ranged from 13% in the Acanthizidae to 56% in the Petroicidae was not uniform across different host families ($F = 3.71$, $df = 7$, $p = 0.002$), however, host family grouping explained only 22% of the total variance in prevalence among different host species. Except in the Ptilonorhynchidae, prevalence of *Plasmodium* was relatively low, and no significant difference was evident among families ($F = 1.39$, $df = 7$, $p = 0.223$). Only about 4% of the variance in prevalence between species could be attributed to host family. Family assignment and frequency of parasite detection for all host species examined is listed in Appendix B.

We detected mixed infections in 29 individuals. Among those with enough sequence data to identify parasite genera present, one individual harbored two *Plasmodium* lineages (66 and 72), 11 harbored two *Haemoproteus* lineages (see below), and four harbored mixed *Plasmodium*/*Haemoproteus* infection (11 and 61, 16 and 72, 21 and 72, 18 and 70). Of the lineages involved in mixed *Haemoproteus* infections, four pairs derived from within well-supported clades composed of non-passerines (3 and 4), Meliphagidae (28 and 29), or Petroicidae (35 and 36, 35 and 37). The remaining pairs (10 and 22, 13 and 37, 14 and 38, 14 and 39 repeatedly) were composed of parasites from each of the two main subclades (see phylogenetic results below). The average LogDet genetic distance between parasite combinations was 0.0623 (n = 1) for mixed *Plasmodium*, 0.0414 (n = 11) for mixed *Haemoproteus*, and 0.1352 (n = 4) for mixed *Haemoproteus/Plasmodium*.

Reliability of Methods

Failure to detect infection by PCR may have been due to low-quality or insufficient template, small daily variation in PCR conditions and reaction composition, and mismatches between the primer and parasite DNA template. To generate a minimum estimate of our detection error, we divided the number of false negatives produced by a given primer set by the total number of samples that were known to be positive by either primer set. By this method, the primer set F2/R2 had an error rate of 30%, while primer set 850F/1024R missed infections at a rate of 17%. Therefore, even under favorable PCR conditions, the chance that both primer sets failed to detect an infection was about 5%.

Estimation of hematozoa presence/absence was identical for 35 of 40 samples analyzed by both PCR and visual inspection of blood smears. PCR screening detected infection in three samples that went undetected by examination of blood smears. Conversely, an initial inspection of blood smears suggested that PCR had missed infections in two samples. Subsequent scanning of the slides by an unbiased second observer (MP), however, suggested that artifacts in these two slides had been misidentified as parasites. In samples where both methods identified a parasite to genus, 7 of 8 matched. The single disparity in genus identification was attributed to a poorly prepared slide and a second appraisal of the slide suggested that the parasite was representative of either *Haemoproteus* or *Plasmodium*. No other hematozoa were observed in blood smears.

Phylogenetics

Among the 165 samples for which we had at least 295 bp of sequence, we found 78 unique mitochondrial lineages: 60 *Haemoproteus* and 18 *Plasmodium* (GenBank accession numbers listed in Appendix A). Lineage 60, isolated from *Macropygia amboienensis*, was included with *Haemoproteus* based on evidence from the restriction assay and morphological assessment of a parasite with a closely related mtDNA sequence (EG unpubl. data). Related lineages have also been found in *Columbina passerina* from North America (unpubl. data) and other doves (S Fallon pers. comm.). Phylogenies developed using ML and LogDet and GTR distances were similar. Because each of these methods yielded similar topologies and for consistency with previous work by Ricklefs and Fallon (2000), we used a tree derived from LogDet distances for tests of host-parasite specificity (Figure 2).

Within *Haemoproteus*, our data could not resolve deep hierarchical relationships, which resulted in a large basal polytomy. Parasites from two non-passerine host families occurred in a unique, well-supported clade (top of Figure 2). Other clades descending from the genus-level polytomy included several which were largely derived from a single host family (Meliphagidae, Petroicidae, Pachycephalidae) and one well-supported clade with diverse host family representation (clade A**)**. Several well-supported host-family-specific clades (Petroicidae, Pachycephalidae, Monarchidae) were nested within clade A. An ML estimate of the phylogeny (Figure 3) identified three major clades within *Haemoproteus*: two lineages derived from passerine hosts (clades A and B) and a third composed of lineages from the two non-passerine families studied. Bootstrap support was relatively low for all but the non-passerine clade. The ML phylogeny also indicated monophyly of all unshared parasites recovered from Meliphagidae.

Deeper level relationships among *Plasmodium* lineages were similarly unresolved in a distance-based phylogeny (Figure 2). Beneath the genus-level polytomy, only a pair of lineages (64 and 65) from Meliphagidae fell into a small well-supported host-specific clade.

Host-Specificity

We found 12 *Haemoproteus* lineages that were each shared by two different host species and we found 6 *Plasmodium* lineages in more than one host species. Three of these *Plasmodium* lineages were each found in three to six host species. Related lineages of *Haemoproteus* were more likely to be found in related hosts than predicted by chance. At average parasite genetic distances of 0 (shared identical

lineages) and 0.014 (1st-step nodes), the probability of related parasites deriving from the same host family was 0.58 (n = 12, P = 0.001) and 0.73 (n = 11, P < 0.001), respectively.

Sample sizes for comparisons within the *Plasmodium* genus were smaller. The probability that a shared *Plasmodium* lineage derived from the same host was 0.13 (average distance = 0, n = 8, P = 0.65). This value was not significant even if pairs of hosts were chosen so as to maximize the probability (probability = 0.38, n = 8, P = 0.11). Similarly, sister lineages joined by 1st-step nodes were not significantly likely to have derived from the same host family (average distance = 0.008, probability = 0.33, n = 3, P = 0.61).

We applied logistic regression to four groups of parasites: all *Plasmodium* lineages, all *Haemoproteus* lineages, *Haemoproteus* clade A, and *Haemoproteus* clade B. Because "region" did not contribute significantly to the regression of host family on distance, we considered Australia/Papua New Guinea to be one region for all logistic regression analyses. Regression coefficients for the genus *Haemoproteus* were significant (a = 0.1909, b = -38.57, P < 0.001) as were coefficients for clade A (a = 1.5701, b = -214.6, P < 0.001) and clade B (a = 1.5513, b = -53.7664, P <0.001). Coefficients for the genus *Plasmodium* were not significant (a = -1.7248, b = -1.8373, P = 0.63). By evaluating the regression equation at P = 0.5, we could estimate the genetic distance at which pairs of parasite lineages were equally likely to have derived from the same or different host family. This distance at which host-family signal was lost was 0.005 for all *Haemoproteus* lineages, 0.007 for clade A, and 0.029 for clade B. Evaluating the regression equation at a distance of zero, the predicted

probability of finding identical parasites in hosts of the same family was 0.55 evaluated over all *Haemoproteus* lineages, 0.83 for clade A, and 0.83 for clade B. Figure 4 depicts the predicted regression curves for *Plasmodium* and *Haemoproteus* clades A and B.

DISCUSSION

Epizootiology

Blood parasites in the genera *Haemoproteus* and *Plasmodium* appear to be nearly ubiquitous in avian communities. We detected one or both of these genera in almost 66% of species and this number would likely rise substantially with deeper sampling of individual species. In the Australo-Papuan region studied, we estimated an overall prevalence of about 44% with no significant differences between northeast Australia and New Guinea lowlands. Estimates of prevalence in tropical regions have ranged from about 10% in Costa Rica and the Neotropics (White et al. 1978, Young et al. 1993; by blood smear) to 28% in the Lesser Antilles (S Fallon pers. comm.), 40% in Central Africa (Richard et al. 2002) and 59% in American Samoa (*Plasmodium* only, Jarvi et al. 2003). Comparison of prevalence across surveys is confounded by differences in sensitivity of the diagnostics employed (Richard et al. 2002) and our PCR technique underestimated infection by at least 5%. Serological tests may provide the most accurate estimate of infection by detecting low-level chronic infections (Jarvi et al. 2002), but interpretation of the assays can be difficult (Jarvi et al. 2003), lineage identification is impossible, and the methods may not be applicable across varied hosts and parasite lineages.

Comparison of prevalence among regions is also likely to be confounded by the host families sampled. Except in the *Ptilonorhynchidae*, which were sampled only sparsely, prevalence of *Plasmodium* was low and fairly uniform among well-represented host families. On the other hand, prevalence of *Haemoproteus* varied significantly among host families, and this could bias regional comparisons in cases where families are not represented equally. Although certain host families such as the Columbidae repeatedly exhibit relatively high prevalence of infection across studies (Atkinson and van Riper 1991), estimates of prevalence, even if accurate, should be considered snapshots in time and host space (Bensch and Akesson 2003, Scheuerlein and Ricklefs 2004). Infection rates can vary dramatically between years and may be more representative of differences in vector abundance and their distribution within different habitats than family-level differences in host immune response or other evolved characters (Bennett and Cameron 1974).

Within the Pachycephalidae, we expected that parasite prevalence might have been lower among pitohuis, a group which produces varying amounts of a toxic alkaloid potentially active in invertebrate vectors (Dumbacher et al. 1992). The overall infection rate in this group (40%), however, was close to both the value for the entire family (35%) and the average prevalence within Papua New Guinea (46%). This suggests little role for the toxin in vector deterrence, however, collection of Papuan birds occurred over several years and the caveats mentioned above may apply.

We uncovered multiple infections from a wide range of hosts. Given that at least 40% of individuals were infected by either *Haemoproteus* or *Plasmodium*, the

prevalence of mixed infections should have been fairly high if not constrained by parasite-parasite interaction (Hatchwell et al. 2000). The 29 cases of multiple infection that we uncovered fell below the expected number of about 60 (based on overall prevalences of about 30% and 12% for *Haemoproteus* and *Plasmodium*, respectively). While this may be indicative of competitive exclusion, the cases of multiple infection observed represent a minimum since we did not recover sequence data from every infected individual and even successful PCR was likely to miss some multiple infections due to primer bias or unequal quantities of parasite DNA. Hematozoan genera may have evolved distinctive antigenic signatures that avoid cross-generic immunity in a common host (Atkinson and van Riper 1991), but the extent to which the evolutionary relatedness of parasites within genera influences inter-lineage competition and thus, the distribution of parasites, should be addressed more carefully in the future.

Host-Parasite Evolution

Parasite lineages found in more than one host have often been cited as evidence of host-switching. While the introduction of parasites into novel hosts is a prerequisite for host-switching, the current distribution of parasites may not reflect long-term co-evolution between the parasite and its vertebrate host, but may be more indicative of the cosmopolitan feeding of its invertebrate vector. Generalist feeders such as mosquitoes or ceratopogonid flies may drive the continuous introduction of varied *Plasmodium* and *Haemoproteus* lineages into diverse hosts. Not all of these interactions will necessarily be stable throughout time. For example, Atkinson (1986) demonstrated that *Haemoproteus meleagridis*, a parasite commonly found in turkeys,

was capable of developing in other Galliformes, but infections were transient and rapidly cleared from these secondary hosts to which the parasite may have been poorly adapted.

We found several lineages of both *Plasmodium* and *Haemoproteus* in multiple host families, however, identical *Haemoproteus* lineages were more likely to derive from related hosts than *Plasmodium*. Even if we assume that these cases represent evolutionarily stable changes in host affinity, recent host-switching by *Haemoproteus* lineages has been relatively constrained to related hosts. The significant signal of host family specificity observed in *Haemoproteus* at greater depths within the phylogeny, however, suggests that not all of the apparent associations between a single parasite lineage and multiple host families represent stable interactions. Given the host-family conservatism at 1st-step nodes and the strong signals from logistic regression, evolutionarily stable jumps between host families are likely to be rare in the genus *Haemoproteus*.

Across the genus *Haemoproteus*, the signal for host family specificity was lost at a parasite divergence of about 0.005. The attenuation in the signal, measured across the entire genus, was probably due to the structure of relationships between lineages within the genus. Analyzed separately, the two large subclades of *Haemoproteus* lineages derived from passerine hosts both exhibited strong host specificity. For clade B, in which the average pairwise divergence among parasites was about 0.075, the host signal extended to a parasite divergence of about 0.029. Within clade A, average pairwise parasite divergence was only about 0.021, and host-specificity was evident up to a parasite divergence of only 0.007.

Lineages within clades A and B may have diversified via periodic host-switching following an early vicariance event in an ancient *Haemoproteus* lineage. In both clades, however, we were largely unable to recover well-supported hierarchical relationships among groups of parasites derived from different host families, suggesting that the common ancestor to each clade spread rapidly across host families. Assuming that rates of nucleotide substitution are similar across various lineages of *Haemoproteus*, the short branch lengths in clade A suggest a relatively recent radiation of parasites across host taxa. Without further sampling, it will remain unclear how frequently lineages have escaped otherwise strong host constraint. If younger parasite radiations have spread broadly across avian hosts in the past, this phenomenon of escape and radiation would continually reset the parasite molecular clock relative to the avian clock. This in turn could help to explain the apparent slow divergence of parasite DNA relative to host DNA noted by Ricklefs and Fallon (2002).

Parasites in the genus *Plasmodium* appeared to be less constrained by the phylogenetic relationships of their hosts and showed no evidence of host-specificity at any depth within the parasite phylogeny. Our relatively small sample of *Plasmodium* may have limited our power to detect a signal, however, we detected host-specificity within the equally small *Haemoproteus* clade A. Interestingly, Ricklefs and Fallon (2002) detected host conservatism across both *Haemoproteus* and *Plasmodium* up to a parasite divergence of 0.026. Because they applied logistic regression across lineages from both genera, however, it is unclear how that value partitioned between genera or between distinct radiations within genera. The evidence

here supports a broad host range for at least some *Plasmodium* parasites and indicates a tendency for a high level of evolutionarily stable host-switching. Of the two parasite genera studied, *Plasmodium* likely presents the greatest threat of colonizing novel hosts and may warrant the most attention when managing the welfare of isolated and naïve hosts.

Most of the avian lineages sampled for parasites derived from a radiation of songbirds unique to the Australo-Papuan region (Sibley and Ahlquist 1985). In addition, birds from tropical Australia and New Guinea may be more isolated than their continental counterparts such that interactions between hosts, vectors, and parasites that would otherwise confound estimates of host-specificity are minimized. Nonetheless, trends in host-specificity observed in the Australo-Papuan region appear to be in line with the picture emerging from many other regional studies (partial summary in Schrenzel et al. 2003). Additional molecular surveys of parasites at the regional level will add further insight into patterns of host-parasite interaction.

TABLE

Table 1. Prevalence of *Plasmodium* and *Haemoproteus* assessed by PCR screening selected avian host families from the Australo-Papuan region. Estimates of prevalence are biased low because identification of genus was not possible for all samples (Genus Unknown).

Host Family	# Species Screened	# Samples Screened	*Plasmodium*		*Haemoproteus*		Genus Unknown	
			# Positive	% of Total	# Positive	% of Total	# Positive	% of Total
Acanthizidae	12	69	9	13	9	13	1	2
Alcedinidae	8	23	1	4	5	22	1	4
Columbidae	8	17	1	6	9	53	1	6
Meliphagidae	15	70	7	10	34	49	1	1
Monarchidae	13	54	13	24	14	26	0	0
Pachycephalidae	17	94	9	10	20	21	7	7
Petroicidae	6	34	1	3	19	56	1	3
Ptilonorhynchidae	2	15	7	47	6	40	0	0
All Families	80	376	48	13	116	31	12	3

FIGURE LEGENDS

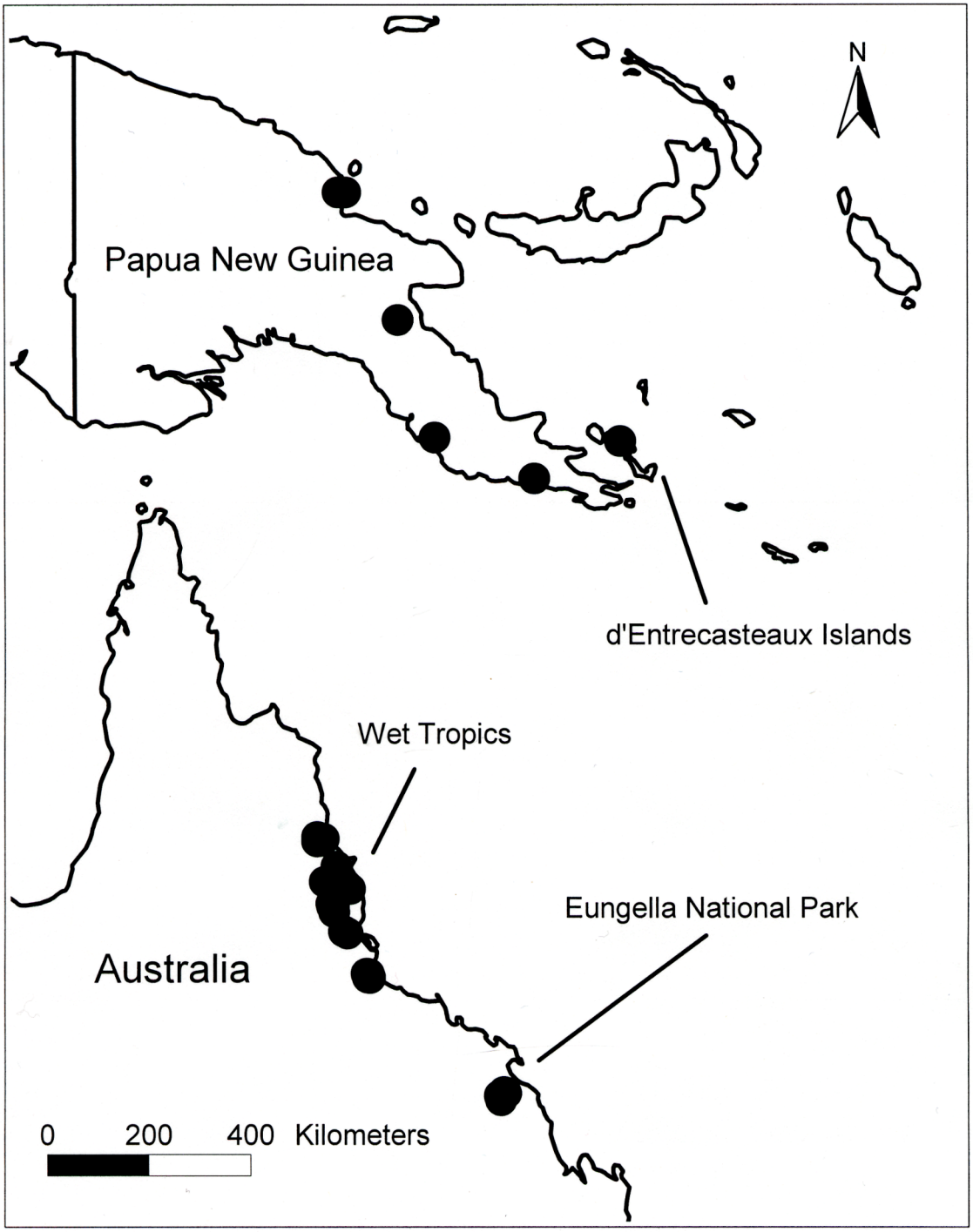
Figure 1. Location of sampling sites in tropical Australia and Papua New Guinea.

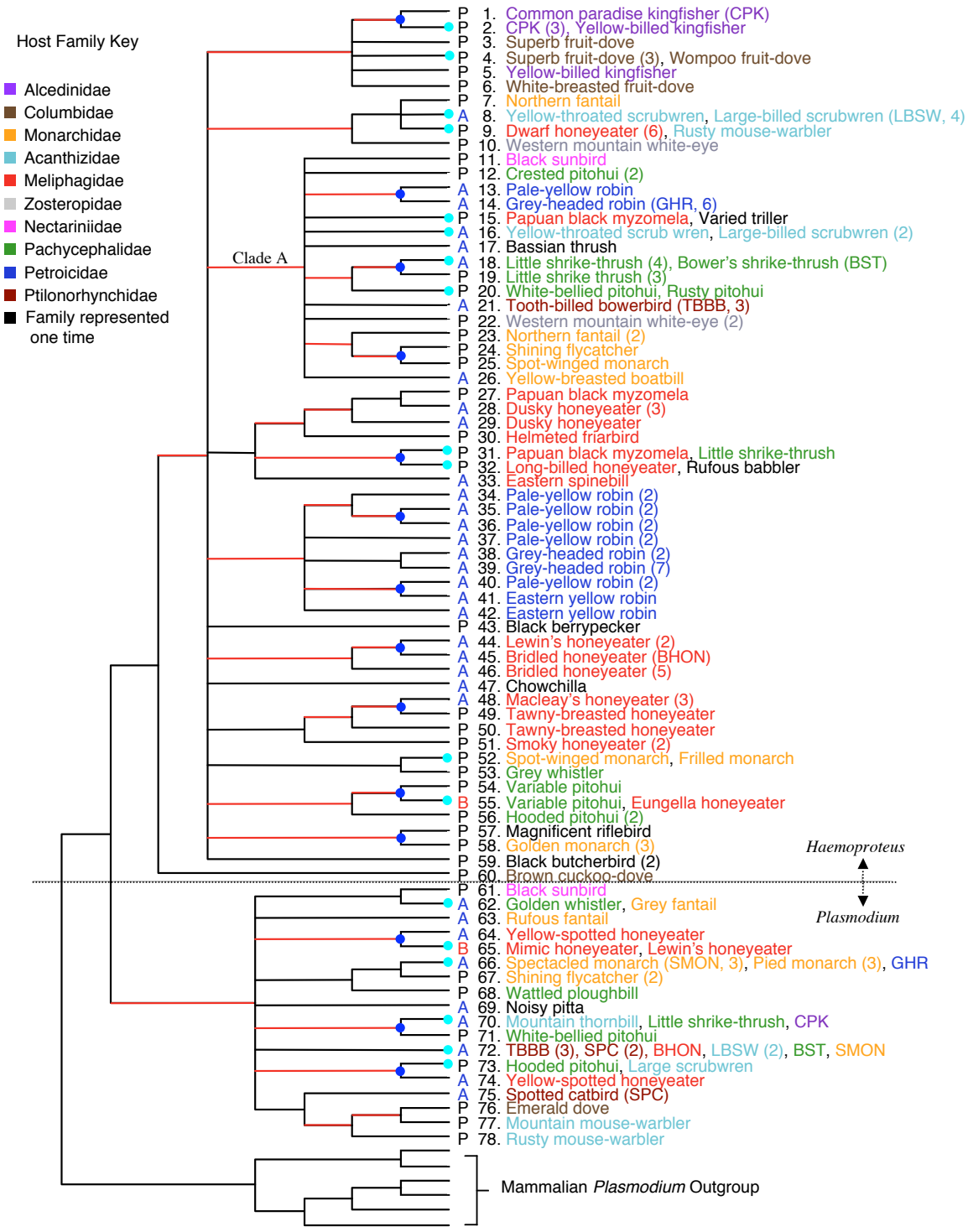
Figure 2. Cladogram depiction of neighbor-joining tree based on LogDet distances between mitochondrial lineages of avian hematozoa. Region of origin (A for Australia, P for Papua New Guinea, B for both regions), lineage number, host species (color-coded for family), and frequency of detection (number in parenthesis when recovered more than once) are indicated at right. Red branches indicate bootstrap support greater than 70% (1000 replicates). Pale blue and dark blue dots indicate lineages used for binomial tests of host conservation.

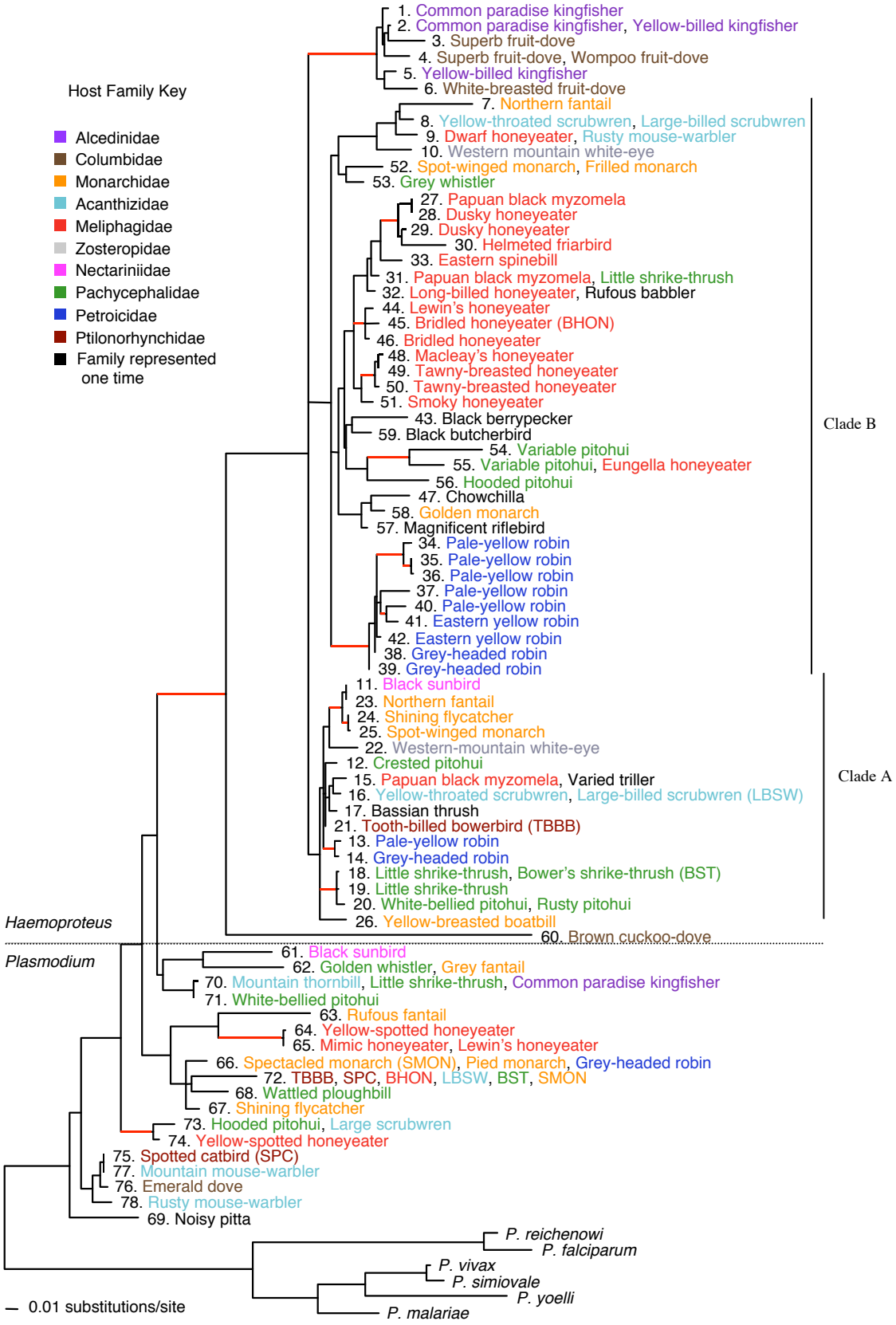
Figure 3. Relationships among hematozoan parasites based on maximum likelihood using the model GTR + I + G. Lineage number and host species (color-coded for family) are indicated at right. Red branches indicate bootstrap support greater than 70% (stepwise addition, 100 replicates).

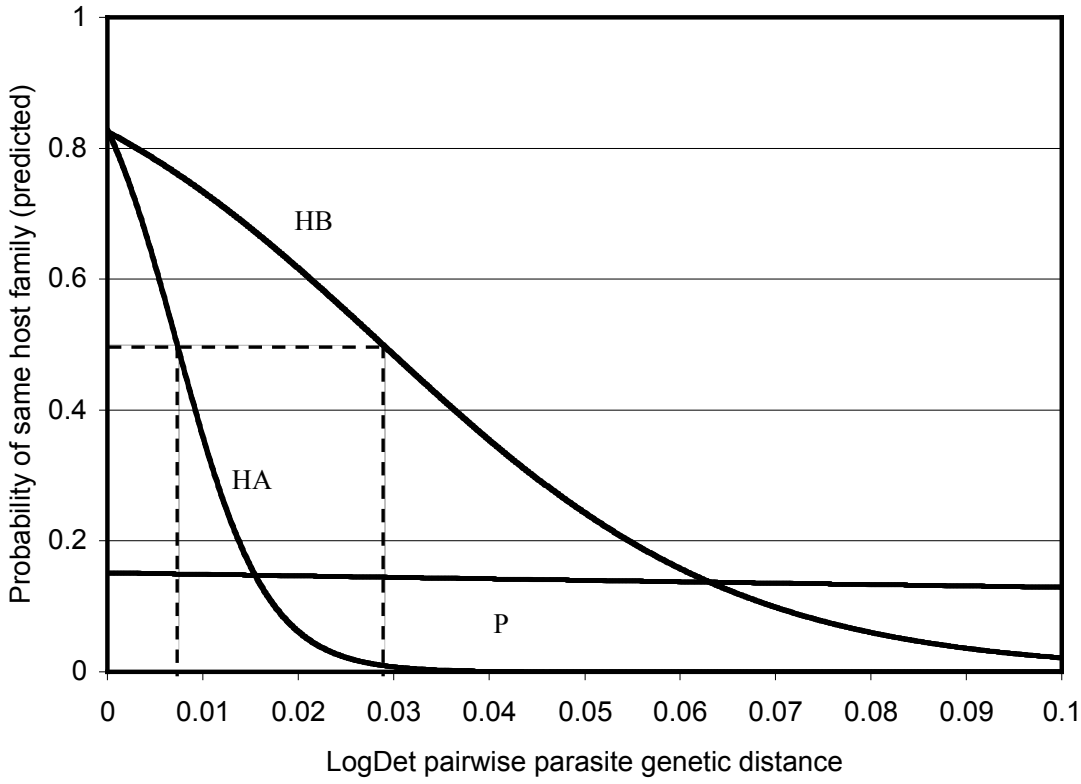
Figure 4. Logistic regression curves relating the predicted probability of host relatedness to genetic differentiation of parasites in the genus *Plasmodium* (P) and *Haemoproteus* clade A (HA) and clade B (HB). Dotted lines indicate the genetic distance at which parasite pairs from clades A and B were equally likely to be found in hosts of the same or different families.

FIGURES


N
Papua New Guinea
d'Entrecasteaux Islands
Wet Tropics
Eungella National Park
Australia
0 200 400 Kilometers


Host Family Key
Alcedinidae
Columbidae
Monarchidae
Acanthizidae
Meliphagidae
Zosteropidae
Nectariniidae
Pachycephalidae
Petroicidae
Ptilonorhynchidae
Family represented one time
Clade A
P 1. Common paradise kingfisher (CPK)
P 2. CPK (3), Yellow-billed kingfisher
P 3. Superb fruit-dove
P 4. Superb fruit-dove (3), Wompoo fruit-dove
P 5. Yellow-billed kingfisher
P 6. White-breasted fruit-dove
P 7. Northern fantail
A 8. Yellow-throated scrubwren, Large-billed scrubwren (LBSW, 4)
P 9. Dwarf honeyeater (6), Rusty mouse-warbler
P 10. Western mountain white-eye
P 11. Black sunbird
P 12. Crested pitohui (2)
A 13. Pale-yellow robin
A 14. Grey-headed robin (GHR, 6)
P 15. Papuan black myzomela, Varied triller
A 16. Yellow-throated scrub wren, Large-billed scrubwren (2)
A 17. Bassian thrush
A 18. Little shrike-thrush (4), Bower's shrike-thrush (BST)
P 19. Little shrike thrush (3)
P 20. White-bellied pitohui, Rusty pitohui
A 21. Tooth-billed bowerbird (TBBB, 3)
P 22. Western mountain white-eye (2)
P 23. Northern fantail (2)
P 24. Shining flycatcher
P 25. Spot-winged monarch
A 26. Yellow-breasted boatbill
P 27. Papuan black myzomela
A 28. Dusky honeyeater (3)
A 29. Dusky honeyeater
P 30. Helmeted friarbird
P 31. Papuan black myzomela, Little shrike-thrush
P 32. Long-billed honeyeater, Rufous babbler
A 33. Eastern spinebill
A 34. Pale-yellow robin (2)
A 35. Pale-yellow robin (2)
A 36. Pale-yellow robin (2)
A 37. Pale-yellow robin (2)
A 38. Grey-headed robin (2)
A 39. Grey-headed robin (7)
A 40. Pale-yellow robin (2)
A 41. Eastern yellow robin
A 42. Eastern yellow robin
P 43. Black berrypecker
A 44. Lewin's honeyeater (2)
A 45. Bridled honeyeater (BHON)
A 46. Bridled honeyeater (5)
A 47. Chowchilla
A 48. Macleay's honeyeater (3)
P 49. Tawny-breasted honeyeater
P 50. Tawny-breasted honeyeater
P 51. Smoky honeyeater (2)
P 52. Spot-winged monarch, Frilled monarch
P 53. Grey whistler
P 54. Variable pitohui
B 55. Variable pitohui, Eungella honeyeater
P 56. Hooded pitohui (2)
P 57. Magnificent riflebird
P 58. Golden monarch (3)
P 59. Black butcherbird (2)
P 60. Brown cuckoo-dove
Haemoproteus
Plasmodium
P 61. Black sunbird
A 62. Golden whistler, Grey fantail
A 63. Rufous fantail
A 64. Yellow-spotted honeyeater
B 65. Mimic honeyeater, Lewin's honeyeater
A 66. Spectacled monarch (SMON, 3), Pied monarch (3), GHR
P 67. Shining flycatcher (2)
P 68. Wattled ploughbill
A 69. Noisy pitta
A 70. Mountain thornbill, Little shrike-thrush, CPK
P 71. White-bellied pitohui
A 72. TBBB (3), SPC (2), BHON, LBSW (2), BST, SMON
P 73. Hooded pitohui, Large scrubwren
A 74. Yellow-spotted honeyeater
A 75. Spotted catbird (SPC)
P 76. Emerald dove
P 77. Mountain mouse-warbler
P 78. Rusty mouse-warbler
Mammalian Plasmodium Outgroup


Host Family Key
Alcedinidae
Columbidae
Monarchidae
Acanthizidae
Meliphagidae
Zosteropidae
Nectariniidae
Pachycephalidae
Petroicidae
Ptilonorhynchidae
Family represented one time
1. Common paradise kingfisher
2. Common paradise kingfisher, Yellow-billed kingfisher
3. Superb fruit-dove
4. Superb fruit-dove, Wompoo fruit-dove
5. Yellow-billed kingfisher
6. White-breasted fruit-dove
7. Northern fantail
8. Yellow-throated scrubwren, Large-billed scrubwren
9. Dwarf honeyeater, Rusty mouse-warbler
10. Western mountain white-eye
52. Spot-winged monarch, Frilled monarch
53. Grey whistler
27. Papuan black myzomela
28. Dusky honeyeater
29. Dusky honeyeater
30. Helmeted friarbird
33. Eastern spinebill
31. Papuan black myzomela, Little shrike-thrush
32. Long-billed honeyeater, Rufous babbler
44. Lewin's honeyeater
45. Bridled honeyeater (BHON)
46. Bridled honeyeater
48. Macleay's honeyeater
49. Tawny-breasted honeyeater
50. Tawny-breasted honeyeater
51. Smoky honeyeater
43. Black berrypecker
59. Black butcherbird
54. Variable pitohui
55. Variable pitohui, Eungella honeyeater
56. Hooded pitohui
47. Chowchilla
58. Golden monarch
57. Magnificent riflebird
34. Pale-yellow robin
35. Pale-yellow robin
36. Pale-yellow robin
37. Pale-yellow robin
40. Pale-yellow robin
41. Eastern yellow robin
42. Eastern yellow robin
38. Grey-headed robin
39. Grey-headed robin
Clade B
11. Black sunbird
23. Northern fantail
24. Shining flycatcher
25. Spot-winged monarch
22. Western-mountain white-eye
12. Crested pitohui
15. Papuan black myzomela, Varied triller
16. Yellow-throated scrubwren, Large-billed scrubwren (LBSW)
17. Bassian thrush
21. Tooth-billed bowerbird (TBBB)
13. Pale-yellow robin
14. Grey-headed robin
18. Little shrike-thrush, Bower's shrike-thrush (BST)
19. Little shrike-thrush
20. White-bellied pitohui, Rusty pitohui
26. Yellow-breasted boatbill
Clade A
Haemoproteus
60. Brown cuckoo-dove
Plasmodium
61. Black sunbird
62. Golden whistler, Grey fantail
70. Mountain thornbill, Little shrike-thrush, Common paradise kingfisher
71. White-bellied pitohui
63. Rufous fantail
64. Yellow-spotted honeyeater
65. Mimic honeyeater, Lewin's honeyeater
66. Spectacled monarch (SMON), Pied monarch, Grey-headed robin
72. TBBB, SPC, BHON, LBSW, BST, SMON
68. Wattled ploughbill
67. Shining flycatcher
73. Hooded pitohui, Large scrubwren
74. Yellow-spotted honeyeater
75. Spotted catbird (SPC)
77. Mountain mouse-warbler
76. Emerald dove
78. Rusty mouse-warbler
69. Noisy pitta
P. reichenowi
P. falciparum
P. vivax
P. simiovale
P. yoelli
P. malariae
0.01 substitutions/site


1
0.8
0.6
0.4
0.2
0
Probability of same host family (predicted)
HB
HA
P
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.1
LogDet pairwise parasite genetic distance

APPENDICES

Appendix A. Avian host names, geographical origin, sequence length and GenBank accession numbers for parasite lineages listed in Figure 2.

	Host Information						
Lineage	Family	Genus	Species	Common Name	Locality	bp	GenBank
1	Alcedinidae	*Tanysiptera*	*galatea*	Common paradise kingfisher	PNG	487	AY714134
2	Alcedinidae	*Tanysiptera*	*galatea*	Common paradise kingfisher	PNG	533	AY714135
2	Alcedinidae	*Halcyon*	*torotoro*	Yellow-billed kingfisher	PNG	533	AY714135
3	Columbidae	*Ptilinopus*	*superbus*	Superb fruit-dove	PNG	533	AY714136
4	Columbidae	*Ptilinopus*	*superbus*	Superb fruit-dove	PNG	533	AY714137
4	Columbidae	*Ptilinopus*	*magnificus*	Wompoo fruit-dove	PNG	533	AY714137
5	Alcedinidae	*Halcyon*	*torotoro*	Yellow-billed kingfisher	PNG	533	AY714138
6	Columbidae	*Ptilinopus*	*rivoli*	White-breasted fruit-dove	PNG	533	AY714139
7	Monarchidae	*Rhipidura*	*rufiventris*	Northern fantail	PNG	295	AY714140
8	Acanthizidae	*Sericornis*	*citreogularis*	Yellow-throated scrubwren	AUS	533	AY714141
8	Acanthizidae	*Sericornis*	*magnirostris*	Large-billed scrubwren	AUS	533	AY714141
9	Meliphagidae	*Oedistoma*	*iliolophus*	Dwarf honeyeater	PNG	533	AY714142
9	Acanthizidae	*Crateroscelis*	*murina*	Rusty mouse-warbler	PNG	533	AY714142
10	Zosteropidae	*Zosterops*	*fuscicapillus*	Western mountain white-eye	PNG	533	AY714143
11	Nectariniidae	*Nectarinia*	*aspasia*	Black sunbird	PNG	295	AY714144
12	Pachycephalidae	*Pitohui*	*cristatus*	Crested pitohui	PNG	533	AY714145
13	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714146
14	Petroicidae	*Heteromyias*	*albispecularis*	Grey-headed robin	AUS	533	AY714147
15	Campephagidae	*Lalage*	*leucomela*	Varied triller	PNG	533	AY714148
15	Meliphagidae	*Myzomela*	*nigrita*	Papuan black myzomela	PNG	533	AY714148
16	Acanthizidae	*Sericornis*	*citreogularis*	Yellow-throated scrubwren	AUS	533	AY714149
16	Acanthizidae	*Sericornis*	*magnirostris*	Large-billed scrubwren	AUS	533	AY714149
17	Muscicapidae	*Zoothera*	*lunulata*	Bassian thrush	AUS	533	AY714150

18	Pachycephalidae	*Colluricincla*	*megarhyncha*	Little shrike-thrush	AUS	533	AY714151
18	Pachycephalidae	*Colluricincla*	*boweri*	Bower's shrike-thrush	AUS	533	AY714151
19	Pachycephalidae	*Colluricincla*	*megarhyncha*	Little shrike-thrush	AUS	533	AY714152
20	Pachycephalidae	*Pitohui*	*incertus*	White-bellied pitohui	PNG	533	AY714153
20	Pachycephalidae	*Pitohui*	*ferrugineus*	Rusty pitohui	PNG	533	AY714153
21	Ptilonorhynchidae	*Scenopoeetes*	*dentirostris*	Tooth-billed bowerbird	AUS	533	AY714154
22	Zosteropidae	*Zosterops*	*fuscicapillus*	Western mountain white-eye	PNG	533	AY714155
23	Monarchidae	*Rhipidura*	*rufiventris*	Northern fantail	PNG	533	AY714156
24	Monarchidae	*Myiagra*	*alecto*	Shining flycatcher	PNG	533	AY714157
25	Monarchidae	*Monarcha*	*guttula*	Spot-winged monarch	PNG	533	AY714158
26	Monarchidae	*Machaerirhynchus*	*flaviventer*	Yellow-breasted boatbill	AUS	271	AY714159
27	Meliphagidae	*Myzomela*	*nigrita*	Papuan black myzomela	PNG	533	AY714160
28	Meliphagidae	*Myzomela*	*obscura*	Dusky honeyeater	AUS	533	AY714161
29	Meliphagidae	*Myzomela*	*obscura*	Dusky honeyeater	AUS	533	AY714162
30	Meliphagidae	*Philemon*	*buceroides*	Helmeted friarbird	PNG	533	AY714163
31	Meliphagidae	*Myzomela*	*nigrita*	Papuan black myzomela	PNG	533	AY714164
31	Pachycephalidae	*Colluricincla*	*megarhyncha*	Little shrike-thrush	PNG	533	AY714164
32	Meliphagidae	*Melilestes*	*megarhynchus*	Long-billed honeyeater	PNG	533	AY714165
32	Pomatostomidae	*Pomatostomus*	*isodorei*	Rufous babbler	PNG	533	AY714165
33	Meliphagidae	*Acanthorhynchus*	*tenuirostris*	Eastern spinebill	AUS	533	AY714166
34	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714167
35	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714168
36	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714169
37	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714170
38	Petroicidae	*Heteromyias*	*albispecularis*	Grey-headed robin	AUS	533	AY714171
39	Petroicidae	*Heteromyias*	*albispecularis*	Grey-headed robin	AUS	533	AY714172
40	Petroicidae	*Tregellasia*	*capito*	Pale-yellow robin	AUS	533	AY714173
41	Petroicidae	*Eopsaltria*	*australis*	Eastern yellow robin	AUS	533	AY714174
42	Petroicidae	*Eopsaltria*	*australis*	Eastern yellow robin	AUS	533	AY714175
43	Melanocharitidae	*Melanocharis*	*nigra*	Black berrypecker	PNG	533	AY714176
44	Meliphagidae	*Meliphaga*	*lewinii*	Lewin's honeyeater	AUS	533	AY714177
45	Meliphagidae	*Lichenostomus*	*frenatus*	Bridled honeyeater	AUS	533	AY714178
46	Meliphagidae	*Lichenostomus*	*frenatus*	Bridled honeyeater	AUS	533	AY714179
47	Orthonychidae	*Orthonyx*	*spaldingii*	Chowchilla	AUS	533	AY714180
48	Meliphagidae	*Xanthotis*	*macleayana*	Macleay's honeyeater	AUS	533	AY714181
49	Meliphagidae	*Xanthotis*	*flaviventer*	Tawny-breasted honeyeater	PNG	533	AY714182

50	Meliphagidae	*Xanthotis*	*flaviventer*	Tawny-breasted honeyeater	PNG	533	AY714183
51	Meliphagidae	*Melipotes*	*fumigatus*	Smoky honeyeater	PNG	533	AY714184
52	Monarchidae	*Monarcha*	*guttula*	Spot-winged monarch	PNG	533	AY714185
52	Monarchidae	*Arses*	*telescophthalmus*	Frilled monarch	PNG	533	AY714185
53	Pachycephalidae	*Pachycephala*	*simplex*	Grey whistler	PNG	533	AY714186
54	Pachycephalidae	*Pitohui*	*kirhocephalus*	Variable pitohui	PNG	533	AY714187
55	Pachycephalidae	*Pitohui*	*kirhocephalus*	Variable pitohui	PNG	533	AY714188
55	Meliphagidae	*Lichenostomus*	*hindwoodi*	Eungella honeyeater	AUS	533	AY714188
56	Pachycephalidae	*Pitohui*	*dichrous*	Hooded pitohui	PNG	533	AY714189
57	Paradisaeidae	*Ptilloris*	*magnificus*	Magnificent riflebird	PNG	533	AY714190
58	Monarchidae	*Monarcha*	*chrysomela*	Golden monarch	PNG	533	AY714191
59	Cracticidae	*Cracticus*	*quoyi*	Black butcherbird	PNG	533	AY714192
60	Columbidae	*Macropygia*	*amboinensis*	Brown cuckoo-dove	PNG	533	AY714193
61	Nectariniidae	*Nectarinia*	*aspasia*	Black sunbird	PNG	295	AY714194
62	Pachycephalidae	*Pachycephala*	*pectoralis*	Golden whistler	AUS	533	AY714195
62	Monarchidae	*Rhipidura*	*fuliginosa*	Grey fantail	AUS	533	AY714195
63	Monarchidae	*Rhipidura*	*rufifrons*	Rufous fantail	AUS	533	AY714196
64	Meliphagidae	*Meliphaga*	*notata*	Yellow-spotted honeyeater	AUS	533	AY714197
65	Meliphagidae	*Meliphaga*	*analoga*	Mimic honeyeater	PNG	533	AY714198
65	Meliphagidae	*Meliphaga*	*lewinii*	Lewin's honeyeater	AUS	533	AY714198
66	Monarchidae	*Monarcha*	*trivirgatus*	Spectacled monarch	AUS	533	AY714199
66	Monarchidae	*Monarcha*	*kaupi*	Pied monarch	AUS	533	AY714199
66	Petroicidae	*Heteromyias*	*albispecularis*	Grey-headed robin	AUS	533	AY714199
67	Monarchidae	*Myiagra*	*alecto*	Shining flycatcher	PNG	533	AY714200
68	Pachycephalidae	*Eulecestoma*	*nigripectus*	Wattled ploughbill	PNG	533	AY714201
69	Pittidae	*Pitta*	*versicolor*	Noisy pitta	AUS	533	AY714202
70	Acanthizidae	*Acanthiza*	*katherina*	Mountain thornbill	AUS	533	AY714203
70	Pachycephalidae	*Colluricincla*	*megarhyncha*	Little shrike-thrush	AUS	533	AY714203
70	Alcedinidae	*Tanysiptera*	*galatea*	Common paradise kingfisher	PNG	533	AY714203
71	Pachycephalidae	*Pitohui*	*incertus*	White-bellied pitohui	PNG	533	AY714204
72	Ptilonorhynchidae	*Scenopoeetes*	*dentirostris*	Tooth-billed bowerbird	AUS	533	AY714205
72	Ptilonorhynchidae	*Ailuroedes*	*melanotis*	Spotted catbird	AUS	533	AY714205
72	Meliphagidae	*Lichenostomus*	*frenatus*	Bridled honeyeater	AUS	533	AY714205
72	Acanthizidae	*Sericornis*	*magnirostris*	Large-billed scrubwren	AUS	533	AY714205
72	Pachycephalidae	*Colluricincla*	*boweri*	Bower's shrike-thrush	AUS	533	AY714205
72	Monarchidae	*Monarcha*	*trivirgatus*	Spectacled monarch	AUS	533	AY714205

73	Pachycephalidae	*Pitohui*	*dichrous*	Hooded pitohui	PNG	533	AY714206
73	Acanthizidae	*Sericornis*	*nouhuysi*	Large scrubwren	PNG	469	AY714206
74	Meliphagidae	*Meliphaga*	*notata*	Yellow-spotted honeyeater	AUS	533	AY714207
75	Ptilonorhynchidae	*Ailuroedes*	*melanotis*	Spotted catbird	AUS	485	AY714208
76	Columbidae	*Chalcophaps*	*indica*	Emerald dove	PNG	295	AY714209
77	Acanthizidae	*Crateroscelis*	*robusta*	Mountain mouse-warbler	PNG	295	AY714210
78	Acanthizidae	*Crateroscelis*	*murina*	Rusty mouse-warbler	PNG	295	AY714211

Appendix B. Frequency of detection of *Haemoproteus* (H), *Plasmodium* (P), unknown genus (U) or mixed infection (M) across host families from Australia (AUS) and Papua New Guinea (PNG). Composition of mixed infections is indicated at right.

Host	Location	Total	H	P	U	M	INF	Mixed
Accipitridae								
Accipiter poliocephalus	PNG	1					0	
Megapodidae								
Megapodius reinwardt	PNG	1					0	
Columbidae								
Chalcophaps indica	PNG	3	1	1		1	3	H
Chalcophaps stephani	PNG	1	1				1	
Ducula pinon	PNG	1					0	
Macropygia amboinensis	PNG	3	1				1	
Ptilinopus magnificus	PNG	2	1				1	
Ptilinopus pulchellus	PNG	1					0	
Ptilinopus rivoli	PNG	2	1				1	
Ptilinopus superbus	PNG	4	2		1	1	4	H
Podargidae								
Podargus ocellatus	PNG	2			1		1	
Aegothelidae								
Aegotheles bennettii	PNG	2					0	
Alcedinidae								
Alcedo azurea	PNG	1					0	
Alcedo pusilla	PNG	1					0	
Halcyon chloris	PNG	1					0	
Halcyon sancta	PNG	1					0	
Halcyon torotoro	PNG	4	2		1		3	
Melidora macrorhina	PNG	1					0	
Tanysiptera danae	PNG	4					0	
Tanysiptera galatea	PNG	10	3	1			4	
Pittidae								
Pitta versicolor	AUS	3		1			1	
Climacteridae								
Cormobates leucophaeus	AUS	3					0	
Ptilonorhynchidae								
Ailuroedus melanotis	AUS	8	3	2		1	6	P
Scenopoeetes dentirostris	AUS	7	2	2		2	6	P,PH
Acanthizidae								
Acanthiza katherina	AUS	8		1			1	

Crateroscelis murina	PNG	5	1	3			4	
Crateroscelis robusta	PNG	6		3			3	
Gerygone mouki	AUS	2					0	
Oreoscopus gutturalis	AUS	6			1		1	
Sericornis citreogularis	AUS	12	2				2	
Sericornis frontalis	AUS	7					0	
Sericornis keri	AUS	4					0	
Sericornis magnirostris	AUS	14	5			1	6	PH
Sericornis nouhuysi	PNG	1		1			1	
Sericornis papuensis	PNG	2					0	
Sericornis perspicillatus	PNG	2					0	
Meliphagidae								
Acanthorhynchus tenuirostris	AUS	7	1		1		2	
Lichenostomus frenatus	AUS	8	6	1			7	
Lichenostomus hindwoodi	AUS	4	1				1	
Melilestes megarhynchus	PNG	3	1				1	
Meliphaga analoga	PNG	1		1			1	
Meliphaga aruensis	PNG	4		1			1	
Meliphaga lewinii	AUS	16	5	2			7	
Meliphaga notata	AUS	3		2			2	
Melipotes fumigatus	PNG	5	2				2	
Myzomela nigrita	PNG	2	1			1	2	H
Myzomela obscura	AUS	3	2			1	3	H
Oedistoma iliolophus	PNG	6	6				6	
Philemon buceroides	PNG	1	1				1	
Xanthotis flaviventer	PNG	2	2				2	
Xanthotis macleayana	AUS	5	4				4	
Petroicidae								
Amalocichla incerta	PNG	2					0	
Eopsaltria australis	AUS	3	2		1		3	
Heteromyias albispecularis	AUS	17	5	1		5	11	H
Melanodryas cucullata	PNG	1					0	
Petroica rosea	PNG	1					0	
Tregellasia capito	AUS	10	3			4	7	H
Orthonychidae								
Orthonyx spaldingii	AUS	4	1				1	
Pomatostomidae								
Pomatostomus isidorei	PNG	5	1				1	
Cinclosomatidae								
Cinclosoma ajax	PNG	2					0	
Psophodes olivaceus	AUS	7					0	
Psophodes olivaceus	PNG	1					0	
Pachycephalidae								
Colluricincla boweri	AUS	8	1	1			2	
Colluricincla harmonica	PNG	1					0	

Colluricincla megarhyncha	AUS	11	4			1	5	PH
Colluricincla megarhyncha	PNG	16	4	1			5	
Colluricincla woodwardi	PNG	1				1	1	?
Eulecestoma nigripectus	PNG	1		1			1	
Falcunculus frontatus	PNG	1			1		1	
Pachycephala melanura	PNG	2					0	
Pachycephala olivacea	PNG	1			1		1	
Pachycephala pectoralis	AUS	11		1			1	
Pachycephala pectoralis	PNG	1					0	
Pachycephala schlegelii	PNG	1					0	
Pachycephala simplex	PNG	3	1		1		2	
Pitohui cristatus	PNG	3	2				2	
Pitohui dichrous	PNG	10	2	1			3	
Pitohui ferrugineus	PNG	14	1		1	1	3	?
Pitohui incertus	PNG	3	1	1			2	
Pitohui kirhocephalus	PNG	5	1		1	2	4	PH
Rhagologus leucostigma	PNG	1					0	
Paradisaeidae								
Cicinnurus magnificus	PNG	1					0	
Paradiseae raggiana	PNG	1			1		1	
Ptilloris magnificus	PNG	1	1				1	
Cracticidae								
Cracticus quoyi	PNG	2	2				2	
Campephagidae								
Lalage leucomela	PNG	1	1				1	
Dicruridae								
Chaetorynchus papuensis	PNG	1					0	
Dicrurus hottentottus	PNG	2					0	
Monarchidae								
Arses telescopthalmus	PNG	3	1			1	2	H
Machaerirynchus flaviventer	AUS	3	1	1			2	
Monarcha chrysomela	PNG	3	3				3	
Monarcha guttula	PNG	5	3				3	
Monarcha kaupi	AUS	3		3			3	
Monarcha trivirgatus	AUS	12		3		1	4	P
Myiagra alecto	PNG	3	1	2			3	
Rhipidura albolimbata	PNG	2					0	
Rhipidura atra	PNG	3					0	
Rhipidura brachyrhyncha	PNG	1					0	
Rhipidura fulginosa	AUS	7		2			2	
Rhipidura rufifrons	PNG	2					0	
Rhipidura rufifrons	AUS	1		1			1	
Rhipidura rufiventris	PNG	6	3			1	5	H
Sylviidae								
Phylloscopus trivirgatus	PNG	1					0	

Muscicapidae						
Zoothera lunulata	AUS	2	1		1	
Melanocharitidae						
Melanocharis nigra	PNG	1	1		1	
Nectariniidae						
Nectarinia aspasia	PNG	2		1	1	PH
Zosteropidae						
Zosterops fuscicapillus	PNG	3	1	1	2	H
Zosterops griseotinctus	PNG	2			0	
Passeridae						
Erythrura trichroa	PNG	1			0	

CHAPTER III

Global phylogeographic limits of Hawaii's avian malaria

ABSTRACT

The introduction of avian malaria (*Plasmodium relictum*) to Hawaii has provided a model system for studying the influence of exotic disease on naïve host populations. Little is known, however, about the origin or genetic variation of Hawaii's malaria and traditional classification methods have confounded attempts to place the parasite within a global ecological and evolutionary context. Using fragments of the parasite mitochondrial gene cytochrome *b* and the nuclear gene DHFR-TS obtained from a global survey of >13,000 avian samples, we show that Hawaii's avian malaria, which can cause high mortality and is a major limiting factor for many species of native passerines, represents just one of numerous lineages composing the morphological parasite species. The single parasite lineage detected in Hawaii exhibits a broad host distribution worldwide and is dominant on several other remote oceanic islands, including Bermuda and Moorea, French Polynesia. Rarity of this lineage in the continental New World and the restriction of closely-related lineages to the Old World suggest limitations to the transmission of reproductively-isolated parasite groups within the morphological species.

INTRODUCTION

The introduction of avian malaria (*Plasmodium relictum*) to the remote Hawaiian Islands has been implicated in the widespread decline and possible extinction of many species within the endemic avian radiation of honeycreepers (Warner 1968; van Riper *et al.* 1986). While mortality in introduced bird species is negligible, mortality in many endemic species can range from 50-90% (Jarvi *et al.* 2001), possibly reflecting their long isolation (ca. 4 million years; Fleischer & McIntosh 2001) from malarial parasites. Although the epidemiology of malaria in Hawaiian birds has been well studied, little is known about the diversity of parasite strains in Hawaii or their origin. Transmission of malaria was impossible until the human-mediated introduction of a competent vector (*Culex quinquefasciatus*) to Hawaii in 1826 (Warner 1968). Since then, resident Hawaiian birds may have been exposed to reservoirs of parasites harbored by the hundreds of exotic birds released in the late 19th and early 20th centuries (Long 1981) and by the thousands of ducks and shorebirds which annually migrate to Hawaii from their breeding grounds in the arctic.

Understanding the host range of *P. relictum* in Hawaii and elsewhere across the globe is essential for the purpose of identifying its original host, for understanding limits to its transmission, and for eventually understanding its extreme virulence in native honeycreepers relative to its effects in other hosts. Unfortunately, classical techniques for identifying parasites may be confounding this understanding. *P. relictum* has been reported to occur in a broad spectrum of hosts from all continents except Antarctica (Bennett *et al.* 1993), however, to date, identification has been based both on morphology, which can vary within strains of the same parasite species

(Peirce 1979; van Riper 1991), and on biological characteristics such as vector, endogenous development and host range (Garnham 1966). These characters may not accurately reflect phylogenetic relationships among parasites (Escalante *et al.* 1998).

The recent detection of extensive genetic diversity across avian malaria parasites (Ricklefs & Fallon 2002; Waldenström *et al.* 2002; Beadell *et al.* 2004) suggests that cryptic structure in parasite populations may underlie differences in host susceptibility, vector competence and parasite virulence. Here, we use molecular markers to characterize Hawaii's avian malaria and to place this genotype in a global context in order to better understand its origin, its current and historic impact, and limits to its transmission.

MATERIAL AND METHODS

Lineage Identification

Although phylogenetic species limits have not been well defined in avian malaria parasites, a previous study indicated that mitochondrial lineages appear to represent reproductively-isolated units (Bensch *et al.* 2004), and therefore, we characterized the Hawaiian strain and its global distribution using cytochrome *b* (cyt *b*). Determination of parasite haplotype frequencies in Europe and Nigeria (table 1) followed PCR methods described in Waldenström *et al.* (2004). Data for the Lesser Antilles and Venezuela were provided by S. Fallon. Detection and identification of all other parasite mitochondrial lineages generally followed the methods described in Beadell *et al.* (2004). Briefly, we screened DNA extracted from blood or tissue for parasites using primers F2/R2, 850F/1024R or 213F/372R (Beadell & Fleischer 2005). For positively infected samples, we then sequenced a larger fragment generated with

primers 3760F/4292rw2 (533 bp) or with primers Fifi/4292rw2 (351 bp; Ishtiaq *et al.* 2006) or F2/4292rw2 (295 bp) in cases where degraded template precluded the amplification of the larger piece (appendix 1). For lineages used in detailed phylogenetic analysis, we also obtained an additional 220 bp of sequence using primers L15368/H15730 (Fallon *et al.* 2003), which generated a 338 bp fragment overlapping previously-generated fragments by 118 bp. Sequences were assembled, aligned and edited using SEQUENCHER v. 4.1. For the purpose of describing the worldwide distribution of Hawaii's parasite lineage (GRW4; Bensch et al. 2000), we identified as GRW4 any sequences that identically matched the largest fragment of parasite cyt *b* isolated from a Hawaiian host (AY733090). In Korea, we detected the presence of GRW4 based on matching a single 91 bp fragment alone, but in all other locations, detection of GRW4 was based on recovery of identical sequences of between 256 and 753 bp (appendix 1). When defining all other lineages, we grouped together only identical sequences exhibiting matching sequence of 256 bp or more. Therefore, within the limits of the sequence examined, parasite lineages are defined by unique mitochondrial haplotypes.

Phylogeny of *Plasmodium* spp.

To examine the concordance between parasite classification based on morphology and on DNA, we assembled cyt *b* sequence from avian *Plasmodium* spp. previously identified by morphology and recognized as valid by Bennett *et al.* (1993). We obtained sequences from Genbank and from DNA extracted from blood smears, which were obtained from the International Reference Collection for Avian Haematozoa (IRCAH; Brisbane, Australia) or from M. Peirce. Classification of

parasites in IRCAH smears were checked by C. Atkinson and M. Peirce and smears exhibiting multiple infections (upon visual inspection or after molecular analysis) were not used. We constructed phylogenetic trees using taxa for which we had recovered between 335 and 753 bp of sequence and rooted trees with sequences from *Haemoproteus* spp., parasites in the sister genus to avian *Plasmodium* (Perkins & Schall 2002). We estimated phylogenies using minimum evolution (ME; on K2P and GTR distances), maximum likelihood (ML), and maximum parsimony (MP) as implemented by PAUP* (Swofford 1999). For ML analysis, we chose the most likely model of base pair substitution (GTR+I+G) and parameters (pinv = 0.5509, shape = 0.6505) based on a likelihood ratio test employed by Modeltest version 3.07 (Posada & Crandall 1998). Bootstrap support was estimated for each method using 1000 replicates.

Phylogenetic Analysis of Lineages Related to GRW4

We assembled a total of 166 unique *Plasmodium* cyt *b* sequences gathered from our global survey and from GenBank. Due to the large number of mitochondrial lineages, we initially constructed an ME tree using K2P distances and PAUP* in order to identify those lineages which shared most recent common ancestry with GRW4 and which were most relevant to tracing the origin of this lineage. The resulting tree (data not shown) exhibited a clade containing GRW4 (lineages 15 through 37), a sister clade (lineages 1 through 14) and two clades immediately ancestral (lineages 38 through 51); combined, these 51 lineages formed a monophyletic clade nested within the other 115 lineages. For subsequent analyses, therefore, we focused only on these 51 lineages plus several lineages with known morphological classification to help

polarize the tree. Lineage 56 was included because a representative (AF254962) was originally classified as *P. nucleophilum*, however, its status is undergoing revision (G. Valkiunas pers. comm.). Employing the longest fragment of cyt *b* available for each lineage (appendix 1), we used ML to re-estimate a phylogeny using a model (GTR+I+G, pinv = 0.5445, shape = 0.7154) chosen by Modeltest. We estimated support for nodes based on 100 replicates. Due to uncertainty about parasite species limits and because a dichotomously-branching tree may not appropriately capture relationships among mitochondrial lineages within a species, we also generated a haplotype network using statistical parsimony as implemented in TCS1.21 (Clement *et al.* 2000). Lineages were joined at the 95% confidence criterion unless noted. Parasite lineages which could not be joined to GRW4 at the 90% level were excluded.

In order to generate a second, independent estimate of relationships among lineages, we followed the protocols in Bensch *et al.* (2004) to amplify and sequence a portion of the nuclear gene dihydrofolate reductase-thymidylate synthase (DHFR-TS; 236 bp) from samples for which we had already recovered mitochondrial sequence (appendix 2). Because nuclear DNA occurs in much lower copy number than mitochondrial DNA, we recovered DHFR-TS from only a fraction of the samples for which we recovered mitochondrial lineages. In addition, we did not include DHFR-TS sequences from samples for which nuclear or mitochondrial sequence provided evidence of multiple infections (e.g., double peaks in the chromatogram or different sequences from different primer sets). Nuclear haplotype Q (AY033582) was derived from *P. gallinaceum* but not necessarily the same strain of *P. gallinaceum* from which mitochondrial lineage 50 (AY099029) was derived. We used the methods

described above to estimate an ML tree using the model GTR+I+G (pinv = 0.5620, shape = 2.0791). Bootstrap support was based on 1000 replicates.

To improve resolution of hierarchical relationships among lineages, we used Bayesian analysis as implemented in MrBayes v3.1.1 (Ronquist & Huelsenbeck 2003) to estimate phylogenetic relationships among lineages for which we could combine both mitochondrial and nuclear markers. Parameters of the GTR+I+G model of DNA substitution were allowed to vary independently for each marker within the concatenated dataset. We performed two runs of 25 million generations, each with one cold and three heated chains and sampled the resulting trees every 1000 generations. Graphical plotting of ML scores suggested that stationarity was reached after approximately 100,000 generations, however, we discarded the first million generations as burn-in. Posterior probabilities of nodes were estimated from the remaining 24,000 trees. Flat priors were assumed for all parameters.

Using a well-supported group of lineages within the Bayesian tree (Clade A) and the program Mesquite v1.05 (Maddison & Maddison 2004), we calculated the likelihood that ancestral parasites were found in the New World. We employed the Mk1 model and considered ancestral state reconstruction to be signficant when raw likelihood scores for the two possible states (in New World or not-in-New World) differed by greater than 2 and the proportional likelihood of the best state was > 0.95.

RESULTS AND DISCUSSION

From 245 introduced and endemic resident forest birds captured during various seasons between 1971 and 1998 on Hawaii, Maui, Molokai, Oahu and Kauai, we recovered only a single mitochondrial lineage of *Plasmodium* (lineage 15; n = 75

sequenced infections; previously identified as GRW4, Bensch et al. 2000) and only one nuclear haplotype (DHFR haplotype G). We detected a second lineage of parasite (lineage 43) in a single migratory golden plover (*Pluvialis fulva*) from the Northwestern Hawaiian Islands, however we found no evidence for transmission of this parasite to Hawaiian forest birds. This result corroborates reports of just a single morphological subspecies of parasite in Hawaii (*P. relictum capistranoae*) (Laird & van Riper 1981; van Riper *et al.* 1986) and suggests that the recent expansion of native host populations into low-elevation forests over the last decade (Woodworth *et al.* 2005) has not been facilitated by the cryptic introduction of different parasite lineages of lower virulence.

While Hawaiian mitochondrial lineages of *P. relictum* were monotypic, pairwise divergence of other parasites identified as *P. relictum* averaged 4.0% (range: 0 to 7.6%), substantially greater than the intraspecific divergence observed in the human parasite *P. falciparum* across the entire mitochondrial genome (0.2%; Joy *et al.* 2003) and the divergence observed between sympatric haematozoan parasites restricted to different avian hosts (0.6%; Ricklefs *et al.* 2004). In addition, phylogenetic analysis of avian malaria parasites classified by morphology indicated that *P. relictum* does not form a monophyletic clade (figure 1). Deep nodes were generally not well supported, however, the close relationship of one isolate of *P. cathemerium* and two isolates of *P. elongatum* to *P. relictum* indicated that either morphology does not reflect evolutionary relationships or that previous classification has been in error. This conflict, as well as the disparity between the broad genetic diversity of parasites identified as *P. relictum* by morphology and the single type

found in Hawaii, suggests that ecological data, such as host and geographical ranges, which have been compiled for parasites classically identified as *P. relictum* are not necessarily applicable to the Hawaiian parasite.

Pinpointing the original host is difficult given the broad host range of *Plasmodium* spp. in general (Bennett *et al.* 1993; Beadell *et al.* 2004; Fallon *et al.* 2005) and of the GRW4 lineage in particular. Worldwide, we recovered GRW4 from 39 species of birds, representing 13 families (appendix I). We found the lineage frequently in continental populations of common mynas (*Acridotheres tristis*) and house sparrows (*Passer domesticus*), both of which were introduced to Hawaii, and also in great reed warblers (*Acrocephalus arundinaceus*). We did not detect GRW4 in a survey of 75 migratory shorebirds sampled from Hawaii, the French Frigate Shoals and Laysan Island, however, the lineage has been detected in a shorebird from Mauritania (Mendes *et al.* 2005).

The apparent lack of host-specificity of GRW4 is reflected by its broad geographic distribution. In addition to Hawaii, we detected GRW4 throughout the Old World, where it was particularly common relative to other malaria lineages in Europe (but only in adults of migratory species), India, and on several Indian Ocean islands (figure 2; table 1). Evidence from our surveys and extensive sampling of thousands of North American birds by R. Ricklefs and coworkers (R. E. Ricklefs, personal communication), however, suggests that the lineage is rare in birds from mainland North and South America. To our knowledge, the only two mainland hosts in which GRW4 has been detected are a house sparrow from California (Schrenzel *et al.* 2003) and a house finch from Arizona (M. Kimura pers. comm.). The only other

New World records of this lineage were derived from two individuals in the Lesser Antilles (Fallon *et al.* 2005). Given the wide host range of GRW4 and its presence in at least some New World host families (Emberizidae and Mimidae), our failure to detect GRW4 more widely in the New World likely reflects its rarity there and not simply an artifact attributable to differences in the composition of hosts sampled in different regions.

In contrast to its rarity relative to other lineages elsewhere in the New World, GRW4 was the only lineage detected in resident passerines of Bermuda, an oceanic island of volcanic origin lying 1000 km off the coast of North America. Colonization of Bermuda by GRW4 is likely to have occurred only recently since mosquitoes were reported as absent from Bermuda by the Spanish sailor Diego Ramirez, who was shipwrecked on the then-uninhabited island in 1603 (account published in Wilkinson 1950). Given subfossil evidence of unique endemic resident passerines existing in Bermuda prior to human colonization (Olson *et al.* 2005), it is possible that, as in Hawaii, the arrival of a competent vector (*C. quinquefasciatus* is currently present) and an Old World lineage of *Plasmodium* may have contributed to the extinction of another island avifauna.

In the Pacific, the Hawaiian form of malaria was also the only lineage of malaria parasite detected in passerines of French Polynesia, though we detected a second lineage exclusively in junglefowl (*Gallus gallus*). In a survey of birds from Moorea, we found GRW4 at low frequency in several introduced species including red-browed firetails (*Neochmia temporalis*; 2 of 34 individuals), silvereyes (*Zosterops lateralis*; 1 of 60 individuals) and common mynas (*Acridotheres tristis*; 2 of 10

individuals). We did not sample any of the native passerines on Moorea because populations of those species, namely the Tahiti reed warbler (*Acrocephalus caffer longirostris*), Pacific swallow (*Hirundo tahitica*) and Polynesian swiftlet (*Aerodramus leucophaeus*), if extant, were extremely small. However, among a small sample of endemic Marquesan reed warblers (*Acrocephalus mendanae*) collected in 1987 on Nuku Hiva, we detected GRW4 in 9 of 11 individuals. Because populations of Marquesan reed warblers remain fairly robust (Holyoak & Thibault 1984; J.-C. Thibault personal communication), this finding presents the possibility that, unlike its effect on Hawaiian honeycreepers, GRW4 may not pose a threat to these endemic French Polynesian passerines, which are relatively recently diverged from a mainland ancestor (ca. 1-2 Ma; Fleischer *et al.* unpublished data) and of Old World descent. Conversely, for older Polynesian endemics such as the *Pomarea* flycatchers, which have likely evolved for a longer time in isolation (ca. 3.6 Ma; Cibois *et al.* 2004), the introduction of GRW4 may represent a previously unrecognized factor driving the decline of these species, most of which are threatened or endangered (BirdLife International 2000). The detection of GRW4 in French Polynesia and the Cook Islands (Ishtiaq *et al.* 2006), and of a closely related parasite in the Marianas (lineage 32), warrants further investigation into the effects of avian malaria on isolated avifaunas outside of Hawaii.

While GRW4 itself exhibited a broad geographical distribution, the distribution of related parasite lineages provided evidence of an Old World ancestry for GRW4. Phylogenetic reconstructions of mitochondrial parasite lineages and associated nuclear haplotypes yielded broadly concordant topologies (figure 3).

Although there was little support for deep nodes when loci were analyzed separately, Bayesian analysis of data from both mitochondrial and nuclear loci combined recovered similar clustering of parasite lineages and provided support for the monophyly of parasite genotypes 1B through 38P (Clade A, figure 4). Within this group, but with the exception of 15G (GRW4), all of the parasite genotypes detected in the New World fell within a well-supported clade (Clade B, genotypes 1B through 14F) that was either sister to or derived from the remainder of genotypes in Clade A. Among the remainder of Clade A, all genotypes except 15G (GRW4) were recovered exclusively from the Old World and likelihood estimation of ancestral origins confirmed that the immediate ancestors of GRW4 likely derived from the Old World (figure 4). Among the 24 parasite mitochondrial lineages composing this group, 18 were recovered from hosts in Africa (figure 3). A haplotype network (figure 5), which may more appropriately describe non-bifurcating relationships among mitochondrial lineages derived from a single species, similarly indicated broad geographical substructure within Clade A and close association of GRW4 (lineage 15) with Old World lineages recovered from Africa (16) and New Guinea (17). The derived position of GRW4 relative to other lineages from the Old World further suggests that its range has only recently expanded to include parts of the New World.

The mitochondrial lineage GRW4 was associated with nuclear haplotype G everywhere except on several Indian Ocean islands where GRW4 was instead associated with haplotype H (figure 3). We identified several additional cases of a single mitochondrial lineage (e.g., 1, 30 and 31) associated with multiple nuclear haplotypes as well as cases of a single DHFR-TS sequence associated with multiple

divergent mitochondrial lineages (e.g., haplotype G and lineages 15, 16, 17, 18, 27, 28, 32, 33; figure 3). If every mitochondrial lineage of *Plasmodium* represents a sexually-isolated unit (Bensch *et al.* 2004), then these results may reflect either incomplete lineage sorting amongst genes in otherwise reproductively-isolated species or insufficient variation in our markers. On the other hand, the sharing of mitochondrial or nuclear haplotypes among different parasite lineages may simply represent intraspecific genetic variation. The resolution of our data precludes investigation of this on a fine scale, however, several cases in which nuclear and mitochondrial haplotypes exhibit similar clustering (e.g., lineages 11, 12, 13 and nuclear haplotypes D, E), combined with an apparent lack of genetic exchange with closely related parasites, provide an indication that species limits may be very narrow. Within the resolution of our data, the complete linkage disequilibrium of mitochondrial and nuclear markers found among parasites in Clade B (average cyt *b* p-dist: 1.5%) relative to other parasites in Clade A (avg. p-dist: 1.9%), and among parasites in Clade A (avg. p-dist: 2.2%) relative to the next most related lineages, suggests that these groups, at least, are reproductively isolated. Except for two parasites, which were described as *P. elongatum* and *P. cathemerium*, all other morphologically-described parasites with associated mitochondrial sequences falling within Clade A were identified as *P. relictum* (lineages 2, 5, 15, 16 and 22; figure 3). Given the results above, the morphological taxon *P. relictum* appears to be composed of at least two, and probably several more, reproductively-isolated groups.

The geographical structuring of parasite lineages within Clade A is surprising in light of the massive commercial and migratory movement of birds worldwide.

Parasites are often lost when their hosts are introduced to novel regions (Torchin *et al.* 2003; Colautti *et al.* 2004) and competence of novel hosts, host migration patterns, and competing strains of parasite may retard the exchange of parasites between hemispheres. Nonetheless, the prominence of GRW4 on several remote oceanic islands and its wide host distribution suggest that these are not primary factors limiting the range of GRW4. Instead, differential vector-parasite compatibility may be limiting transmission of GRW4 and driving genetic isolation between populations of *P. relictum*. Vector incompatibility may be preventing the transmission of GRW4 in northern Europe (Waldenström *et al.* 2002) and appears to be responsible for the isolation of New World and Old World forms of *P. vivax* (Li *et al.* 2001), the dominant form of malaria in humans.

We found further evidence for transmission limits in Bermuda, where we recovered three lineages of *Plasmodium*, but GRW4 was the only lineage of *Plasmodium* detected in blood from resident Bermuda passerines (n = 42 sequenced infections) sampled between 2002 and 2004. Among resident birds, we detected GRW4 in both introduced Old World hosts [house sparrows (*P. domesticus*) and European starlings (*Sturnus vulgaris*)] and New World hosts [grey catbirds (*Dumetella carolinensis*) and Eastern bluebirds (*Sialia sialis*), but never in white-eyed vireos (*Vireo griseus*; n=16) or great kiskadees (*Pitangus sulphuratus*; n= 33)]. As in Hawaii, Bermuda provides a wintering ground for numerous North American migrants, some of which may exhibit transmissible erythrocytic-stage malaria infections. Assuming that winter parasitemias are not low enough to prevent transmission, the absence of all lineages except GRW4 in Bermuda residents suggests

that either resident Bermuda birds are not competent hosts for most North American *Plasmodium* lineages or that the local vector is refractory to these lineages. The former is unlikely to be true for all resident passerines since many of these species colonized or were introduced to the island from the New World only within the last several hundred years (C. E. McIntosh *et al.*, unpublished work). In addition, we found one of the non-GRW4 lineages (lineage 1) in a migratory ovenbird in Bermuda and in house sparrows from continental North America, but never in resident house sparrows from Bermuda (n = 15 sequenced infections). The other non-GRW4 lineage in Bermuda was recovered from two migratory yellow-throated warblers (*Dendroica dominica*). This sequence matched a parasite lineage recovered in several other North American species and was only distantly related to lineages in Clades A. Given the distinct parasite lineages in resident and migratory species, it appears that refractoriness of the local strain of *C. quinquefasciatus* to parasites carried by migrants may be important in structuring the parasite community in Bermuda.

If transmission of GRW4 was initially limited to the Old World, as seems possible given the distribution of lineages most closely related to it, the spread and admixture of Old World populations of *C. quinquefasciatus* with genetically-differentiated New World populations (Fonseca *et al.* 2006) may be facilitating the expansion of GRW4 into new locations. Future experimental infections of New World mosquitoes with isolates of *P. relictum* from different regions could shed light on the mechanism underlying the current rarity of GRW4 in the New World. Given the diversity of lineages encompassed by the morphological taxon *P. relictum*, future assessment of the ecological and evolutionary impacts of GRW4 and other avian

malarias will require a molecular characterization of the pathogen in question. This will be particularly valuable, for example, when identifying an independent source of GRW4 with which to assess co-evolutionary models of virulence change in Hawaii. Previous hypotheses of virulence change (van Riper 1991; Atkinson *et al.* 1995) have been based in part on comparisons of pathogenicity between the Hawaiian parasite and a North American strain that was presumed to be its closest counterpart (van Riper 1991). Our results, which provide evidence of cryptic population structure within *Plasmodium relictum* and an Old World origin for the Hawaiian parasite, should provide a more robust foundation for understanding the evolution of virulence and the dynamics of host-parasite-vector interactions in Hawaii's model system.

TABLE

Table 1. Sampling effort and frequency with which the Hawaiian lineage of *Plasmodium* (GRW4, lineage 15) was recovered from regions shown in figure 2. Lineages defined as unique (differing by at least 1 bp) within a given region may be shared between regions.

#	region	total host individuals sampled (n)	total species sampled (n)	host species with *Plasmodium* (n)	*Plasmodium* sequences recovered (n)	sequences matching GRW4 (n)	minimum unique lineages (n)
1.	Hawaiian Archipelago	320	17	8	79	78	2
2.	French Polynesia[a]	161	8	4	14	14	1
3.	USA	161	21	10	61	0	12
4.	Bermuda	142	14	7	42	39	3
5.	Antilles/Venezuela	5553	169	47	303	2	17
6.	Guyana	195	53	22	42	0	23
7.	Uruguay	322	111	33	57	0	13
8.	Northern Europe[b]	2835	26	19	305	131	36
9.	Southern Europe[c]	1151	9	8	206	4	16
10.	Nigeria	827	71	33	101	7	30
11.	Western Africa[d]	656	105	62	174	0	44
12.	South Africa	171	15	8	60	1	15
13.	Indian Ocean Islands[e]	150	20	15	48	23	8
14.	India	259	43	23	71	18	23
15.	Burma	344	133	42	60	0	28
16.	Japan/Korea	209	58	26	48	1	15

17.	Australia/Papua New Guinea	454	106	30	56	3	22

[a]French Polynesia: Moorea (Society Islands), Nuku Hiva (Marquesas). [b]Northern Europe: Belarus, Belgium, England, Germany, Lithuania, Sweden. [c]Southern Europe: France, Israel, Italy, Spain, Ukraine. [d]Western Africa: Annabon, Bioko, Cameroon, Gabon, Principe, Sao Tome. [e]Indian Ocean Islands: Anjouan, Fregate, Grand Comore, Madagascar, Mauritius, Mayotte, Moheli, Praslin, Reunion, Rodrigues.

FIGURE LEGENDS

Figure 1. Phylogenetic relationships among morphologically-identified species of *Plasmodium* estimated using ML, MP and ME with cytochrome *b* sequences. Numbers above branches indicate bootstrap support based on 1000 replicates. Numbers before species names correspond to mitochondrial lineage numbers in figure 3. Sequences were obtained directly from Genbank (accession number in italics) or from extracts of blood smears obtained from the International Reference Collection for Avian Haematozoa (IRCAH) and M. Peirce.

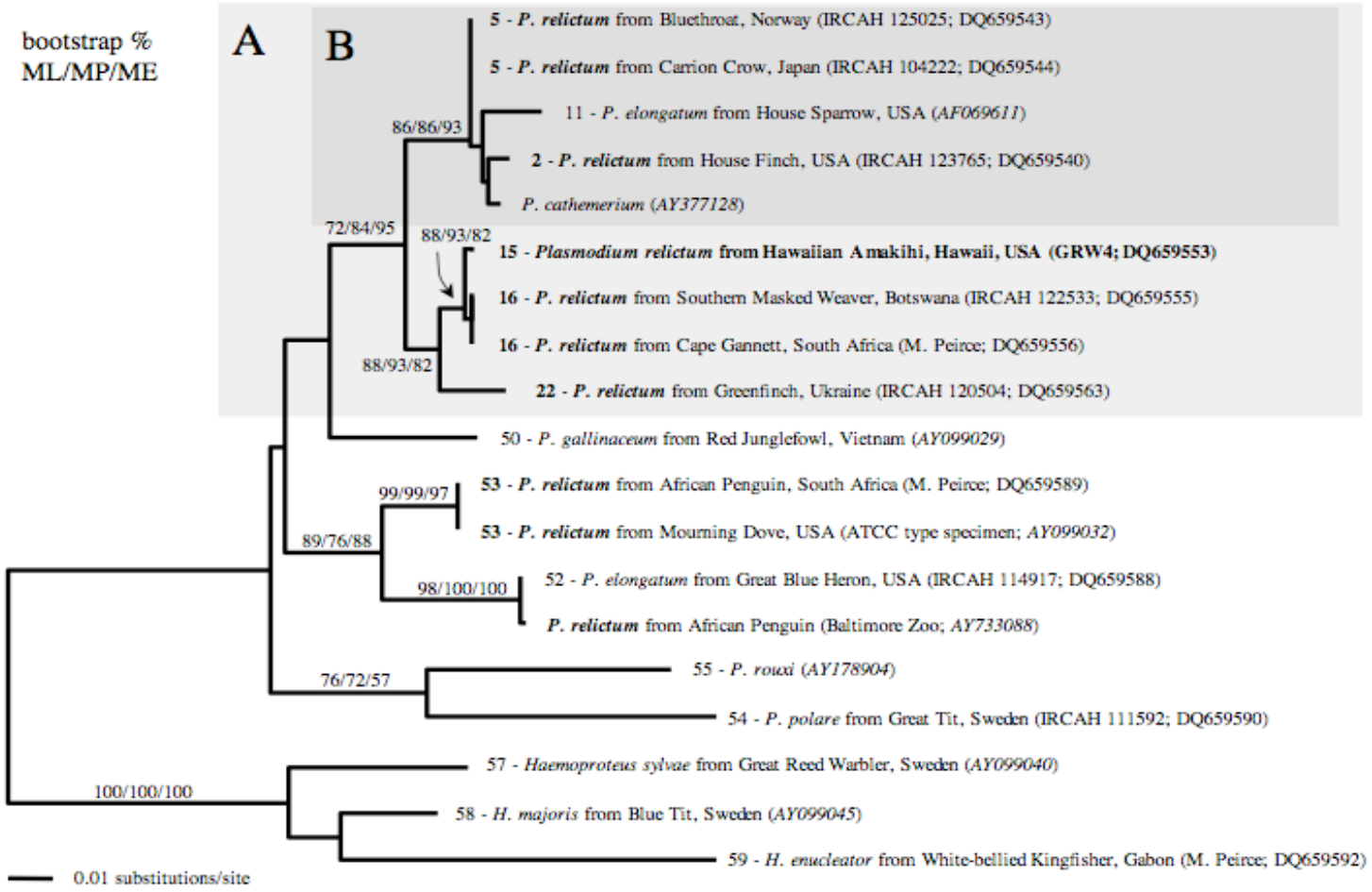
Figure 2. Map depicting the global distribution of the single mitochondrial lineage of malaria parasite (GRW4) found in resident Hawaiian passerines. Pie charts indicate the proportion of all sequenced *Plasmodium* infections in a given region that were identical to GRW4 (red). Details concerning locations and sampling effort are in table 1. Red dots indicate additional locations in which GRW4 has been reported previously (Ishtiaq *et al.*; Mendes e*t al.* 2005; Schrenzel *et al.* 2003; M. Kimura pers. comm.) or in which GRW4 was recovered from a relatively small group of samples (Kazakhstan).

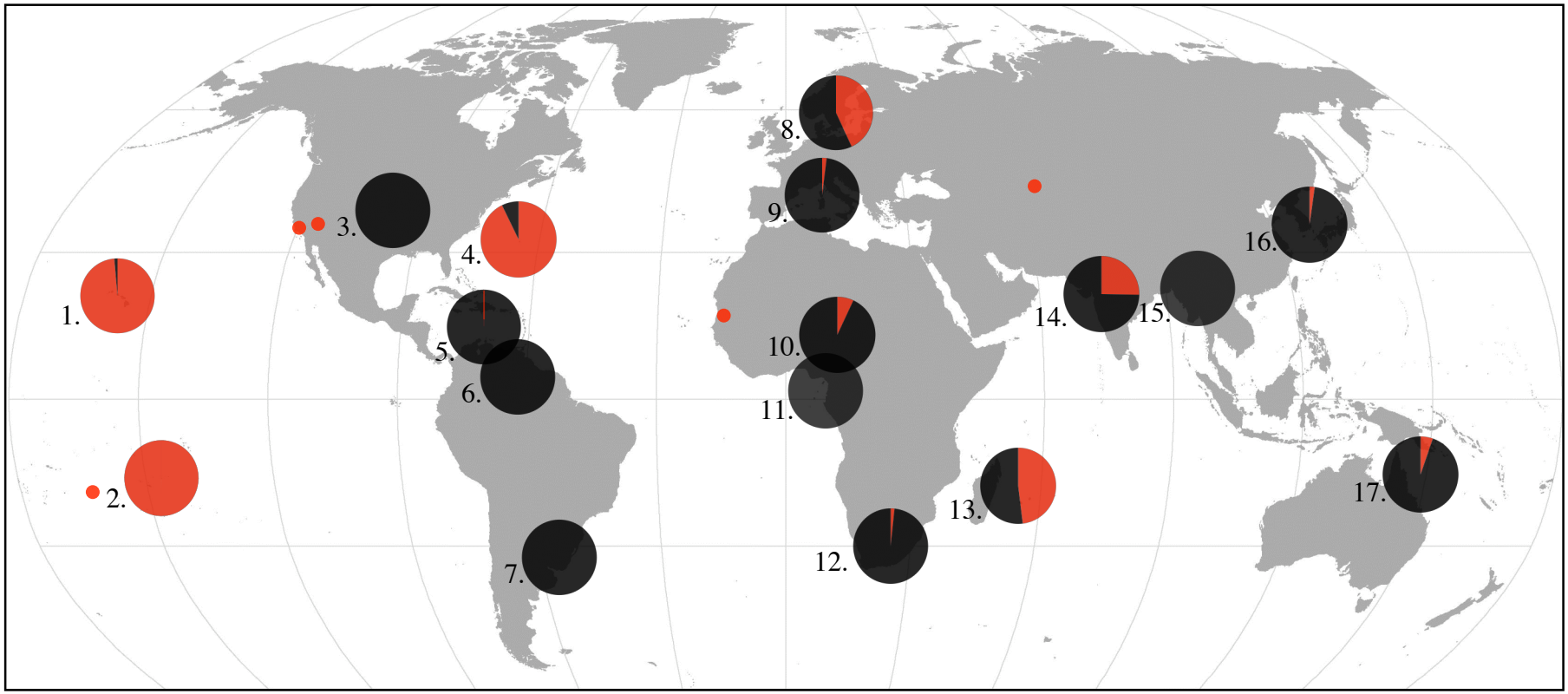
Figure 3. Phylogenetic trees of parasite mitochondrial lineages (cyt *b*; left; numbered) and associated nuclear haplotypes (DHFR-TS; right; lettered), constructed using maximum likelihood (GTR+I+G for both markers). The distribution of mitochondrial lineages across global regions is indicated with squares, color-coded to help identify the associated DHFR-TS sequence (when available, otherwise black).

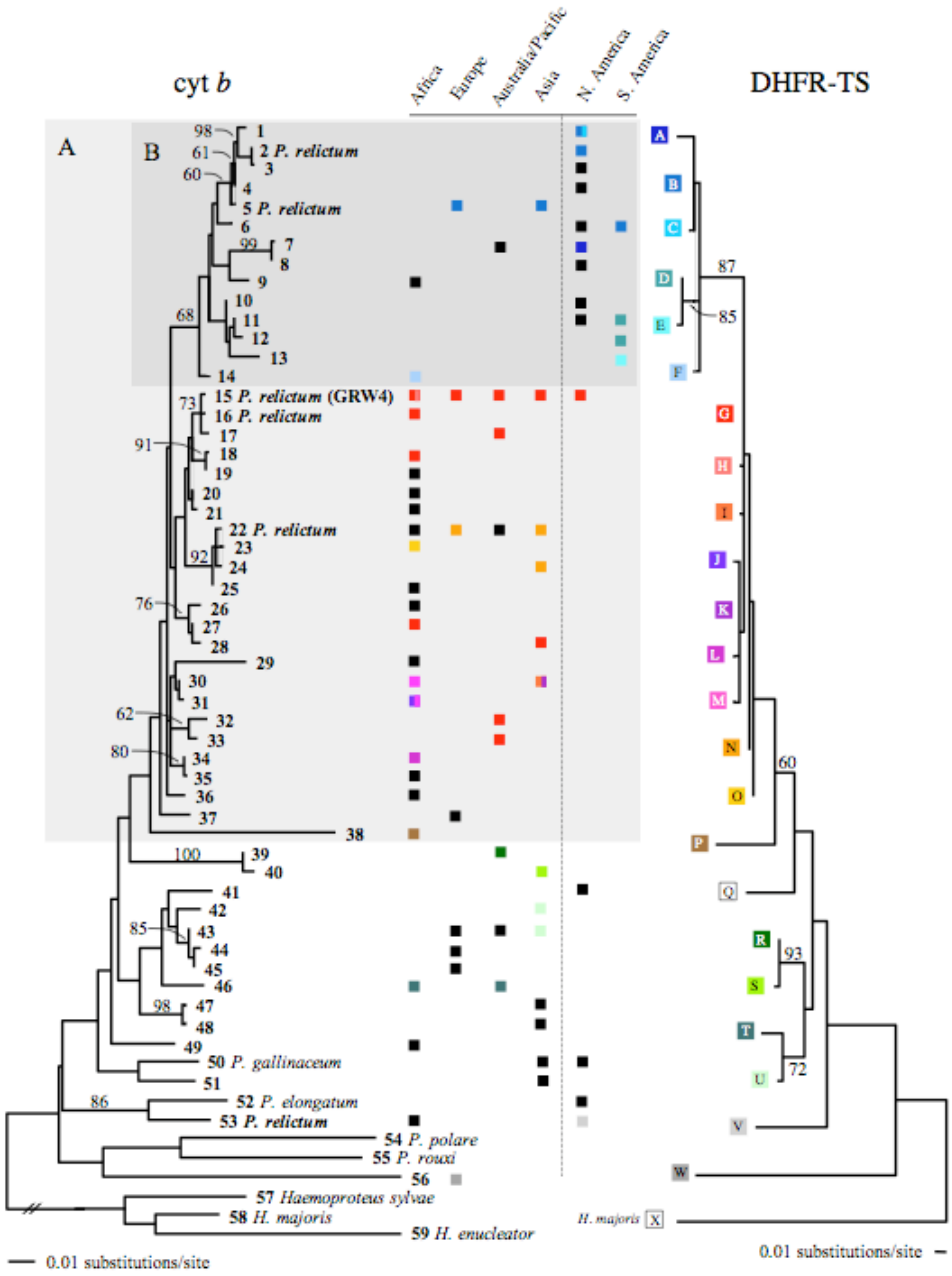
Background shading reflects the limits of two clades with good support in analysis of combined data (figure 4). Mitochondrial lineages which derived from at least one parasite identified as *P. relictum* by morphology are indicated in bold. Bootstrap support values (>60) are indicated above branches.

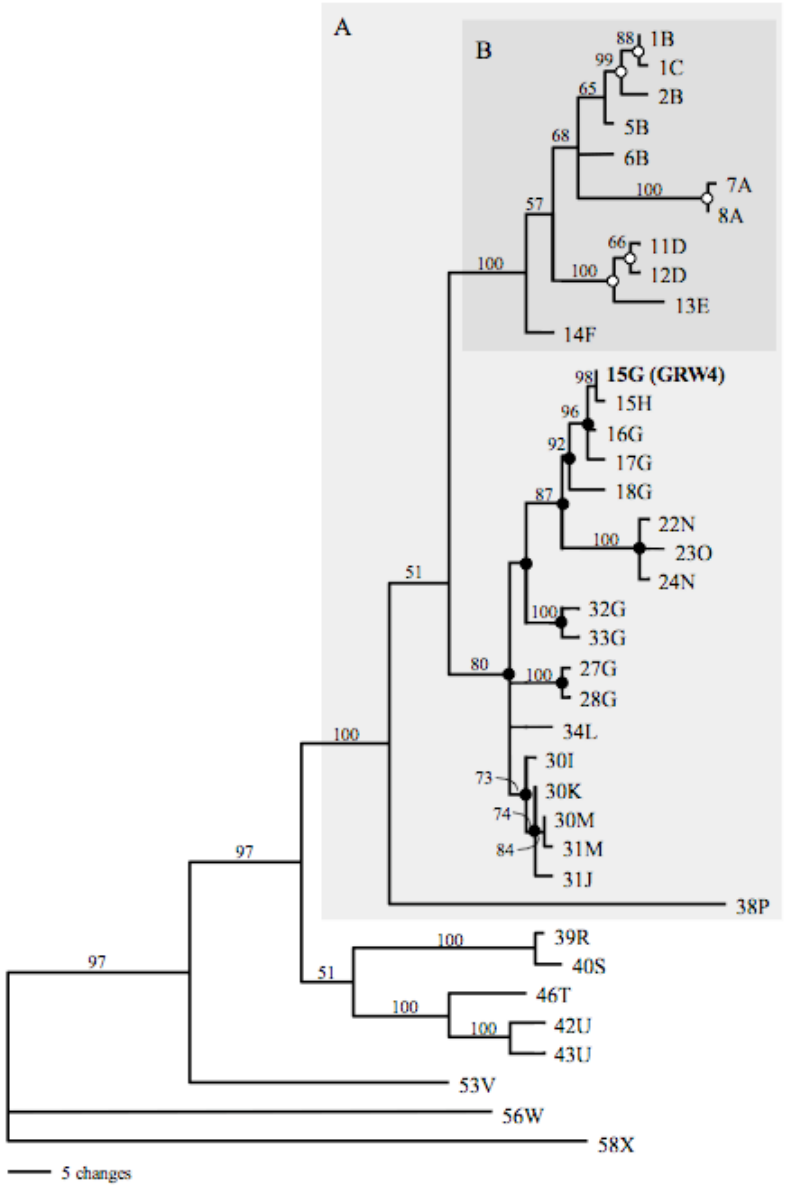
Figure 4. Majority rule consensus tree of avian malaria parasite lineages generated by Bayesian analysis of combined mitochondrial (cytb *b*) and nuclear (DHFR-TS) sequence. Parasite genotypes are identified by their respective cytb *b* lineage (number) and DHFR-TS haplotype (letter), which are depicted separately in figure 3. Clade credibility values are indicated above branches. Background shading identifies two well-supported clades (A and B) referenced in the text. Dots within Clade A indicate nodes for which New World (open circle) or non-New World (black) origin could be confidently assigned based on ancestral trait reconstruction performed with Mesquite.

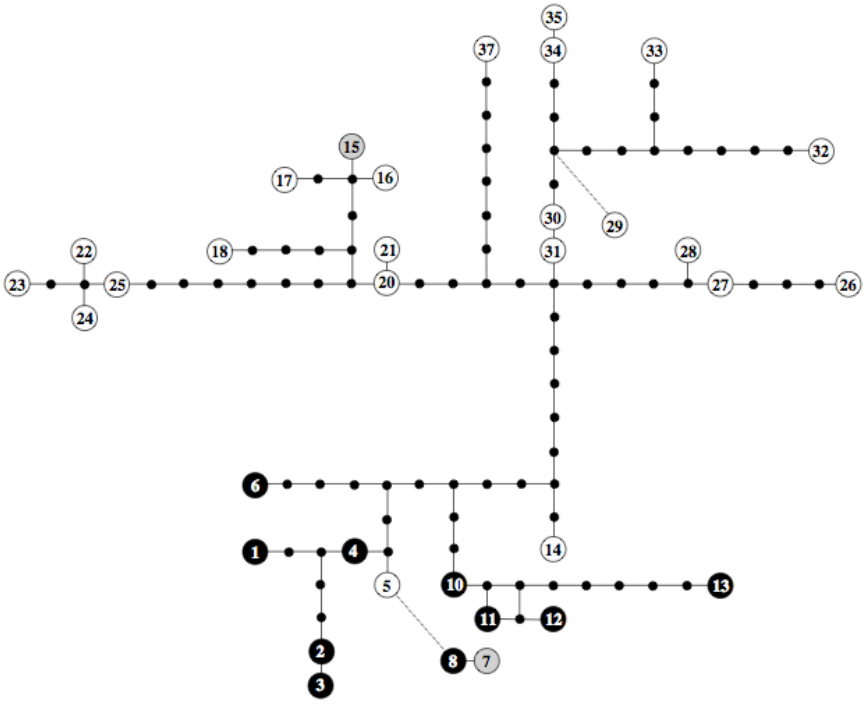
Figure 5. Statistical parsimony network of *Plasmodium* mitochondrial lineages related to the Hawaiian strain (lineage 15). Sampled haplotypes are numbered as in figure 3 and inferred haplotypes are indicated by black dots. Shading indicates whether the lineage was detected in the Old World (white), New World (black) or in both regions (grey). Lineages 9, 19, and 36 were not included due to missing sequence. Lineages 7, 8, and 29 were joined at the 90% connection limit (13 substitutions).


bootstrap %
ML/MP/ME
A
B
5 - P. relictum from Bluethroat, Norway (IRCAH 125025; DQ659543)
5 - P. relictum from Carrion Crow, Japan (IRCAH 104222; DQ659544)
11 - P. elongatum from House Sparrow, USA (AF069611)
2 - P. relictum from House Finch, USA (IRCAH 123765; DQ659540)
P. cathemerium (AY377128)
15 - Plasmodium relictum from Hawaiian Amakihi, Hawaii, USA (GRW4; DQ659553)
16 - P. relictum from Southern Masked Weaver, Botswana (IRCAH 122533; DQ659555)
16 - P. relictum from Cape Gannett, South Africa (M. Peirce; DQ659556)
22 - P. relictum from Greenfinch, Ukraine (IRCAH 120504; DQ659563)
50 - P. gallinaceum from Red Junglefowl, Vietnam (AY099029)
53 - P. relictum from African Penguin, South Africa (M. Peirce; DQ659589)
53 - P. relictum from Mourning Dove, USA (ATCC type specimen; AY099032)
52 - P. elongatum from Great Blue Heron, USA (IRCAH 114917; DQ659588)
P. relictum from African Penguin (Baltimore Zoo; AY733088)
55 - P. rouxi (AY178904)
54 - P. polare from Great Tit, Sweden (IRCAH 111592; DQ659590)
57 - Haemoproteus sylvae from Great Reed Warbler, Sweden (AY099040)
58 - H. majoris from Blue Tit, Sweden (AY099045)
59 - H. enucleator from White-bellied Kingfisher, Gabon (M. Peirce; DQ659592)
86/86/93
72/84/95
88/93/82
88/93/82
99/99/97
89/76/88
98/100/100
76/72/57
100/100/100
0.01 substitutions/site


1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
13.
14.
15.
16.
17.


cyt b
DHFR-TS
Africa
Europe
Australia/Pacific
Asia
N. America
S. America
A
B
1
2 P. relictum
3
4
5 P. relictum
6
7
8
9
10
11
12
13
14
15 P. relictum (GRW4)
16 P. relictum
17
18
19
20
21
22 P. relictum
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50 P. gallinaceum
51
52 P. elongatum
53 P. relictum
54 P. polare
55 P. rouxi
56
57 Haemoproteus sylvae
58 H. majoris
59 H. enucleator
H. majoris X
0.01 substitutions/site
0.01 substitutions/site


A
B
88
1B
99
1C
65
2B
5B
68
6B
100
7A
8A
57
66
11D
100
100
12D
13E
14F
98
15G (GRW4)
15H
96
16G
92
17G
18G
87
22N
100
23O
24N
51
100
32G
33G
80
100
27G
28G
34L
100
30I
73
30K
74
30M
84
31M
31J
38P
97
100
39R
40S
97
51
46T
100
100
42U
43U
53V
56W
58X
5 changes


35
37
34
33
15
32
17
16
30
29
22
18
21
31
28
23
25
20
27
26
24
6
1
4
14
5
10
13
11
12
2
8
7
3

APPENDICES

Appendix 1. Avian hosts, geographical origin, frequency of detection and GenBank accession numbers for parasite mitochondrial (cyt *b*) lineages. GenBank numbers for sequences obtained from previously published data are in italics.

lineage	host information					n	length	GenBank
	family	genus	species	common name	locality[a]		(bp)	no.
1	Fringillidae	*Carpodacus*	*mexicanus*	House finch	USA	1	753	DQ659538
1	Parulidae	*Seiurus*	*aurocapillus*	Ovenbird	BER	1	256	DQ838987
1	Passeridae	*Passer*	*domesticus*	House sparrow	USA	4	533	DQ838988
1	Troglodytidae	*Thryomanes*	*bewickii*	Bewick's wren	USA	1	533	DQ838989
1	Cardinalidae	*Cardinalis*	*cardinalis*	Northern cardinal	USA	28	533	DQ838990
1	Emberizidae	*Melospiza*	*georgiana*	Swamp sparrow	USA	-	551	*AY640130*
1	Hirundinidae	*Tachycineta*	*bicolor*	Tree swallow	USA	-	551	*AY640130*
2	Icteridae	*Molothrus*	*ater*	Brown-headed cowbird	USA	1	753	DQ659539
2	Fringillidae	*Carpodacus*	*mexicanus*	House finch	USA	2	653	DQ659540
3	Fringillidae	*Carpodacus*	*mexicanus*	House finch	USA	1	753	DQ659541
4	Parulidae	*Geothlypis*	*trichas*	Common yellowthroat	USA	1	753	*DQ659542*
5	Muscicapidae	*Luscinia*	*svecica*	Bluethroat	NOR	1	753	DQ659543
5	Phasianidae	*Phasianus*	*colchicus*	Ring-necked pheasant	KOR	1	533	DQ838991
5	Motacillidae	*Anthus*	*hodgsoni*	Olive-backed pipit	KOR	2	753	DQ838992
5	Corvidae	*Corvus*	*corone*	Carrion crow	JAP	1	433	DQ659544
6	Icteridae	*Icterus*	*cayanensis*	Epaulet oriole	URU	1	753	DQ659545

6	Emberizidae	*Poospiza*	*lateralis*	Red-rumped warbling finch	URU	1	256	DQ838993
6	Emberizidae	*Loxigilla*	*noctis*	Lesser Antillean bullfinch	DOM	-	312	AF465558
6	Troglodytidae	*Troglodytes*	*aedon*	House wren	URU	1	515	DQ838994
7	Parulidae	*Icteria*	*virens*	Yellow-breasted chat	USA	2	753	DQ659546
7	Pachycephalidae	*Eulacestoma*	*nigropectus*	Wattled ploughbill	PNG	-	533	*AY714201*
8	Parulidae	*Icteria*	*virens*	Yellow-breasted chat	USA	2	753	*DQ659547*
9	Muscicapidae	*Cercotrichas*	*podobe*	Black scrub-robin	NIG	-	478	AF495549
10	Tyrannidae	*Myiarchus*	*tyrannulus*	Brown-crested flycatcher	USA	1	753	DQ659548
11	Parulidae	*Geothlypis*	*trichas*	Common yellowthroat	USA	1	753	DQ659549
11	Passeridae	*Passer*	*domesticus*	House sparrow	USA	-	533	AF069611
11	Icteridae	*Gnorimopsar*	*chopi*	Chopi blackbird	URU	2	322	DQ838995
11	Turdidae	*Turdus*	*rufiventris*	Rufous-bellied thrush	URU	1	313	DQ838996
11	Troglodytidae	*Troglodytes*	*aedon*	House wren	URU	3	753	DQ838997
11	Icteridae	*Pseudoleistes*	*guirahuro*	Yellow-rumped marshbird	URU	1	533	DQ838998
11	Thraupidae	*Tangara*	*preciosa*	Chestnut-backed tanager	URU	1	533	DQ838999
11	Thraupidae	*Stephanophorus*	*diadematus*	Diademed tanager	URU	1	335	DQ839000
12	Emberizidae	*Emberizoides*	*herbicola*	Wedge-tailed grass-finch	GUY	1	753	DQ659550
12	Icteridae	*Sturnella*	*militaris*	Red-breasted blackbird	GUY	1	256	DQ839001
13	Icteridae	*Sturnella*	*supercilliaris*	White-browed	URU	2	753	DQ659551

				blackbird				
14	Turdidae	*Alethe*	*diademata*	Fire-crested alethe	GAB	4	753	DQ659552
15	Drepanididae	*Hemignathus*	*virens*	Hawaii amakihi	HI	12	753	DQ659553
15	Drepanididae	*Hemignathus*	*flavus*	Oahu amakihi	HI	6	533	DQ839002
15	Drepanididae	*Himatione*	*sanguinea*	Apapane	HI	4	315	DQ839003
15	Drepanididae	*Vestiaria*	*coccinea*	I'iwi	HI	2	338	DQ839004
15	Emberizidae	*Carpodacus*	*mexicanus*	House finch	HI	2	351	DQ839005
15	Emberizidae	*Loxigilla*	*violacea*	Greater Antillean bullfinch	DOM	1	533	DQ839006
15	Estrildidae	*Lonchura*	*punctulata*	Nutmeg mannikin	HI	1	351	DQ839007
15	Estrildidae	*Lonchura*	*malacca*	Black-headed munia	IND	1	256	DQ839008
15	Estrildidae	*Neochmia*	*temporalis*	Red-browed firetail	FP	2	753	DQ839009
15	Mimidae	*Dumetella*	*carolinensis*	Grey catbird	BER	7	533	DQ839010
15	Mimidae	*Mimus*	*gilvus*	Tropical mockingbird	GRE	1	533	DQ839011
15	Muscicapidae	*Cercomela*	*fusca*	Indian chat	IND	1	351	DQ839012
15	Muscicapidae	*Humblotia*	*flavirostris*	Grand Comoro flycatcher	GCO	1	295	DQ839013
15	Muscicapidae	*Luscinia*	*svecica*	Bluethroat	SWE	5	478	DQ839014
15	Nectariniidae	*Nectarinia*	*humbloti*	Humblot's sunbird	GCO	1	295	DQ839015
15	Nectariniidae	*Nectarinia*	*notata*	Long-billed green sunbird	GCO	1	533	DQ839016
15	Nectariniidae	*Cinnyris*	*sovimanga*	Souimanga sunbird	MAD	1	91	DQ839017
15	Paradoxornithidae	*Paradoxornis*	*webbianus*	Vinous-throated parrotbill	KOR	1	91	DQ839018
15	Passeridae	*Passer*	*domesticus*	House sparrow	BER, HI, IND	67	533	DQ839019
15	Ploceidae	*Foudia*	*madagascarensis*	Red fody	GCO, MAD, PRA	5	533	DQ839020
15	Ploceidae	*Foudia*	*eminentissima*	Red-headed fody	GCO	2	533	DQ839021
15	Ploceidae	*Foudia*	*flavicans*	Rodrigues fody	ROD	1	533	DQ839022

15	Ploceidae	*Quelea*	*quelea*	Red-billed quelea	SAF	1	295	DQ839023
15	Sturnidae	*Acridotheres*	*tristis*	Common myna	AUS, HI, IND, FP	19	533	DQ839024
15	Sturnidae	*Sturnus*	*vulgaris*	European starling	BER	13	533	DQ839025
15	Sylviidae	*Acrocephalus*	*arundinaceus*	Greet reed-warbler	BEL, ISR, NIG, SWE,UKR	133	478	DQ839026
15	Sylviidae	*Acrocephalus*	*baeticatus*	African reed-warbler	NIG	1	478	DQ839027
15	Sylviidae	*Acrocephalus*	*gracilirostris*	Lesser swamp-warbler	NIG	1	478	DQ839028
15	Sylviidae	*Acrocephalus*	*schoenobaenus*	Sedge warbler	NIG	1	478	DQ839029
15	Sylviidae	*Acrocephalus*	*mendanae*	Marquesan reed-warbler	FP	9	753	DQ839030
15	Sylviidae	*Hippolais*	*pallida*	Olivaceous warbler	NIG	1	478	DQ839031
15	Sylviidae	*Megalurus*	*palustris*	Striated grassbird	IND	1	351	DQ839032
15	Sylviidae	*Orthotomus*	*cuculatus*	Common tailorbird	IND	1	351	DQ839033
15	Timaliidae	*Stachyris*	*pyrrhops*	Black-chinned babbler	IND	1	351	DQ839034
15	Zosteropidae	*Zosterops*	*borbonicus*	Mascarene white-eye	MAU, REU	3	533	DQ839035
15	Zosteropidae	*Zosterops*	*mouroniensis*	Comoro white-eye	GCO	2	295	DQ839036
15	Zosteropidae	*Zosterops*	*maderaspatanus*	Madagascar white-eye	GCO, MAD, MAY	5	533	DQ839037
15	Zosteropidae	*Zosterops*	*choloronothos*	Mauritius white-eye	MAU	1	121	DQ839038
15	Zosteropidae	*Zosterops*	*lateralis*	Silvereye	FP	1	351	DQ839039
16	Fringillidae	*Linurgus*	*olivaceus*	Oriole finch	BIO	1	753	DQ659554
16	Fringillidae	*Serinus*	*atrogularis*	Black-throated canary	SAF	2	753	DQ839040
16	Ploceidae	*Ploceus*	*velatus*	African masked-	BOT, SAF	3	653	DQ659555

				weaver				
16	Ploceidae	*Quelea*	*quelea*	Red-billed quelea	SAF, ZIM	19	533	DQ839041
16	Ploceidae	*Euplectes*	*orix*	Red bishop	SAF	1	295	DQ839042
16	Cisticolidae	*Cisticola*	*fulvicapillus*	Piping cisticola	SAF	1	295	DQ839043
16	Sulidae	*Sula*	*capensis*	Cape gannet	SAF	1	533	DQ659556
17	Monarchidae	*Myiagra*	*alecto*	Shining flycatcher	PNG	2	753	*DQ659557*
18	Ploceidae	*Ploceus*	*velatus*	African masked weaver	SAF	1	753	DQ659558
18	Ploceidae	*Quelea*	*quelea*	Red-billed quelea	BOT, SAF, ZIM	15	533	DQ839044
19	Ploceidae	*Quelea*	*quelea*	Red-billed quelea	BOT	1	351	DQ659559
20	Nectariniidae	*Cinnyris*	*coquerellii*	Mayotte sunbird	MAY	3	753	DQ659560
20	Nectariniidae	*Cinnyris*	*sovimanga*	Souimanga sunbird	MAD	1	533	DQ839045
21	Ploceidae	*Foudia*	*seychellarum*	Seychelles Fody	FRE	1	753	DQ659561
21	Nectariniidae	*Cinnyris*	*dussumieri*	Seychelles sunbird	FRE	1	533	DQ839046
22	Sylviidae	*Acrocephalus*	*arundinaceus*	Great reed warbler	SWE	1	753	DQ659562
22	Sylviidae	*Sylvia*	*atricapilla*	Blackcap	SPA	-	478	*AF495571*
22	Passeridae	*Passer*	*luteus*	Sudan golden-sparrow	NIG	-	478	AF495571
22	Fringillidae	*Carduelis*	*chloris*	European greenfinch	UKR	1	456	DQ659563
22	Paridae	*Parus*	*major*	Great tit	KOR	1	351	DQ839047
22	Paridae	*Sittiparus*	*varius*	Varied tit	KOR	1	256	DQ839048
22	Corvidae	*Garrulus*	*glandarius*	Eurasian jay	KOR	1	753	DQ839049
22	Sturnidae	*Acridotheres*	*tristis*	Common myna	IND, NZ	3	256	DQ839050
23	Passeridae	*Passer*	*melanurus*	Mossie	SAF	1	753	DQ659564
24	Motacillidae	*Motacilla*	*alba*	White wagtail	KOR	1	753	DQ659565
24	Sylviidae	*Acrocephalus*	*orientalis*	Oriental reed warbler	KOR	1	256	DQ839051
25	Ploceidae	*Quelea*	*quelea*	Red-billed quelea	SAF	1	753	DQ659566
26	Zosteropidae	*Zosterops*	*senegalensis*	African yellow	SAF	1	753	DQ659567

				white-eye				
27	Nectariniidae	*Cyanomitra*	*oritis*	Cameroon sunbird	BIO	1	753	DQ659568
27	Ploceidae	*Ploceus*	*melanogaster*	Black-billed weaver	BIO	1	517	DQ839052
28	Alaudidae	*Alauda*	*arvensis*	Sky lark	KOR	1	753	DQ659569
28	Paridae	*Parus*	*major*	Great tit	KOR	2	256	DQ839053
29	Nectariniidae	*Cyanomitra*	*olivacea*	Olive sunbird	GAB, CAM, PRI	6	753	DQ659570
29	Nectariniidae	*Cinnyris*	*chloropygius*	Olive-bellied sunbird	GAB	3	533	DQ839054
30	Ploceidae	*Ploceus*	*nigerrimus*	Vieillot's weaver	GAB	1	753	DQ659571
30	Ploceidae	*Ploceus*	*nigricollis*	Black-necked weaver	GAB	1	533	DQ839055
30	Muscicapidae	*Copsychus*	*malabaricus*	White-rumped shama	BUR	1	533	DQ839056
30	Muscicapidae	*Copsychus*	*saularis*	Oriental magpie-robin	BUR	1	533	DQ839057
30	Estrildidae	*Lonchura*	*punctulata*	Nutmeg manikin	IND	1	351	DQ839058
30	Monarchidae	*Hypothymis*	*azurrea*	Black-naped monarch	BUR	1	295	DQ839059
31	Ploceidae	*Foudia*	*madagascariensis*	Red fody	MAD, MAY, MAU	5	753	DQ659572
31	Ploceidae	*Ploceus*	*grandis*	Giant weaver	SAO	2	533	DQ839060
31	Ploceidae	*Ploceus*	*princeps*	Principe golden-weaver	PRI	1	491	DQ839061
32	Rhipiduridae	*Rhipdiura*	*rufifrons*	Rufous fantail	MAR	2	753	DQ659573
33	Monarchidae	*Monarcha*	*kaupi*	Pied monarch	AUS	3	753	DQ659574
33	Monarchidae	*Monarcha*	*trivirgatus*	Spectacled monarch	AUS	3	533	AY714199
33	Petroicidae	*Heteromyias*	*albispecularis*	Grey-headed robin	AUS	1	533	*AY714199*
34	Ploceidae	*Ploceus*	*princeps*	Principe golden-weaver	PRI	1	753	DQ659575

34	Nectariniidae	*Hedydipna*	*collaris*	Collared sunbird	GAB	1	533	DQ839062
34	Nectariniidae	*Hedydipna*	*platura*	Pygmy sunbird	NIG	-	478	AF495566
34	Sturnidae	*Lamprotornis*	*splendidus*	Splendid glossy-starling	PRI	1	518	DQ839063
35	Muscicapidae	*Fraseria*	*cinerascens*	White-browed forest flycatcher	GAB	1	753	DQ659576
36	Nectariniidae	*Cinnyris*	*chloropygius*	Olive-bellied sunbird	CAM	1	533	DQ659577
37	Sylviidae	*Phylloscopus*	*trochilus*	Willow warbler	SWE	1	753	DQ659578
38	Nectariniidae	*Cyanomitra*	*olivacea*	Olive sunbird	GAB	1	753	DQ659579
39	Ptilonorhynchidae	*Scenopoeetes*	*dentirostris*	Tooth-billed bowerbird	AUS	3	753	*DQ659580*
39	Ptilonorhynchidae	*Ailuroedes*	*melanotis*	Spotted catbird	AUS	2	533	AY714205
39	Acanthizidae	*Sericornis*	*magnirostris*	Large-billed scrubwren	AUS	2	533	AY714205
39	Meliphagidae	*Lichenostomus*	*frenatus*	Bridled honeyeater	AUS	1	533	AY714205
39	Monarchidae	*Monarcha*	*trivirgatus*	Spectacled monarch	AUS	1	533	AY714205
39	Pachycephalidae	*Colluricincla*	*boweri*	Bower's shrike-thrush	AUS	1	533	AY714205
40	Aegithinidae	*Aegithina*	*tiphia*	Common iora	BUR	2	753	DQ659581
41	Turdidae	*Turdus*	*migratorius*	American robin	USA	-	753	AY099033
42	Emberizidae	*Emberiza*	*rutila*	Chestnut bunting	KOR	1	753	DQ659582
42	Emberizidae	*Emberiza*	*spodocephala*	Black-faced bunting	KOR	2	533	DQ839064
43	Charadriidae	*Pluvialis*	*fulva*	Pacific golden plover	HI	1	753	DQ659583
43	Fringillidae	*Carpodacus*	*erythrinus*	Common rosefinch	KOR	1	753	DQ839065
43	Paridae	*Parus*	*major*	Great tit	SWE	-	413	AF254978
44	Paridae	*Parus*	*major*	Great tit	SWE	-	447	AF495564
45	Turdidae	*Turdus*	*philomelos*	Song thrush	SWE	-	472	AF495576
46	Cisticolidae	*Camaroptera*	*brachyura*	Green-backed	GAB	1	753	DQ659584

				camaroptera				
46	Dicruridae	*Dicrurus*	*adsimilis*	Fork-tailed drongo	GAB	1	334	DQ839066
46	Estrildidae	*Pyrenestes*	*ostrinus*	Black-bellied seedcracker	GAB	1	335	DQ839067
46	Estrildidae	*Estrilda*	*astrild*	Common waxbill	TAN	1	533	DQ839068
46	Estrildidae	*Spermophaga*	*haematina*	Western bluebill	GAB	1	334	DQ839069
46	Muscicapidae	*Cossypha*	*niveicapilla*	Snowy-crowned robin-chat	GAB	1	333	DQ839070
46	Muscicapidae	*Muscicapa*	*olivascens*	Olivaceous flycatcher	GAB	1	533	DQ839071
46	Muscicapidae	*Stiphrornis*	*erythrothorax*	Forest robin	GAB	2	533	DQ839072
46	Nectariniidae	*Chalcomitra*	*rubescens*	Green-throated sunbird	GAB	1	334	DQ839073
46	Nectariniidae	*Cyanomitra*	*olivacea*	Olive sunbird	GAB	1	334	DQ839074
46	Nectariniidae	*Hedydipna*	*collaris*	Collared sunbird	GAB	1	533	DQ839075
46	Ploceidae	*Malimbus*	*nitens*	Grey's malimbe	GAB	1	334	DQ839076
46	Ploceidae	*Ploceus*	*cucullatus*	Village weaver	GAB	1	503	DQ839077
46	Pycnonotidae	*Andropadus*	*gracilis*	Grey greenbul	GAB	1	533	DQ839078
46	Pycnonotidae	*Andropadus*	*virens*	Little greenbul	CAM	2	533	DQ839079
46	Pycnonotidae	*Bleda*	*notata*	Lesser bristlebill	GAB	1	334	DQ839080
46	Pycnonotidae	*Bleda*	*syndactyla*	Common bristlebill	GAB	2	334	DQ839081
46	Pycnonotidae	*Criniger*	*calurus*	Red-tailed greenbul	GAB	1	533	DQ839082
46	Pycnonotidae	*Criniger*	*chloronotus*	Eastern bearded greenbul	GAB	1	334	DQ839083
46	Pycnonotidae	*Nicator*	*chloris*	Yellow-spotted nicator	GAB	1	315	DQ839084
46	Rhipiduridae	*Rhipidura*	*rufifrons*	Rufous fantail	MAR	2	753	DQ839085
46	Turdidae	*Alethe*	*poliocephala*	Brown-chested alethe	GAB	4	533	DQ839086
46	Turdidae	*Neocossyphus*	*fraseri*	Rufous thrush	GAB	4	533	DQ839087

46	Turdidae	*Neocossyphus*	*poensis*	White-tailed ant-thrush	GAB	2	334	DQ839088
46	Turdidae	*Neocossyphus*	*rufus*	Red-tailed ant-thrush	GAB	1	533	DQ839089
46	Turdidae	*Zoothera*	*cameronensis*	Black-eared ground-thrush	GAB	1	533	DQ839090
46	Turdidae	*Cyanomitra*	*olivacea*	Olive sunbird	GAB	1	533	DQ839091
47	Timaliidae	*Pomatorhinus*	*ferruginosus*	Coral-billed scimitar-babbler	BUR	1	753	DQ659585
47	Timaliidae	*Alcippe*	*morrisonia*	Grey-cheeked fulvetta	BUR	1	295	DQ839092
47	Muscicapidae	*Cinclidium*	*leucurum*	White-tailed robin	BUR	1	533	DQ839093
48	Muscicapidae	*Niltava*	*sundara*	Rufous-bellied niltava	BUR	1	533	DQ659586
49	Nectariniidae	*Cyanomitra*	*olivacea*	Olive sunbird	GAB	1	533	DQ659587
50	Icteridae	*Quiscalus*	*quiscula*	Common grackle	USA	-	753	AY099031
50	n/a	*n/a*	*n/a*	n/a	VIE	-	753	AY099029
51	Strigidae	*Ninox*	*scutulata*	Brown hawk-owl	SIN	-	753	*AY099035*
52	Ardeidae	*Ardea*	*herodias*	Great blue heron	USA	1	753	DQ659588
53	Columbidae	*Zenaida*	*macroura*	Mourning dove	USA	-	753	*AY099032*
53	Spheniscidae	*Spheniscus*	*demursus*	African penguin	SAF	1	335	DQ659589
54	Paridae	*Parus*	*major*	Great tit	SWE	1	753	DQ659590
55	n/a	*n/a*	*n/a*	n/a	n/a	-	478	*AY178904*
56	Sylviidae	*Acrocephalus*	*arundinaceus*	Great reed warbler	SWE	1	753	DQ659591
57	Sylviidae	*Acrocephalus*	*arundinaceus*	Great reed warbler	SWE	-	753	*AY099040*
58	Paridae	*Cyanistes*	*caeruleus*	Blue tit	SWE	-	753	*AY099045*
59	Alcedinidae	*Alcedo*	*leucogaster*	White-bellied kingfisher	GAB	1	753	DQ659592

[a]AUS, Australia; BER, Bermuda; BEL, Belarus; BIO, Bioko; BOT, Botswana; BUR, Burma; CAM, Cameroon; DOM, Dominican Republic; FRE, Fregate; FP, French Polynesia; GAB, Gabon; GCO, Grand Comore; GRE, Grenada; GUY, Guyana; HI, Hawaii; IND, India; ISR, Israel; KOR, Korea; MAD, Madagascar; MAU, Mauritius; MAR, Marianas; MAY, Mayotte; NIG, Nigeria; NOR, Norway; NZ, New Zealand; PNG, Papua New Guinea; PRA, Praslin; PRI, Principe; REU, Reunion; ROD, Rodrigues Island; SAF, South Africa; SAO, Sao Tome; SIN, Singapore; SWE, Sweden; TAN, Tanzania; UKR, Ukraine; URU, Uruguay; USA, United States; VIE, Vietnam; ZIM, Zimbabwe

Appendix 2. Avian hosts, geographical origin, frequency of detection, associated mitochondrial lineage and GenBank accession numbers for DHFR-TS haplotypes shown in figure 3. DHFR-TS did not amplify from all samples for which a mitochondrial lineage was recovered.

lineage	host	location[a]	n	associated mtDNA lineage	GenBank no.
A	Yellow-breasted chat	USA	2	7	DQ659597
A	Yellow-breasted chat	USA	3	8	DQ659597
B	House finch	USA	1	1	DQ659598
B	Brown-headed cowbird	USA	1	2	DQ839094
B	Bluethroat	NOR	1	5	DQ839095
B	Carrion crow	JAP	1	5	DQ839096
B	Olive-backed pipit	KOR	2	5	DQ839097

B	Ring-necked pheasant	KOR	1	5	DQ839098
B	Epaulet oriole	URU	1	6	DQ839099
B	Red-rumped warbling finch	URU	1	6	DQ839100
B	House wren	URU	1	6	DQ839101
C	Northern cardinal	USA	2	1	DQ659599
D	Chestnut-backed tanager	URU	1	11	DQ659600
D	Wedge-tailed grass-finch	GUY	1	12	DQ839102
E	White-browed blackbird	URU	1	13	DQ659601
F	Fire-crested alethe	GAB	2	14	DQ659602
G	Hawaii amakihi	HI	4	15	DQ659603
G	Gray catbird	BER	4	15	DQ839103
G	Greater Antillean bullfinch	DOM	1	15	DQ839104
G	Red-browed firetail	FP	2	15	DQ839105
G	Marquesan reed-warbler	FP	2	15	DQ839106
G	European starling	BER	4	15	DQ839107
G	House sparrow	BER, HI	7	15	DQ839108
G	Iiwi	HI	2	15	DQ839109
G	Oahu amakihi	HI	1	15	DQ839110
G	Shining flycatcher	PNG	1	17	DQ839111
G	African masked weaver	SAF	1	18	DQ839112
G	Red-billed quelea	SAF	1	18	DQ839113
G	African masked weaver	SAF	1	16	DQ839112
G	Oriole finch	BIO	1	16	DQ839114
G	Black-billed weaver	BIO	1	27	DQ839115
G	Sky lark	KOR	1	28	DQ839116
G	Rufous fantail	MAR	2	32	DQ839117
G	Spectacled monarch	AUS	2	33	DQ839118
H	Madagascar white-eye	GCO, MAD	4	15	DQ659604
I	Black-naped monarch	BUR	1	30	DQ659605
J	Red fody	MAD, MAY	2	31	DQ659606

K	White-rumped shama	BUR	1	30	DQ659607
L	Collared sunbird	GAB	1	34	DQ659608
M	Vieillot's weaver	GAB	1	30	DQ659609
M	Giant weaver	SAO	1	31	DQ839119
M	Principe golden-weaver	PRI	1	31	DQ839120
N	Eurasian jay	KOR	1	22	DQ659610
N	Great tit	KOR	1	22	DQ839121
N	White wagtail	KOR	1	24	DQ839122
N	Blackcap	SPA	1	22	*AY560372*
O	Mossie	SAF	1	23	DQ659611
P	Olive sunbird	GAB	1	38	DQ659612
Q	n/a – *Plasmodium gallinaceum*	n/a	-	-	*AY033582*
R	Bower's shrike thrush	AUS	1	39	DQ659613
R	Bridled honeyeater	AUS	1	39	DQ839123
R	Spotted catbird	AUS	1	39	DQ839124
S	Common iora	BUR	1	40	DQ659614
T	Black-eared ground thrush	GAB	1	46	DQ659615
T	Little greenbul	CAM	1	46	DQ839125
T	Rufous fantail	MAR	2	46	DQ839126
U	Common rosefinch	KOR	1	43	DQ659616
U	Black-faced bunting	KOR	2	42	DQ839127
U	Chestnut bunting	KOR	1	42	DQ839128
V	Mourning dove	USA	1	53	DQ659617
W	Great reed-warbler	SWE	-	56	*AY560373*
X	Blue tit	SWE	-	58	*AY560369*

[a]AUS, Australia; BER, Bermuda; BIO, Bioko; BUR, Burma; CAM, Cameroon; DOM, Dominican Republic; FP, French Polynesia; GAB, Gabon; GCO, Grand Comore; GUY, Guyana; HI, Hawaii; JAP, Japan; KEN, Kenya; KOR, Korea; MAD, Madagascar; MAR, Marianas; MAY, Mayotte; NOR, Norway; PNG, Papua New Guinea; PRI, Principe; SAF, South Africa; SAO, Sao Tome; URU, Uruguay; USA, United States

CHAPTER IV

Immunological change in island birds: no uniform evidence of an "island syndrome"

ABSTRACT

Dramatic declines of native Hawaiian avifauna due to the human-mediated emergence of avian malaria and pox prompted an examination of whether reduced immune response is a generalized component of an island syndrome, potentially driven by increased inbreeding and reduced exposure to parasites. We tested this hypothesis by characterizing parasite exposure, genetic diversity and several measures of immune response in both recently-introduced and endemic island taxa and by comparing the results to those observed in closely-related mainland counterparts. We show that significant reorganization of the inflammatory cell-mediated response as well as levels of innate immune compounds such as natural antibodies and complement may occur following island colonization. However, we did not find evidence of uniformly reduced immune response in island taxa, even amongst those taxa with the longest residence times. Our results point to the potential importance of small differences in the pathogenic landscape and the stochastic history of mutation and genetic drift in shaping the immunological profiles of small isolated populations. Consequently, predicting the impact of introduced disease on the many other endemic faunas of the remote Pacific will remain a challenge.

INTRODUCTION

Emerging disease in wildlife is an important force driving the decline and extinction of threatened populations (Cooper 1993, Lyles and Dobson 1993, Wikelski et al. 2004) and may pose a threat to worldwide biodiversity (Daszak et al. 2000). Human-mediated environmental changes are often the root cause of disease emergence (Friend et al. 2001) and may have particularly dire consequences in island ecosystems. In Hawaii, for example, the introduction of non-native songbirds and the mosquito vector *Culex quinquefasciatus* has led to the emergence of avian malaria and avian poxvirus in endemic honeycreepers (Drepanididae), contributing to the dramatic declines and contracting range limits of several species (Warner 1968, Atkinson et al. 1995, 2000, Yorinks and Atkinson 2000). While host species that have been introduced to Hawaii from continental sources over the last several centuries are largely unaffected by avian malaria, endemic species may exhibit mortalities ranging as high as 100% (Atkinson et al. 2001, summary in Jarvi et al. 2001). This suggests that the introduced strain of malaria is not unusually virulent; instead, it appears that at least some long-term island residents are unusually susceptible to this parasite.

High susceptibility of island endemics to infectious disease has been proposed as a component of an "island syndrome" (Hochberg and Moller 2001, Matson 2006), which seeks to codify typical changes observed in body size (Millien 2006), life history traits such as survival and fecundity (Wiggins et al. 1998, Goltsman et al. 2005) and other features associated with insular organisms (Blondel 2000). Several factors common to insular life could be driving susceptibility in island endemics. First, the strength of selection exerted by infectious agents on the immune systems of

remote island taxa is likely to be lower than that experienced by mainland birds over their evolutionary history. Because the parasites that are successfully transported to an island by avian colonists are only a subsample of those present in the source host population and because even those parasites may go extinct due to reduced transmission probabilities while the small island host population becomes established (Colautti et al. 2004), parasite richness is typically low on islands compared to the mainland (Fromont et al. 2001, Beadell et al. 2006), though prevalence may vary depending on relative transmission efficiency and host densities (Dobson 1988). On the remote islands of the Pacific in particular, even accounting for recent extinctions (Steadman 1997), bird communities are extremely depauperate and unlikely to sustain the diversity or abundance of parasites observed in large and diverse mainland host communities. Given the physiological costs associated with developing, maintaining, and using an immune system (Klasing and Barnes 1988, Scrimshaw 1991, Lochmiller and Deerenberg 2000, Zuk and Stoehr 2002, Martin et al. 2003), in a parasite-impoverished environment, selection should favor birds that maximize fitness by allocating resources away from the immune system and perhaps towards other fitness-related traits such as reproductive effort (Gustafsson et al. 1994, Deerenberg et al. 1997, Nordling et al. 1998), survival (Gonzalez et al. 1999) or the expression of sexual ornaments (Hillgarth and Wingfield 1997, Peters et al. 2004).

A second factor which may contribute to susceptibility of island fauna is the low genetic diversity typically associated with small population sizes (Frankham 1997). Theory (Wright 1931, Nei et al. 1975) and observations on natural avian systems (Baker and Moeed 1987, Tarr et al. 1998) suggest that bottlenecks, such as

those experienced upon colonization of an island, are most likely to decrease allelic diversity (due to the loss of rare alleles) while heterozygosity will decline only if the bottleneck is severe and the growth rate of the population is low. Additional diversity may be lost due to serial bottlenecks (Clegg et al. 2002) if island populations, already constrained to be small by island size, are repeatedly reduced due to demographic stochasticity. This latter effect may be important in driving the differences in disease susceptibility observed in recently introduced versus endemic species. Observations in wild populations have confirmed the deleterious impacts of bottlenecks and inbreeding on immunological parameters (Reid et al. 2003, Hawley et al. 2005, Hale and Briskie 2007) and parasite susceptibility (Acevedo-Whitehouse et al. 2006, Pearman and Garner 2005, Whiteman et al. 2006), however, drift is unlikely to affect all populations similarly and thus, the impact of inbreeding on disease susceptibility is not likely to be uniform (Spielman et al. 2004).

The Hawaiian honeycreepers have become a model for understanding the susceptibility of a naïve fauna to exotic disease, but given a relative paucity of data on disease prevalence and consequences in island taxa (but see Goltsman et al. 1996, Wikelski et al. 2004, Gottdenker et al. 2005, Smits et al. 2005, Clifford et al. 2006), the extent to which this model applies elsewhere across the globe is not obvious. For example, in contrast to Hawaii, the avifauna of American Samoa is characterized by stable native communities exhibiting relatively high prevalence of chronic infection with possibly indigenous blood parasites (Jarvi et al. 2003, Atkinson et al. 2007). Lack of clear parallels to the Hawaiian model may reflect Hawaii's unique position as the most remote archipelago in the world or the unique susceptibility of the

Drepanidine radiation to exotic disease. Alternatively, introduced pathogens may have decimated similarly susceptible species so quickly that parallel declines have gone unrecorded elsewhere in the world. In the Pacific region especially, which harbors 24% of all threatened birds species (BirdLife International 2006), Hawaii, the Galapagos (Wikelski et al. 2004, Gottdenker et al. 2005, Parker et al. 2006) and New Zealand (Tompkins and Gleeson 2006) have received the vast majority of attention, to the neglect of numerous other archipelagoes, many of which are extremely isolated and home to small populations of endemics.

To investigate the evolution of immunity in island taxa and to test for a common immunological signature of an island syndrome, we characterized immunological responses in endemic and recently-introduced bird populations on remote islands of the Pacific and compared the results to closely-related taxa from mainland Australia. Because vertebrate immunity depends on a diversity of defenses of variable specificity and inducibility (Schmid-Hempel and Ebert 2003) and because successful immune defense may emphasize just a single component of those defenses (Zuk and Stoehr 2002), we characterized multiple components of immunity using techniques that were applicable to wild and, in some cases, vulnerable populations. As a measure of constitutive innate immunity, we assayed levels of natural antibodies and complement in plasma (Matson et al. 2005). Natural antibodies are germ-line encoded molecules that are important in initial recognition of pathogens (Ochsenbein et al.1999) and may be linked to activation of the B-cell mediated production of specific antibodies (Parmentier et al. 2004). They are also integral for initiating the action of complement, a suite of enzymes that function together to lyse foreign cells

(Janeway et al. 2005). As an index of cell-mediated immunity, we measured the delayed-type hypersensitivity response to injection with the plant-derived mitogen PHA. The swelling that results reflects the action of T-lymphocytes, which secrete cytokines and direct the recruitment of macrophages, basophils, heterophils and B-lymphocytes to the site of injection (Janeway et al. 2005). This response is potentially important in the defense against intracellular parasites such as viruses and haemosporidia (Gonzalez et al. 1999). A strong response has been linked to increased probability of survival (Gonzalez et al. 1999, Merino et al. 2000, Moller and Saino 2004) and may be indicative of high exposure to parasites over evolutionary time (Martin et al. 2001).

If evolution on remote, parasite-impoverished islands necessarily leads to increased susceptibility to exotic parasites, then we would expect immune responses to be lower in island populations relative to their mainland counterparts. In addition, we would expect this pattern to be most evident in island endemics relative to species that have been introduced to an island only recently. Alternatively, island colonization may lead to variable upregulation or downregulation of immune components depending on the costs of those components in a new environment, their lability in the face of genetic changes, and the particular parasites with which they are challenged. While a previous study pointed to defined patterns of immunological reorganization characteristic of an island syndrome (Matson 2006), here, we find no evidence for a uniformly reduced immune response or for other fixed patterns of change in island taxa. We explore this outcome in light of the genetic and parasitological context in which immunity has evolved and, through the consideration

of both recently-introduced and endemic island species, we address the timescale on which immunological changes have occurred in an extremely isolated avifauna.

METHODS

Avian System

We characterized immune response, genetic variability, and parasite exposure in mainland populations of three species of songbirds and compared the results to closely related island populations or species representing isolation at two different time scales. As a model of short-term isolation on islands, we sampled populations of Red-browed firetails (*Neochmia temporalis*) and Silvereyes (*Zosterops lateralis*) from their native range in Australia (Blakers et al. 1985) and also from French Polynesia. *N. temporalis* was introduced to French Polynesia in the late 19th century and may have been reintroduced in 1938, while *Z. lateralis* was most likely introduced in 1938 (Long 1981). As a model of long-term evolution in an island environment, we compared two island endemic species of *Acrocephalus* reed warbler to their most closely related mainland form (*A. australis,* Fleischer et al., unpublished manuscript). We sampled the Rimitara reed warbler (*A. rimitarae*) on Rimitara, Austral Islands, French Polynesia and the Bokikokiko (*A. aequinoctialis*) on Kiritimati, Line Islands, Kiribati as these species likely represent two distinct lineages of Pacific warblers (Fleischer et al., unpublished manuscript) and their populations were sufficiently large and accessible to accommodate sampling.

Australian populations of *N. temporalis, Z. lateralis* and *A. australis* were sampled using mistnets between June and July, 2005 at several sites in the region from Brisbane south to the Clarence River. Island populations of *N. temporalis* and *Z.*

lateralis were sampled at two sites on Moorea, French Polynesia in July and August, 2005. Rimitara reed warblers were sampled in August 2005 and Bokikokiko were sampled in March 2006. The timing of sampling insured that birds were not breeding, except in the case of the Bokikokiko which may breed opportunistically throughout the year given evidence of terrritoriality and nesting by some individuals in both March (pers. obs.) and mid-July (Milder and Schreiber 1982). In this case, we did not perform immune assays on females that were obviously attending a nest (i.e. females carrying an egg or possessing a well-developed brood patch). Protocols for handling birds were approved by Animal Care and Use Committees at the University of Maryland (R-05-19) and the Smithsonian National Zoological Park (05-10).

Parasite Screening

All captured birds were visually inspected for evidence of exposure to *Avipoxvirus* spp. (wartlike lesions on exposed skin). In addition, we screened blood smears for trypanosomes and microfilaria. For each slide, we scanned 30 fields at 100x and 50 fields at 500x magnification. Finally, we screened DNA, extracted from blood samples using DNeasy kits (Qiagen), for evidence of haematozoa in the genera *Haemoproteus, Leucocytozoon,* and *Plasmodium* using molecular methods described previously (Beadell et al. 2004, Beadell and Fleischer 2005). PCR methods have proven more likely to detect haematozoon infection than microscopy alone (Richard et al. 2002). Briefly, we used primers F2/R2 and 213F/372R to detect parasite infections. The latter includes restriction sites that are diagnostic for the three different parasite genera. In order to evaluate the diversity of parasite lineages present in any population, we used forward primers F2, Fifi, or 3760F with reverse primer

4292rw2 to amplify a 295 to 533 bp fragment of cytochrome b, which was then sequenced and compared to sequences on GenBank to confirm parasite identification. We tested for significant differences in prevalence between island and mainland populations using Fisher's exact test, since all comparisons involved cells with low values.

Genetic Variability

We quantified levels of genetic diversity using microsatellites designed by previous authors for use with taxa related to *N. temporalis* (6 loci; Sefc et al. 2001), *Z. lateralis* (12 loci; Degnan et al. 1999, Frentiu et al. 2003), and *Acrocephalus* spp. (12 loci; Hansson et al. 2000, Richardson et al. 2000). Loci chosen for use across species of Pacific *Acrocephalus* were originally isolated from distantly related taxa (*A. arundinaceus* or *A. seychellensis*) and therefore, ascertainment bias should not contribute to any differences in diversity observed between species. Generally, PCR reactions were carried out in a total volume of 10 uL with 1x PCR buffer, 1 U of AmpliTaq DNA polymerase (Applied Biosystems), 0.2 mM each dNTP (NEB), 0.5 uM each primer, and concentrations of MgCl and/or betaine and other conditions as indicated in the online appendix. Products were separated on an ABI 3100 Genetic Analyzer (Applied Biosystems). Alleles were aligned and scored using Genotyper 2.5 (Perkin-Elmer) and manually binned. All loci were tested for significant deviations from Hardy-Weinberg equilibrium (HWE) in Genepop. Samples that yielded homozygotes at any locus showing significant departures from HWE were rerun at less stringent conditions to reduce the likelihood of allelic dropout.

Values for observed heterozygosity were obtained from Genepop while values for allelic richness, genic diversity and the coefficient of inbreeding (F_{IS}) were calculated using FSTAT v 2.9.3. Differences between mainland and island values were tested for significance across loci within each species (i.e., *N. temporalis, Z. lateralis*) or species group (i.e., *Acrocephalus* spp.) using a Wilcoxon signed-ranks test implemented with SAS v 9.1. In addition, we calculated internal relatedness (IR) for each individual using microsatellite allele data and the Excel spreadsheet IR_macroN3 (Amos et al. 2001). IR provides a measure of inbreeding (i.e. parental relatedness), which is similar to standardized heterozygosity but weights alleles by their frequency in the population. Thus, an individual homozygous for a rare allele will be scored as more inbred than an individual homozygous for a common allele. To test for a significant effect of inbreeding on immune measures, we reran the analysis of immune differences (described below), but added IR, as well as the interaction of IR with population, as fixed effects in the ANCOVA.

Immunological Tests

We characterized multiple components of the avian immune system. As a measure of investment in the cell-mediated immune response, we challenged a subsample of captured birds with the plant-derived mitogen phytohaemagglutinin (PHA). Following the basic protocol of Smits et al. (1999), we measured the patagium of captured birds to the nearest 0.01 mm with a digital small-face spline micrometer (Fowler) prior to and 24 hours after injection with PHA. Each measurement was repeated three times and averaged. Resultant swelling, which is widely used as a measure of the cell-mediated response (reviewed in Martin et al. 2001), reflects the

recruitment and proliferation of T-lymphocytes and macrophages at the site of injection, as well as the action of other leucocytes (Martin et al. 2006). For *N. temporalis* and *Z. lateralis*, we injected 20uL of a 1.5mg/mL solution of PHA (Sigma L9017) in PBS buffer (Sigma P4417) into the patagium. For all *Acrocephalus* spp., which were larger, we injected 25ul of a 3mg/mL solution. Birds were housed in portable cages in the shade and provided with *ad libitum* water and food (seeds, fruit, or larval-stage invertebrates depending on the species).

We also characterized two components of constitutive innate immunity in plasma sampled from island and mainland populations. We measured levels of natural antibodies, as indicated by the agglutination of foreign red blood cells, and we measured levels of complement, as indicated by the lysis of these foreign cells (Matson et al. 2005). In the case of the island endemics *A. aequinoctialis* and *A. rimitarae*, as well as island and mainland populations of *N. temporalis* and *Z. lateralis*, plasma was obtained from blood samples taken immediately after capture of individuals that did not undergo further immunological testing. In the case of *A. australis*, several blood samples (n = 15) were collected after completion of the PHA assay (see results). Plasma was stored in a minus 20°C freezer in the field and then transferred to a -80°C freezer until assayed (June 2006). We performed serial dilutions (in PBS, Sigma P-4417) of plasma samples across 12 wells of a 96-well plate (Corning 3798) and then added an equal volume (25 uL) of 1% washed rabbit red blood cells (Hemostat R83546). Each plate included a dilution series of 7 randomly chosen plasma samples plus a row of negative controls (PBS only) to aid in scoring agglutination and lysis endpoints. Plates were covered with parafilm, mixed

for 2 minutes on a rotary shaker, then placed in a 37°C incubator for 90 minutes. Following tilting of the plate at 45 degrees for 20 minutes, we created a computerized image for future scoring of agglutination, and then incubated the plate at 37°C for an additional 70 minutes. At this point, plates were again scanned to record the extent of lysis. Agglutination titer, scored blindly with respect to species and location, was determined by the last plasma dilution at which aggregated blood cells showed "peaking" relative to the negative control. Lysis titer was determined by the lowest concentration of plasma at which >50% of rabbit red blood cells had ruptured. Assays were repeated on samples for which sufficient plasma was available and scores for these samples were averaged.

Least squares means for mainland and island immune responses were generated and tested separately for each taxa using contrasts in PROC MIXED (SAS v 9.1). We employed an ANCOVA framework with immune response as the dependent variable, population (or species in the case of *Acrocephalus*) as the main effect, and body condition (the residuals of the regression of mass on tarsus length) as a covariate. In all cases, we tested for a significant interaction of condition with population before proceeding with a model that did not include the interaction term.

RESULTS

Parasite Prevalence and Diversity

We did not detect trypanosomes, microfilarae or pox lesions in any individuals and therefore, parasite analyses are limited to haematozoan infections (fig. 1). Across all individuals of the three species surveyed in Australia (n = 165), we detected 8 unique lineages of blood parasite. In contrast, we detected only a single lineage of blood

parasite among the four island species (n = 174) surveyed on Kiritimati, Moorea and Rimitara.

Within *Z. lateralis*, prevalence of *Haemoproteus* spp. in the introduced population on Moorea, French Polynesia (0%, n = 59) was significantly lower than that observed in Australia (39.1%, n = 64; $p < 0.001$), where only a single lineage was detected. Prevalence of *Plasmodium* spp. did not differ significantly between island (1.7%) and mainland (6.3%) populations of *Z. lateralis*, however, mainland populations harbored at least 2 lineages of *Plasmodium*, both of which were divergent from the single lineage detected in just one individual from the French Polynesian population (see below).

No significant differences were observed in the prevalence of any blood parasites between island (n = 34) and mainland (n = 67) populations of *N. temporalis*. We detected a single individual infected with *Leucocytozoon* spp. in Australia and this parasite was not detected in the introduced population on Moorea, French Polynesia. In addition, *Plasmodium* spp. was detected in individuals from both populations at low prevalence (1.5 to 5.9%), however the lineage in Australia was different from that found in *N. temporalis* from French Polynesia. Interestingly, the only lineage of blood parasite recovered from any forest bird sampled on Moorea (including introduced species *N. temporalis*, *Z. lateralis*, *Pycnonotus cafer* (n = 10), *Lonchura castaneothorax* (n = 24), *Acridotheres tristis* (n = 10), *Estrilda astrild* (n = 10), *Geopelia striata* (n = 8) and the endemic dove *Ptilinopus purpuratus* (n = 5)) identically matched the strain of avian malaria introduced to Hawaii (Beadell et al. 2006), suggesting that it is a relatively recent introduction.

Plasmodium spp. was the only blood parasite detected in the mainland taxon *A. australis*. The prevalence of *Plasmodium* spp. in *A. australis* was 17.7% (n = 34) and we detected three distinct lineages of parasite. In contrast, we did not detect any blood parasites in the endemic reed warblers *A. aequinoctialis* (n = 25) or *A. rimitarae* (n = 56). The difference in the prevalence of *Plasmodium* spp. between island and mainland taxa was significant (p = 0.03 for both comparisons).

Genetic Diversity

No locus exhibited a significant departure from HWE within any island or mainland population when p-values were Bonferroni corrected for multiple comparisons (p > 0.004 for *Acrocephalus* spp. and *Z. lateralis*; p > 0.008 for *N. temporalis*). Ase13 and Ase58 exhibited significant linkage disequilibrium, but only within *A. aequinoctialis* (p < 0.0001). This is likely an artifact of low diversity given that these loci have been mapped to distinct linkage groups in the related warbler *A. arundinaceus* (B. Hansson pers. comm.) and that we did not detect linkage disequilibrium between these loci in either *A. australis* or *A. rimitarae*. All other pairs of loci appeared to segregate independently within each population when p-values were corrected for multiple comparisons (p > 0.0001 for *Acrocephalus* spp. and *Z. lateralis*; p > 0.003 for *N. temporalis*) and therefore, we treated locus-specific indices of diversity as independent samples when comparing genetic diversity between populations.

All loci examined were polymorphic in mainland populations of *Z. lateralis* and *N. temporalis* as well as recently introduced island populations of these species. In contrast, while 100% of loci were polymorphic in the mainland species *A. australis*, 3 of 12 loci were fixed in the island endemic *A. aequinoctialis* and 7 of 12

loci were fixed in *A. rimitarae* (genetic diversity indices summarized in table 1). Allelic richness tended to be lower in island populations of all species (or species groups) and the difference was significant in the case of the recently introduced island population of *Z. lateralis* (minimum n = 59, difference = -4.3 alleles, S = 33, p = 0.001) as well as the endemic warblers *A. aequinoctialis* (minimum n = 25, difference = -5.8 alleles, S = 39, p = 0.001) and *A. rimitarae* (minimum n = 25, difference = -6.6 alleles, S = 39, p = 0.001) relative to mainland counterparts. For *Z. lateralis* and *N. temporalis*, which were recently introduced to French Polynesia and in which allele frequency changes were unlikely to have been altered by mutation events, we tested whether low allelic diversity in French Polynesian populations could be attributed to the loss of rare alleles. For each species, we divided alleles recovered from the Australian source population into two classes depending on whether they had been retained or lost upon founding of the French Polynesian population. We excluded loci in which all alleles had been retained. For the remaining loci, we calculated the average frequency of alleles in each class and compared the difference across loci using a Wilcoxon signed-ranks test. As expected, the average frequency of alleles (in the Australian population) that were lost in French Polynesia tended to be lower than the frequency of alleles that were retained following colonization in *N. temporalis* (n = 5, Δ frequency = -0.14, S = 7.5, p = 0.063) and this difference was signficant in *Z. lateralis* (n = 11, Δ frequency = -0.226, S = 33, p = 0.001).

No significant differences were detected in either gene diversity (H_E) or observed heterozygosity (H_o) between recently introduced taxa and their mainland counterparts. However, both measures of heterozygosity were significantly lower in

the island endemics *A. aequinoctialis* (ΔH_E = -0.34, S = 36, p = 0.002, ΔH_o = -0.42, S = 39, p = 0.001) and *A. rimitarae* (ΔH_E = -0.50, S = 39, p = 0.001, ΔH_o = -0.51, S = 39, p = 0.001) compared to *A. australis*. F_{IS}, a measure of the overall level of inbreeding in a population, tended to be slightly, but not significantly, lower in recently introduced populations of *Z. lateralis* and *N. temporalis* relative to their mainland source (table 1). Conversely, F_{IS} was higher in both island endemic warblers compared to the mainland taxon however this difference was not significant. Our power to detect a significant difference among warblers was impaired by high levels of fixation across microsatellite loci, which allowed for the comparison of F_{IS} at just 4 polymorphic loci across all three species.

Immune Response

Across all immune tests, we did not observe consistent changes in island birds relative to their mainland counterparts, nor did we observe consistent trends even when island birds were grouped by island residence time (fig. 2). With regard to recently-introduced island residents, *Z. lateralis* in French Polynesia exhibited a significant decrease in cell-mediated response relative to the Australian population (difference = -0.34 mm, t = -4.37, df = 60, p < 0.001), however, no difference was evident between the innate response of island and mainland populations as measured by agglutination. The French Polynesian population of *N. temporalis*, on the other hand, showed no significant differences in either cell-mediated or innate immune response when compared to a mainland population. Neither species demonstrated a measurable lysis response in either island or mainland populations.

The two *Acrocephalus* species representing long-term island evolution exhibited strikingly divergent patterns of immune response across all three tests. A Wilcoxon-Mann-Whitney test for differences in innate immune responses between *A. australis* that had been treated with PHA (n = 15) and those that were untreated (n = 4) revealed no significant differences (agglutination, $Z = -1.18$, $p = 0.24$; lysis, $Z = -1.52$, $p = 0.13$), and therefore data were combined to provide a baseline for comparison to responses in endemic warblers. *A. aequinoctialis* exhibited little change in cell-mediated immune response relative to *A. australis*, however both measures of innate immunity were significantly lower (agglutination difference = -1.1 titers, $t = -2.42$, $df = 40$, $p = 0.020$; lysis difference = -1.1 titers, $t = -3.15$, $df = 41$, $p = 0.003$). In contrast, *A. rimitarae* exhibited significantly increased immune responses relative to *A. australis* across all three assays (patagial swelling difference = 0.26 mm, $t = 3.66$, $df = 62$, $p < 0.001$; agglutination difference = 1.3 titers, $t = 3.91$, $df = 40$, $p < 0.001$; lysis difference = 0.6 titers, $t = 2.38$, $df = 41$, $p = 0.022$).

Across *Acrocephalus* spp., agglutination titers and lysis titers were significantly correlated ($n = 46$, $r = 0.69$, $p < 0.001$). Among the 15 individuals of *A. australis* that were assayed for both cell-mediated and innate components of immunity, no correlation was evident between degree of patagial swelling and either agglutination or lysis titers.

The effect of body condition on immune response was significant in the models of cell-mediated ($F = 8.47$, $df = 60$, $p = 0.005$) and innate ($F = 4.96$, $df = 21$, $p = 0.037$) responses observed in *Z. lateralis*. Condition did not contribute significantly to any other model of immune response in either *N. temporalis* or *Acrocephalus* spp.

Individual measures of breeding (IR) did not account for a significant proportion of variance in any immune response in any of the taxa assayed, nor did we recover a significant interaction between IR and population (or species in the case of *Acrocephalus* spp.).

We examined the effect of infection status on immune response in *A. australis* and *Z. lateralis* from Australia, two populations in which infection rates were high enough to warrant tests. In *A. australis*, cell-mediated response tended to be lower in individuals infected with *Plasmodium* spp. (mean patagial swelling = 0.22, n = 5) compared to those that were uninfected (mean = 0.38, n = 19) but this difference was not signficant based on a t-test ($t = 1.36$, $df = 22$, $p = 0.19$). Similarly, no significant differences were observed in innate immune responses between infected and uninfected *A. australis*. Cell-mediated responses in *Z. lateralis* that were infected with *Haemoproteus* spp. (mean patagial swelling = 0.471, n = 9) also showed a decline relative to uninfected individuals (mean = 0.7088, n = 22) but this difference was not significant ($t = 1.79$, $df = 29$, $p = 0.084$), and the relationship became less obvious when comparing cell-mediated responses of mainland *Z. lateralis* infected (mean = 0.55, n = 11) or uninfected (mean = 0.69, n = 20) with any blood parasite ($t = 1.10$, $df = 29$, $p = 0.28$). Again, no significant differences were observed in innate immune response between infected and uninfected individuals.

DISCUSSION

Our results do not uniformly support the hypothesis that island taxa exhibit reduced immunological capacity compared to mainland relatives, despite the fact that our system incorporated two key components that could theoretically drive an island

syndrome: reduced exposure to pathogens and reduced genetic diversity. Those taxa with the longest history of evolution on islands were no more likely to exhibit reduced immune responses than taxa with shorter island residence times. Furthermore, changes in the response of island taxa relative to their mainland counterparts were not consistent among taxa with similar island residency times. These results, along with similar reports of significant population-level variation in immune response (Lindström et al. 2004, Matson 2006, Whiteman et al. 2006), suggest that this response may be more appropriately considered in light of the particular parasite communities to which a population is exposed and the particular genetic background in which immunity is evolving.

Recently Introduced Island Populations

In keeping with the prediction of an island syndrome, the island population of *Z. lateralis* showed a significant decrease in cell-mediated immunity relative to its mainland source population, although innate immunity was unchanged. In contrast to *Z. lateralis*, however, the immunological profile of the other recently-introduced island resident *N. temporalis* was largely similar to that found in mainland individuals, though our sample size for innate immunity provided low power with which to detect a difference. The differential effects of island life on the cell-mediated immune response in these two species may be attributable to the degree to which the island forms have escaped mainland parasites. While the island population of *Z. lateralis* appeared to have lost a common mainland parasite (*Haemoproteus* spp.), prevalence of haemosporidian parasites in *N. temporalis* was largely unchanged in the island population relative to the mainland. *N. temporalis* exhibited a low prevalence

of infection with avian malaria (*Plasmodium* spp.) both on the mainland and in French Polynesia and the one parasite that may have been lost by the island population occurred only infrequently on the mainland (*Leucocytozoon* spp.).

Haemosporidia represent only one class of parasite to which island birds may be differentially exposed. Given their potentially deleterious effects on reproduction and survival (Bennett et al. 1993, Merino et al. 2000, Valkiunas 2005), however, they are likely to be important factors structuring host immunity. The absence of *Haemoproteus* spp. in the island population of *Z. lateralis* may represent a relaxation of selection on the immune system, allowing for a shift in resources away from a costly nonspecific inflammatory cell-mediated response. Given that we did not detect any difference in response to PHA between mainland individuals that were infected or uninfected with *Haemoproteus* spp, it is unlikely that the population-level reduction in cell-mediated response observed in French Polynesia can be attributed to direct effects of parasitism on the response of mainland individuals. Parasitized individuals on the mainland tended to have lower cell-mediated response and thus, if these individuals were removed from consideration, the difference in response between island and mainland populations would have been even larger.

Among recently-introduced taxa, the effects of reduced parasite exposure on immune response cannot be fully decoupled from the potential effects of reduced genetic diversity. As with blood parasite exposure, genetic composition was similar between the immunologically indistinguishable island and mainland populations of *N. temporalis*. On the other hand, the island population of *Z. lateralis*, which exhibited lower prevalence of parasites, also exhibited reduced allelic richness. Conforming to

theoretical expectation, the alleles that were lost were rare in the mainland source population. If this reduction in allelic richness at neutral loci was accompanied by a parallel loss of functional alleles (e.g., antigen-binding motifs encompassed by the major histocompatibility locus and expressed on T-cells, Hansson and Richardson 2005; but see Aguilar et al. 2004), then this loss might be reflected by reduced sensitivity to a novel antigen such as PHA. Increased inbreeding has also been demonstrated to be negatively correlated to cell-mediated immune response within bottlenecked populations of song sparrows (Reid et al. 2003) and house finches (Hawley et al. 2005). In *Z. lateralis*, however, we observed similar levels of heterozygosity in island and mainland populations and found no support for a relationship between immune response and individual measures of inbreeding. Therefore, while we cannot exclude the possibility that the loss of a particular allele has contributed to a population-level effect of reduced cell-mediated immune response, inbreeding is unlikely to have affected this response

Island Endemics

While the change observed in cell-mediated response across populations of *Z. lateralis* could simply reflect plasticity in the immune system, the changes observed in the two island endemics relative to their mainland counterpart may represent long-term evolutionary responses to their isolated environment. *A. aequinoctialis* and *A. rimitarae* exhibit average pairwise cytochrome b divergences of 1.7% and 2.3%, respectively, from their closest mainland relative *A. australis* (based on 551 bp of cytb, K2P distances, data not shown). Using a molecular clock calibrated for passerine cytb (1.6% per million years; Fleischer et al. 1998) and correcting for

ancestral polymorphism (Nei and Li 1979, Avise et al. 1998) by subtracting the mean intraspecific divergence observed in the continental form (0.4%), these divergences equate to approximate separation times of 0.8 and 1.2 million years. Given this period of isolation, we expected that the strongest signal of an island syndrome would be found in the endemic reed warblers. Interestingly, the immunological profiles observed in *A. aequinoctialis* and *A. rimitarae* were extremely divergent and did not support this hypothesis, even though both species exhibited significant reductions in genetic diversity and reduced exposure to parasites. While *A. aequinoctialis* exhibited only reduced innate immune response relative to the baseline provided by *A. australis*, *A. rimitarae* exhibited significantly higher innate and cell-mediated responses than the mainland control.

If investment in immunity is costly, then the latter result is particularly surprising given the degree to which *A. rimitarae* has likely been exposed to pathogens. *A. rimitarae*, like *A. aequinoctialis*, appears to have escaped the avian malaria parasites present in its mainland congener, though the presence of a recently introduced lineage of parasite on adjacent archipelagoes suggests that this may soon change. Considering the isolation of Rimitara (ca. 3200 km east of Fiji, 6400 km east of Australia), the small island size (ca. 9km^2), and the paucity of alternative terrestrial reservoirs for pathogens (four species including junglefowl and one introduced finch), our failure to detect haematozoa in *A. rimitarae* (as well as in the co-occurring finch *L. castaneothorax*, n = 10) may reflect an impoverished pathogen community in general. The same is likely true for Kiritimati, which is substantially larger, but similarly isolated (ca. 6400 km east-northeast of Australia) and host to just two

additional terrestrial bird species (a native lorikeet and junglefowl). On the other hand, native passerines may be exposed to at least some of the pathogens known to be present in semi-domesticated junglefowl in both French Polynesia and Kiribati (Pacific Animal Health Information System http://www.spc.int/rahs, Gottdenker et al. 2005). We surveyed only a small fraction of the total pathogen community that may occur on these islands and therefore, it is possible that differential exposure to just a handful of unmeasured pathogens could be driving differential immune response. Futhermore, even if parasite communities on both islands are currently impoverished, slightly different histories of pathogen colonization and extinction on Rimitara and Kiritimati could be sufficient to drive differential investment in the immune system as well as differential partitioning of resources between arms of immunity.

Another possible explanation for the generally high response observed in *A. rimitarae* may be that the particular immune reponses that we measured are not extremely costly to maintain and use. For example, Matson (2006) has proposed that insular birds may actually favor innate immune responses over adaptive humoral immunity. Likewise, while the inflammatory cell-mediated response can be costly in terms of both nutrients required (Lochmiller and Deerenberg 2000) and potential damage inflicted to the organism itself (Janeway et al. 2005), it is not well understood how these costs compare to those required to support the adaptive antibody mediated response, which we did not measure. Adaptive responses are generally cheap to use but can incur substantial developmental costs associated with the time and resources required to produce a diverse B-cell repertoire (Humphrey et al. 2002). Immunocompetence in the face of a particular challenge may manifest itself by the

absence of a response (Boots and Bowers 2004), or at least by varied emphasis on any particular arm of the immune system (Zuk and Stoehr 2002). Therefore, our results for *A. rimitarae* would support the hypothesis of an island syndrome if the high responses observed were coupled to downregulation of a much more costly adaptive immune response. The increased cell-mediated response observed in *A. rimitarae* is consistent with the gradient of increasing inflammatory response observed in Galapagos finches exposed to fewer pathogens on increasingly smaller islands (Lindström et al. 2004). However, we did not observe a concomitant reduction in natural antibody titers, which was observed in the Galapagos finches and which may be indicative of adaptive antibody immune capacity (Parmentier et al. 2004). In addition, the contrasting immunological profile of *A. aequinoctialis* highlights the fact that immunological reorganization in island taxa, if it occurs at all, is not uniform.

One factor underlying this lack of uniformity in immune response may be the stochastic nature of genetic drift acting on regulatory regions or functional genes associated with immunity. As observed in *Z. lateralis*, an initial bottleneck associated with island colonization may result in a loss of allelic diversity across the genome, which if associated with a concomitant loss of MHC diversity (Richardson and Hansson 2005) could lead to reduced surveillance for foreign antigens. Over the longer-term, small island populations may exhibit further erosion of allelic diversity, as well as reduced heterozygosity and higher levels of inbreeding, as evidenced in *A. aequinoctialis* and *A. rimitarae*. Changes in immune response observed in bottlenecked or inbred populations (Reid et al. 2003, Hawley et al. 2005, Hale and Briskie 2007) may be linked to indirect effects of inbreeding, the random loss of

resistance alleles, and also the loss of any advantages that may be associated with overdominance. In addition, deleterious mutations in immunologically-important regions of DNA may become fixed given the increasing strength of drift over selection in small populations and the fact that selection may be reduced if the pathogenicity of the island environment is indeed reduced. Fixation of a mutation affecting regulation of the cell-mediated immune pathway could explain the unusually strong response observed in *A. rimitarae*. An optimal immune response is not necessarily a maximal response (Viney et al. 2005) and therefore, the strong immune response that we observed may be more indicative of a damaging allergic reaction than increased investment in that particular arm of immunity or increased ability to fight off disease.

Conclusions

Our results indicate that 1) significant reorganization of the inflammatory cell-mediated response as well as levels of innate immune compounds such as natural antibodies and complement may occur following island colonization and 2) immunological reorganization in island taxa is not uniform, even among taxa with similar residence times. Variation in immunological profiles likely reflect small differences in the pathogenic landscape and the stochastic history of mutation and genetic drift in small populations. Unfortunately, it is not clear to what extent high or low immune response can be linked to the phenotypes in which we are most interested: disease resistance or susceptibility (Adamo 2004). Similarly, while numerous studies have equated reduced genetic diversity to increased disease susceptibility (O'brien et al. 1985, Roelke et al. 1993, Acevedo-Whitehouse 2006,

Reid et al. 2003, Hawley et al. 2005, Pearman and Garner 2005), this relationship is not universally applicable (Reid et al. 2003, Spieleman 2004). Therefore, predicting the susceptibility of the many small and threatened populations of birds residing on islands of the remote Pacific may not be feasible. In the case of the genus *Acrocephalus*, the detection of a Hawaiian-like strain of avian malaria in populations of *A. mendanae* from the Marquesas, French Polynesia (Beadell et al. 2006) has provided the basis for a natural experiment. This species, like *A. aequinoctialis* and *A. rimitarae*, is a long-term island resident and exhibits reduced genetic diversity similar to its congeners studied here (unpubl. data). Interestingly, *A. mendanae* is apparently robust to infection with the introduced parasite and provides some hope that related members of the widely-distributed and highly endemic Pacific genus *Acrocephalus* will not be decimated by this parasite. We should be careful, however, in extrapolating even this far, considering that even the Hawaiian honeycreepers exhibit substantial variation in susceptibility to malaria (Jarvi et al. 2001) although they share a common evolutionary background. Immunity integrates not only energetic investments, which may be constrained by variable physiological demands of alternate life histories and differential parasite exposure, but also genetically-determined molecular recognition and regulation systems which are subject to random, population-specific effects of drift. Therefore, immunity in isolated fauna is unlikely to follow the simple heuristic of an island syndrome and may be best assayed with experimental challenges employing the particular pathogen of interest.

TABLE

Table 1. Genetic diversity indices for introduced and endemic island taxa compared to their mainland counterpart.

Population	n	% Loci polymorphic (total surveyed)	Allelic richness	H_E	H_o	F_{IS}[a]
Introduced						
Z. lateralis						
Mainland	64	100 (12)	8.1	0.57	0.54	0.048
Island	59	100 (12)	**3.8**	0.52	0.53	-0.027
N. temporalis						
Mainland	68	100 (6)	10.1	0.69	0.67	0.032
Island	34	100 (6)	8.0	0.70	0.69	0.007
Endemic						
Acrocephalus spp.						
Mainland (*A. australis*)	34	100 (12)	8.3	0.69	0.68	-0.005
Island (*A. aequinoctialis*)	25	75 (12)	**2.5**	**0.35**	**0.28**	0.124
Island (*A. rimitarae*)	56	42 (12)	**1.7**	**0.19**	**0.17**	0.145

Bold face indicates significant differences in the island index relative to the mainland ($p < 0.05$).

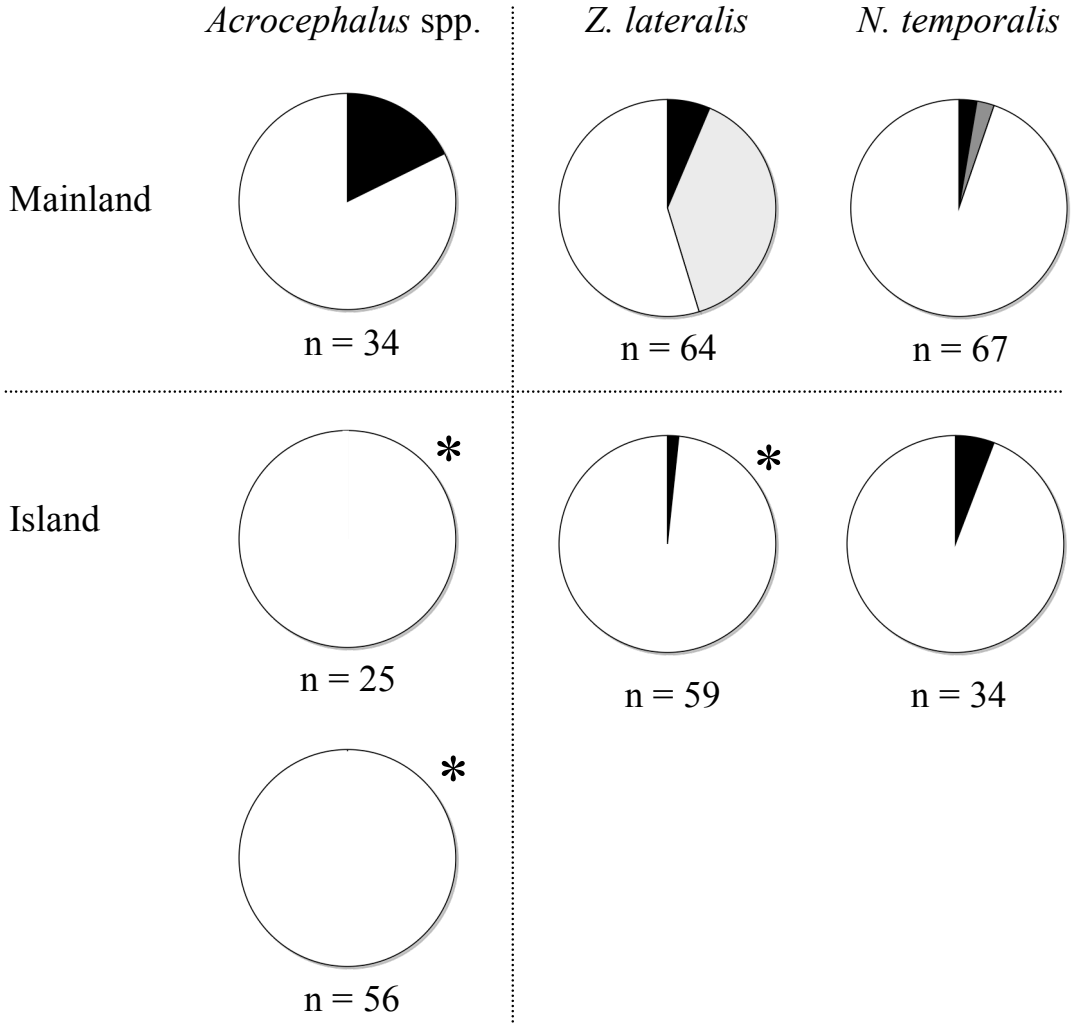
[a] For comparative purposes, F_{IS} for *Acrocephalus* is based on only 4 loci for which gene diversity (i.e., expected heterozygosity) was non-zero in all three species.

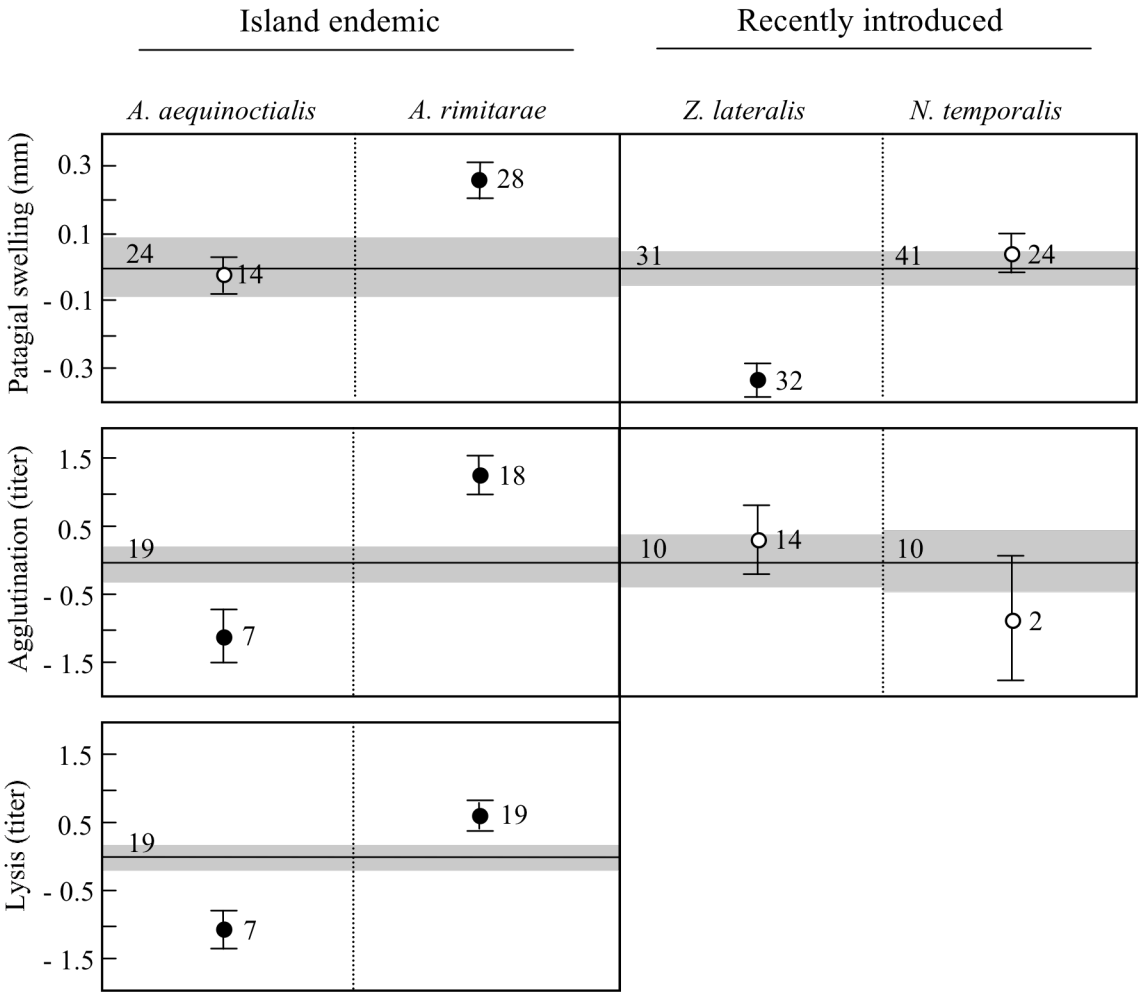
FIGURE LEGENDS

Figure 1. Prevalence of the haematozoan parasites *Plasmodium* spp. (black), *Haemoproteus* spp. (light grey) and *Leucocytozoon* spp. (dark grey) across mainland and island populations of *Z. lateralis*, *N. temporalis*, and *Acrocephalus* spp. Asterisks indicate significantly lower parasite prevalences in recently introduced island populations (*Haemoproteus* spp. in *Z. lateralis*) or island endemic species (*Plasmodium* spp. in *A. aequinoctialis*, above, and *A. rimitarae*, below) relative to their mainland counterparts.

Figure 2. Cell-mediated (PHA-induced patagial swelling) and innate (agglutination and lysis of rabbit red blood cells) measures of immune response in endemic and recently-introduced island taxa (circles) relative to a mainland control (centered at zero). Numbers indicate sample size and standard error is depicted by vertical bars, or by grey shading in the case of the mainland control. Filled circles indicate a significant difference from the mainland control.

FIGURES


Acrocephalus spp.
Z. lateralis
N. temporalis
Mainland
n = 34
n = 64
n = 67
Island
*
*
n = 25
n = 59
n = 34
*
n = 56


Island endemic
Recently introduced
A. aequinoctialis
A. rimitarae
Z. lateralis
N. temporalis
Patagial swelling (mm)
0.3
0.1
- 0.1
- 0.3
24
14
28
31
32
41
24
Agglutination (titer)
1.5
0.5
- 0.5
- 1.5
19
7
18
10
14
10
2
Lysis (titer)
1.5
0.5
- 0.5
- 1.5
19
7
19

APPENDIX

Appendix 1. PCR conditions for microsatellite loci.

Locus	Tm (°C)	Mg (mM)	Betaine (%)	BSA (mg/mL)	Cycles	Source
N. temporalis						
IND7	54	1.5	--	--	35	Sefc et al. 2001
IND8	56	1.0	--	--	35	Sefc et al. 2001
IND28	56	1.5	--	--	35	Sefc et al. 2001
IND29	56	1.5	--	--	35	Sefc et al. 2001
IND38	54	1.5	--	--	35	Sefc et al. 2001
IND41	54	1.5	--	--	35	Sefc et al. 2001
Z. lateralis						
ZL12	58	1.5	10	--	35	Degnan et al. 1999
ZL14	58	2.0	--	1.0	35	Degnan et al. 1999
ZL18	58	1.5	10	1.0	35	Degnan et al. 1999
ZL22	57	1.5	10	--	35	Degnan et al. 1999
ZL35	60	1.5	10	--	35	Degnan et al. 1999
ZL38	56	2.0	--	--	35	Degnan et al. 1999
ZL41	53	2.0	--	1.0	35	Frentiu et al. 2003
ZL44	53	1.0	10	--	30	Frentiu et al. 2003
ZL45	58	1.5	--	--	39	Frentiu et al. 2003
ZL46	54	2.0	10	1.0	35	Frentiu et al. 2003
ZL50	59	1.5	--	--	35	Frentiu et al. 2003
ZL54	58	1.5	10	--	35	Frentiu et al. 2003
Acrocephalus spp.						
Aar2	60	1.5	--	--	35	Hansson et al. 2000
Ase7	60	1.0	--	--	35	Richardson et al. 2000

Ase9	60	1.0	--	--	35	Richardson et al. 2000
Ase11	62	1.5	--	--	35	Richardson et al. 2000
Ase12	60	1.5	--	--	35	Richardson et al. 2000
Ase13	62	1.5	--	--	35	Richardson et al. 2000
Ase34	60	1.5	--	--	35	Richardson et al. 2000
Ase48	60	2.0	--	--	35	Richardson et al. 2000
Ase51	60	1.5	--	--	35	Richardson et al. 2000
Ase56	60	1.5	--	--	35	Richardson et al. 2000
Ase57	TD[a]	1.5	--	--	35	Richardson et al. 2000
Ase58	60	2.0	--	--	35	Richardson et al. 2000

[a] Touch-down cycle as in Richardson et al. 2000.

Bibliography

Acevedo-Whitehouse, K., Gulland, F., Greig, D. & Amos, W. 2006. Inbreeding: disease susceptibility in California sea lions. *Nature*, **422**, 35.

Adamo, S. A. 2004. How should behavioural ecologists interpret measurements of immunity? *Animal Behaviour*, **68**, 1443-1449.

Aguilar, A., Roemer, G., Debenham, S., Binns, M., Garcelon & Wayne, R. K. 2004. High MHC diversity maintaine by balancing selection in an otherwise genetically monomorphic mammal. *Proceedings of the National Academy of Sciences U. S. A.*, **101**, 3490-3494.

Amos, W., Wilmer, J. W., Fullard, K., Burg, T. M., Croxall, J. P., Block, D. & Coulson, T. 2001. The influence of parental relatedness on reproductive success. *Proceedings of the Royal Society London B*, **268**, 2021-2027.

Anderson, R. M. & May, R. M. 1992. Infectious diseases of humans, dynamics and control. Oxford University Press, New York.

Atkinson, C. T. 1986. Host specificity and morphometric variation of *Haemoproteus meleagridis* Levine, 1961 (Protozoa: Haemosporina) in gallinaceous birds. *Canadian Journal of Zoology*, **64**, 2634-2638.

Atkinson, C. T., Dusek, R. J., Woods, K. L. & Iko, W. M. 2000. Pathogenecity of avian malaria in experimentally-infected Hawaii Amakihi. *Journal of Wildlife Diseases*, **36**, 197-204.

Atkinson, C. T., Lease, J. K., Drake, B. M. & Shema, N. P. 2001. Pathogenicity, serological responses, and diagnosis of experimental and natural malarial infections in native Hawaiian thrushes. *The Condor*, **103**, 209-218.

Atkinson, C. T., Utzurrum, R. C., Seamon, J. O., Savage, A. F. & LaPointe, D. A. 2007. Hematozoa of forest birds in American Samoa – evidence for a diverse, indigenous parasite fauna from the South Pacific. *Pacific Conservation Biology*, **13**, 229-237.

Atkinson, C. T., & van Riper III, C. 1991. Pathogenicity and epizootiology of avian haematozoa: *Plasmodium*, *Leucocytozoan*, and *Haemoproteus*. *In* Bird-parasite interactions, ecology, evolution and behavior, J. E. Loye & M. Zuk (eds.). Oxford University Press, Oxford, U.K., p. 19-48.

Atkinson, C. T., Woods, K. L., Dusek, R. J., Sileo, L. S. & Iko, W. M. 1995. Wildlife

disease and conservation in Hawaii: pathogenecity of avian malaria (*Plasmodium relictum*) in experimentally infected Iiwi (*Vestiaria coccinea*). *Parasitology*, **111**, S59-S69.

Avise, J. C., Walker, D. & Johns, G. C. 1998. Speciation durations and Pleistocene effects on vertebrate phylogeography. *Proceedings of the Royal Society London B*, **265**, 1707-1712.

Baker, A. J. & Moeed, A. 1987. Rapid genetic differentiation and founder effect in colonizing populations of common mynas (*Acridotheres tristis*). *Evolution*, **41**, 525-538.

Beadell, J. S. & Fleischer, R. C. 2005. A restriction enzyme-based assay for distinguishing between avian haematozoa. *Journal of Parasitology*, **91**, 683-685.

Beadell, J. S., Gering, E., Austin, J., Dumbacher, J. P., Peirce, M., Pratt, T. K., Atkinson, C. T. & Fleischer, R. C. 2004. Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. *Molecular Ecology*, **13**, 3829-3844.

Beadell, J. S., Ishtiaq, F., Covas, R., Melo, M., Warren, B. H., Atkinson, C. T., Bensch, S., Graves, G. R., Jhala, Y. V., Peirce, M. A., Rahmani, A. R., Fonseca, D. M. & Fleischer, R. C. 2006. Global phylogeographic limits of Hawaii's avian malaria. *Proceedings of the Royal Society London B*, **273**, 2935-2944.

Bennett, G. F., Bishop, M. A. & Peirce, M. A. 1993. Checklist of the avian species of *Plasmodium* Marchiafava & Celli, 1885 (Apicomplexa) and their distribution by avian family and Wallacean life zones. *Systematic Parasitology*, **26**, 171-179.

Bennett, G. F. & Cameron, M. 1974. Seasonal prevalence of avian hematozoa in passeriform birds of Atlantic Canada. *Canadian Journal of Zoology*, **52**, 1259-1264.

Bennett, G. F. & Campbell, A. G. 1973 Avian Haemoproteidae. III. Description of *Haemoproteus fusca* sp. N. and re-description of *Haemoproteus halcyonis* De Mello, 1935, parasites of the kingfisher family Alcedinidae. *Journal of Parasitology*, **59**, 337-343.

Bennett, G. F., Montgomerie, R. & Seutin, G. 1992. Scarcity of haematozoa in birds breeding on the Arctic tundra of North America. *Condor*, **94**, 289–292.

Bennett, G. F. & Peirce, M. A. 1988 Morphological form in the avian

Haemoproteidae and an annotated checklist of the genus *Haemoproteus* Kruse, 1890. *Journal of Natural History*, **22**, 1683-1696.

Bennett, G. F., Peirce, M. A. & Ashford, R. W. 1993. Avian haematozoa: mortality and pathogenicity. *Journal of Natural History*, **27**, 993-1001.

Bensch, S. & Akesson, S. 2003 Temporal and spatial variation of hematozoans in Scandinavian willow warblers. *Journal of Parasitology*, **89**, 388-391.

Bensch, S., Pérez-Tris, J., Waldenström, J. & Hellgren, O. 2004. Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation? *Evolution*, **58**, 1617-1621.

Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H. & Torres Pinheiro, R. 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proceedings of the Royal Society London B*, **267**, 1583-1589.

Bensch, S., Waldenström, J., Jonzén, N., Westerdahl, H., Hansson, B., Sejberg, D. & Hasselquist, D. 2007. Temporal dynamics and diversity of avian malaria parasites in a single host species. *Journal of Animal Ecology*, **76**, 112-122.

Bentz, S., Rigaud, T., Barroca, M., Martin-Laurent, F., Bru. D., Moreau, J. & Faivre, B. 2006. Sensitive measure of prevalence and parasitaemia of haemosporidia from European blackbird (*Turdus merula*) populations: value of PCR-RFLP and quantitative PCR. *Parasitology*, **133**, 685-692.

BirdLife International. 2000. *Threatened birds of the world*. Lynx Edicions, Barcelona, and BirdLife International, Cambridge, UK.

Blakers, M., Davies, S. J. J. F. & Reilly, P. N. 1985. The atlas of Australian birds. International Scholarly Book Service, Inc., Beaverton. 738pp.

Blondel, J. 2000. Evolution and ecology of birds on islands: trends and prospects. *Vie et Mileu*, **50**, 205-220.

Boots, M. & Bowers, R. G. 2004. The evolution of resistance through costly acquired immunity. *Proceedings of the Royal Society London B*, **271**, 715-723.

Cann, R. L., Feldman, R. A., Agullana, L. & Freed, L. A. 1996. A PCR approach to detection of malaria in Hawaiian birds. *In* Molecular genetic approaches in conservation, T. B. Smith & R. K. Wayne (eds.). Oxford University Press, New York, New York, p. 202-213.

Cibois, A., Thibault, J.-C. & Pasquet, E. 2004. Biogeography of eastern Polynesian

monarchs (*Pomarea*): an endemic genus close to extinction. *Condor*, **106**, 837-851.

Cleaveland, S., Laurenson, M. K. & Taylor, L. H. 2001. Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. *Philosophical Transaction of the Royal Society London B*, **356**, 991-999.

Clegg, S. M., Degnan, S. M., Kikkawa, J., Moritz, C., Estoup, A. & Owens, I. P. F. 2002. Genetic consequences of sequential founder events by an island-colonizing bird. *Proceedings of the National Academy of Sciences*, **99**, 8127-8132.

Clement, M., Posada, D. & Crandall, K. A. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657-1660.

Clifford, D. L., Mazet, J. A. K., Dubovi, E. J., Garcelon, D. K., Coonan, T. J., Conrad, P. A. & Munson, L. 2006. Pathogen exposure in endangered island fox (*Urocyon littoralis*) populations: implications for conservation management. *Biological Conservation*, **131**, 230-243.

Colautti, R. I., Ricciardi, A., Grigorovich, I. A. & MacIsaac, H. J. 2004. Is invasion success explained by the enemy release hypothesis? *Ecology Letters*, **7**, 721-733.

Conway, D. J., Fanello, C., Lloyd, J. M., Al-Joubori, B. M. A., Baloch, A. H., Somanath, S. D., Roper, C., Oduola, A. M. J., Mulder, B., Povoa, M. M., Singh, B. & Thomas, A. W. 2000. Origin of *Plasmodium falciparum* malaria is traced by mitochondrial DNA. *Molecular and Biochemical Parasitology*, **111**, 163-171.

Cooper, J. E. 1993. Historical survey of disease in birds. *Journal of Zoo and Wildlife Medicine*, **24**, 256-264.

Cunningham, A. A. & Daszak, P. 1998. Extinction of a species of land snail due to infection with a microsporidian parasite. *Conservation Biology*, **12**, 1139-1141.

Daszak, P., Cunningham, A. A. & Hyatt, A. D. 2000. Emerging infectious diseases of wildlife – threats to biodiversity and human health. *Science*, **287**, 443-449.

Daszak, P., Cunningham, A. A. & Hyatt, A. D. 2003. Infectious disease and amphibian population declines. *Diversity and Distributions*, **9**, 141-150.

de Castro, F. & Bolker, B. 2005. Mechanisms of disease-induced extinction. *Ecology Letters*, **8**, 117-126.

Deem, S. L., Karesh, W. B. & Weisman, W. 2001. Putting theory into practice: wildlife health in conservation. *Conservation Biology*, **15**, 1224-1233.

Deerenberg, C., Arpanius, V., Daan, S. & Bos, N. 1997. Reproductive effort decreases antibody responsiveness. *Proceedings of the Royal Society London B*, **264**, 1021-1029.

Degnan, S. M., Robertson, B. C., Clegg, S. M. & Moritz, C. 1999. Microsatellite primers for the study of gene flow and mating systems in white-eyes (*Zosterops*). *Molecular Ecology*, **8**, 157-158.

Dobson, A. P. 1988. Restoring Island Ecosystems: the potential of parasites to control introduced mammals. *Conservation Biology*, **2**, 31-39.

Dobson, A. & Foufopoulos, J. 2001. Emerging infectious pathogens of wildlife. *Philosophical Transactions of the Royal Society London B*, **356**, 1001-1012.

Dumbacher, J. P., Beehler, B. M., Spande, T. F., Garraffo, H. M. & Daly, J. W. 1992. Homobatrachotoxin in the genus *Pitohui*: chemical defense in birds? *Science*, **258**, 799-801.

Dumbacher, J. P., Pratt, T. K. & Fleischer, R. C. 2003. Phylogeny of the owlet-nightjars (Aves: Aegothelidae) based on mitochondrial DNA sequence. *Molecular Phylogenetics and Evolution*, **29**, 540-549.

Escalante, A. A., Freeland, D. E., Collins, W.E. & Lal, A. A. 1998 The evolution of primate malaria parasites based on the gene encoding cytochrome *b* from the linear mitochondrial genome. *Proceedings of the National Academy of Science*, **95**, 8124-8129.

Ewers, W. H. 1967. The blood parasites of some New Guinea birds. *Nova Guinea*, **38**, 427-432.

Fallon, S. M., Bermingham, E. & Ricklefs, R. E. 2003. Island and taxon effects in parasitism revisited: avian malaria in the Lesser Antilles. *Evolution*, **57**, 606-615.

Fallon, S. M., Bermingham, E. & Ricklefs, R. E. 2005. Host specialization and geographic localization of avian malaria parasites: a regional analysis in the Lesser Antilles. *American Naturalist*, **165**, 466-480.

Fallon, S. M., Fleischer, R. C. & Graves, G. R. 2006. Malarial parasites as geographical markers in migratory birds? *Biology Letters*, **2**, 213-216.

Fallon, S. M., Ricklefs, R. E., Latta, S. C. & Bermingham, E. 2004. Temporal

stability of insular avian malarial parasite communities. *Proceedings of the Royal Society London B*, **271**, 493-500.

Fallon, S. M., Ricklefs, R. E., Swanson, B. L. & Bermingham, E. 2003. Detecting avian malaria: an improved polymerase chain reaction diagnostic. *Journal of Parasitology*, **89**, 1044-1047.

Feagin, J. E. 1992. The 6-kb element of *Plasmodium falciparum* encodes mitochondrial cytochrome genes. *Molecular and Biochemical Parasitology*, **52**, 145-148.

Feldman, R. A., Freed, L. A. & Cann, R. L. 1995. A PCR test for avian malaria in Hawaiian birds. *Molecular Ecology*, **4**, 663-673.

Fleischer, R. C. & McIntosh, C. E. 2001. Molecular systematics and biogeography of the Hawaiian avifauna. *Studies in Avian Biology*, **22**, 51-60.

Fleischer, R. C., McIntosh, C. E. & Tarr, C. L. 1998. Evolution on a volcanic conveyor belt: Using phylogenetic reconstructions and K-Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates. *Molecular Ecology*, **7**, 533-545.

Fleischer, R. C., Olson, S., James, H. F. & Cooper, A. C. 2000. The identity of the extinct Hawaiian eagle (*Haliaeetus*) as determined by mitochondrial DNA sequence. *Auk***, 117**, 1051-1056.

Fonseca, D., Smith, J., Wilkerson, R. & Fleischer, R. 2006. Pathways of expansion and multiple introductions illustrated by large genetic differentiation among worldwide populations of the southern house mosquito. *American Journal of Tropical Medicine and Hygiene*, **74**, 284-289.

Frankham, R. 1997. Do island populations have less genetic variation than mainland populations? *Heredity*, **78**, 311-327.

Freed, L. A. & Cann, R. L. 2003. On polymerase chain reaction tests for estimating prevalence of malaria in birds. *Journal of Parasitology*, **89**, 1261-1264.

Frentiu, F. D., Lange, C. L., Burke, T. & Owens, I. P. F. 2003. Isolation of microsatellite loci in the Capricorn silvereye, *Zosterops lateralis chlorcephalus* (Aves: Zosteropidae). *Molecular Ecology Notes*, **3**, 462-464.

Friend, M., McLean, R. G. & Dein, F. J. 2001. Disease emergene in birds: challenges for the twenty-first century. *The Auk*, **118**, 290-303.

Fromont, E., Morvilliers, L., Artois, M. & Pontier, D. 2001. Parasite richness and

abundance in insular and mainland feral cats: insularity or density? *Parasitology*, **123**, 143-151.

Garnham, P. C. C. 1966. Malaria parasites and other Haemosporidia. Blackwell Scientific Publishers, Oxford, U.K., 1114 p.

Goltsman, M., Kruchenkova, E. P. & Macdonald, D. W. 1996. The Mednyi Arctic foxes: treating a population imperilled by disease. *Oryx*, **30**, 251-258.

Goltsman, M., Kruchenkova, E. P., Sergeev, S., Volodin, I. & Macdonald, D. W. 2005. 'Island syndrom' in a population of Arctic foxes (*Alopex lagopus*) from Mednyi Island. *Journal of Zoology*, **267**, 405-418.

Gonzalez, G., Sorci, G., Moller, A. P., Ninni, P., Haussy, C. & de Lope, F. 1999. Immunocompetence and condition-dependent sexual advertisement in male house sparrows (*Passer domesticus*). *Journal of Animal Ecology*, **68**, 1225-1234.

Gottdenker, N. L., Walsh, T., Vargas, H., Merkel, J., Jimenez, G. U., Miller, R. E., Dailey, M. & Parker, P. G. 2005. Assessing the risks of introduced chickens and their pathogens to native birds in the Galapagos archipelago. *Biological Conservation*, **126**, 429-439.

Gustafsson, L., Nordling, D., Andersson, M. S., Sheldon, B. C. & Qvarnström, A. 1994. Infectious diseases, reproductive effort and the cost of reproduction in birds. *Philosophical Transactions of the Royal Society London B*, **346**, 323-331.

Hale, K. A. & Briskie, J. V. 2007. Decreased immunocompetence in a severely bottlenecked population of an endemic New Zealand bird. *Animal Conservation*, **10**, 2-10.

Handbook of the Birds of the World Volume 8 Broadbills to Tapaculos. 2003. J. del Hoyo, A. Elliot & D. Christie (eds.). Lynx Edicions, Barcelona, Spain.

Hansson, B., Bensch, S., Hasselquist, D., Lillandt, B.-G., Wennerberg, L. & von Schantz, T. 2000. Increase of genetic variation over time in a recently founded population of great reed warblers (*Acrocephalus arundinaceus*) revealed by microsatellites and DNA fingerprinting. *Molecular Ecology*, **9**, 1529-1538.

Hansson, B. & Richardson, D. S. 2005. Genetic variation in two endangered *Acrocephalus* species compared to a widespread congener: estimates based on functional and random loci. *Animal Conservation*, **8**, 83-90.

Hatchwell, B. J., Wood, M. J., Anwar, M. A., Chamberlain, D. E. & Perrins, C. M.

2001. The haematozoan parasites of Common Blackbirds *Turdus merula*: associations with host condition. *Ibis*, **143**, 420-426.

Hatchwell, B. J., Wood, M. J., Anwar, M. A. & Perrins, C. M. 2000. The prevalence and ecology of the haematozoan parasites of European blackbirds, *Turdus merula*. *Canadian Journal of Zoology*, **78**, 684-687.

Hawley, D. M., Sydenstricker, K. V., Kollias, G. V. & Dhondt, A. A. 2005. Genetic diversity predicts pathogen reisistance and cell-mediated immunocompetence in house finches. *Biology Letters*, **1**, 326-329.

Hillgarth, N. & Wingfield, J. C. 1997. Parasite-mediated sexual selection: endocrine aspects. *In* Host-parasite evolution, D. H. Clayton & J. Moore (eds.). Oxford University Press, Oxford, p. 78-104

Hochachka, W. M. & Dhondt, A. A. 2000. Density-dependent decline of host abundance resulting from a new infectious disease. *Proceedings of the National Academy of Sciences U. S. A.*, **97**, 5303-5306.

Hochberg, M. E. & Moller, A. P. 2001. Insularity and adaptation in coupled victim-enemy association. *Journal of Evolutionary Biology*, **14**, 539-551.

Holyoak, D. T. & Thibault, J.-C. 1984. Contribution à l'étude des oiseaux de Polynésie orientale. Mémoires Museum National Histoire Naturelle, Paris (sér. A). *Zoologie*, **127**, 1-209.

Humphrey, B. D., Koutsos, E. A. & Klasing, K. C. 2002. Requirements and priorities of the immune system for nutrients. *In* Nutrition biotechnology in the feed and food industries: Proceedings of Alltech's 18th annual symposium, T. P. Lyons & K. A. Jasques (eds.). Nottingham University Press, Nottingham, p. 69-77.

Ishtiaq, F., Beadell, J., Baker, A. J., Rahmani, A. R., Jhala, Y. V. & Fleischer, R. 2006. Prevalence and evolutionary relationships of haematozoan parasites in native versus introduced populations of common myna *Acridotheres tristis*. *Proceedings of the Royal Society London B*, **273**, 587-594.

Janeway, C. A., Travers, P., Walport, M. & Sclomchik, M. J. 2005. Immunobiology, 6th ed. Garland Science Publishing, New York.

Janovy, J. 1997 Protozoa, helminths, and arthropods of birds. *In* D. Clayton & J. Moore (eds.). Host-parasite evolution. New York: Oxford University Press.

Jarvi, S. I., Atkinson, C. T. & Fleischer, R. C. 2001. Immunogenetics and resistance to avian malaria in Hawaiian honeycreepers (Drepanidinae). *Studies in Avian Biology*, **22**, 254-263.

Jarvi, S. I., Farias, M. E. M., Baker, H., Freifeld, H. B., Baker, P., van Gelder, E., Massey, J. G. & Atkinson, C. T. 2003. Detection of avian malaria (*Plasmodium* spp.) in native land birds of American Samoa. *Conservation Genetics*, **4**, 629-637.

Jarvi, S. I., Schultz, J. J. & Atkinson, C. T. 2002. PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. *Journal of Parasitology*, **88**, 153-158.

Jones, H. I. 1985. Hematozoa from montane forest birds in Papua New Guinea. *Journal of Wildlife Diseases*, **21**, 7-10.

Joy, D. A., Feng, X., Mu, J., Furuya, T., Chotivanich, K., Krettli, A. U., Ho, M., Wang, A., Suh, E., Beerli, P. & Su, X.-Z. 2003. Early origin and recent expansion of *Plasmodium falciparum*. *Science*, **300**, 318-321.

Kimura, M., Dhondt, A. A. & Lovette, I. J. 2006. Phylogeographic structuring of *Plasmodium* lineages across the North American range of the house finch (*Carpodacus mexicanus*). *Journal of Parisitology*, **92**, 1043-1049.

Klasing, K. C. & Barnes, D. M. 1988. Decreased amino acid requirements of growing chicks due to immunologic stress. *Journal of Nutrition*, **118**, 1158-1164.

Laird, M. & van Riper III, C. 1981. Questionable reports of *Plasmodium* from birds in Hawaii, with the recognition of *P. relictum* ssp. capistranoae (Russell, 1932) as the avian malaria parasite there. *In* Parasitological Topics, E. V. Canning (ed.). Allen Press, Lawrence, pp. 159-165.

Li, J., Collins, W. E., Wirtz, R. A., Rathore, D., Lal, A. & McCutchan, T. R. 2001. Geographic subdivision of the range of the malaria parasite, *Plasmodium vivax*. *Emerging Infectious Disease*, **7**, 35-42.

Lindström, K. M., Foufopoulos, J., Pärn, H. & Wikelski, M. 2004. Immunological investments reflect parasite abundance in island populations of Darwin's finches. *Proceedings of the Royal Society London B*, **271**, 1513-1519.

Lochmiller, R. L. & Deerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos*, **88**, 87-98.

Lockhart, P. J., Steel, M. A., Hendy, M. D. & Penny, D. 1994. Recovering evolutionary trees under a more realistic model of sequence evolution. *Molecular Biology and Evolution*, **11**, 605-612.

Long, J. L. 1981. Introduced birds of the world. Universe Books, New York. 528 p.

Lyles, A. M. & Dobson, A. P. 1993. Infectious disease and intensive management:

population dynamics, threatened hosts, and their parasites. *Journal of Zoo and Wildlife Medicine*, **24**, 315-326.

Maddison, W. P. & Maddison, D. R. 2004. Mesquite: a modular system for evolutionary analysis. Version 1.05 http://mesquiteproject.org.

Manwell, R. D. 1936. The problem of species, with special reference to the malaria parasites. *Annals of Tropical Medicine*, **30**, 435-439.

Martin II, L. B., Han, P., Lewittes, J., Kuhlman, J. R., Klasing, K.C. & Wikelski, M. 2006. Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. *Functional Ecology*, **20**, 290-299.

Martin, L. B., Scheuerlein, A. & Wikelski, M. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? *Proceedings of the Royal Society of London B*, **270**, 153-158.

Martin, T. E., Moller, A. P., Merino, S. & Clobert, J. 2001. Does clutch size evolve in response to parasites and immunocompetence? *Proceedings of the National Academy of Sciences U. S. A.*, **98**, 2071-2076.

Matson, K. D. 2006. Are there differences in immune function between continental and insular birds? *Proceedings of the Royal Society London B*, **273**, 2267-2274.

Matson, K. D., Ricklefs, R. E. & Klasing, K. C. 2005. A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Developmental and Comparative Immunology*, **29**, 275-286.

May, R. M. & Anderson, R. M. 1983. Epidemiology and genetics in the coevolution of parasites and hosts. *Proceedings of the Royal Society London B*, **219**, 281-313.

McConkey, G. A., Li, J., Rogers, M. J., Seeley Jr., D. C., Graczyk, T. K., Cranfield, M. R. & McCutchan, T. F. 1996. Parasite diversity in an endemic region for avian malaria and identification of a parasite causing penguin mortality. Journal of Eukaryotic *Microbiology*, **43**, 393-399.

Mendes, L., Piersma, T., Lecoq, M., Spaans, B. & Ricklefs, R. E. 2005. Disease-limited distributions? Contrasts in the prevalence of avian malaria in shorebird species using marine and freshwater habitats. *Oikos*, **109**, 396-404.

Merino, S., Moreno, J., Sanz, J. J. & Arriero, E. 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proceedings of the Royal Society London B*, **267**, 2507-2510.

Milder, S. L. & Schreiber, R. W. 1982. Notes on the nesting behavior of *Acrocephalus aequinoctialis*. *Bulletin of the British Ornithological Council*,**102**, 20-22.

Millien, V. 2006. Morphological evolution is accelerated among island mammals. *PLoS Biology*, **10**, 1863-1868.

Moller, A. P. & Saino, N. 2004. Immune response and survival. *Oikos*, **104**, 299-304.

Nei, M., Maruyama, T. & Chakraborty, R. 1975. The bottleneck effect and genetic variability in populations. *Evolution*, **29**, 1-10.

Nei, M. & Li, W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences U. S. A.*, **76**, 5269-5273.

Nordling, D., Andersson, M., Zohari, S. & Gustafsson, L. 1998. Reproductive effort reduces specific immune respone and parasite resistance. *Proceedings of the Royal Society London B*, **265**, 1291-1298.

O'Brien, S. J., Roelke, M. E., Marker, L., Newman, A., Winkler, C. A., Meltzer, D., Colly, L., Evermann, J. F., Bush, M. & Wildt, D. E. 1985. Genetic basis for species vulnerability in the cheetah. *Science*, **227**, 1428-1434.

Ochsenbein, A. F., Fehr, T., Lutz, C., Suter, M., Brombacher, F., Hengartner, H. & Zinkernagel, R. M. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. *Science*, **286**, 2156-2159.

Olson, S. L., Wingate, D. B., Hearty, P. J. & Grady, F. V. 2005. Prodromus of vertebrate paleontology and geochronology of Bermuda. *In* Insular Vertebrate Evolution: the Palaeontological Approach, J. Alcover & P. Bover (eds.). Proceedings of the International Symposium "Insular Vertebrate Evolution: the Paleontological Approach." *Monografies de la Societat d"Historia Natural de les Balears*, **12**, 219-232.

Parker, P. G., Whiteman, N. K. & Miller, R. E. 2006. Conservation medicine on the Galapagos Islands: partnerships among behavioral, population, and veterinary scientists. *Auk*, **123**, 625-638.

Parmentier, H. K., Lammers, A., Hoekman, J. J., Reilingh, G. de V., Zaanen, I. T. A. & Savelkoul, H. F. J. 2004. Different levels of natural antibodies in chickens divergently selected for specific antibody responses. *Developmental and Comparative Immunology*, **28**, 39-49.

Pearce-Duvet, J. M. C. 2006. The origin of human pathogens: evaluating the role of

agriculture and domestic animals in the evolution of human disease. *Biological Reviews*, **81**, 369-382.

Pearman, P. B. & Garner, T. W. J. 2005. Susceptibility of Italian agile frog populations to an emerging strain of *Ranavirus* parallels population genetic diversity. *Ecology Letters*, **8**, 401-408.

Peirce, M. A. 1979. Some additional observations on haematozoa of birds in the Mascarene Islands. *Bulletin of the British Ornithological Council*, **99**, 68-71.

Perkins, S. L. 2000. Species concepts and malaria parasites: detecting a cryptic species of *Plasmodium*. *Proceedings of the Royal Society London B*, **267**, 2345-2350.

Perkins, S. L. & Schall, J. J. 2002. A molecular phylogeny of malarial parasites recovered from cytochrome *b* gene sequences. *Journal of Parasitology*, **88**, 972-978.

Peters, A., Delhey, K., Denk, A. G. & Kempenaers, B. 2004. Trade-offs between immune investment and sexual signaling in male mallards. *American Naturalist*, **164**, 51-59.

Posada, D. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**, 817-818.

Price, P. 1991. *In* Host-parasite interactions, J. E. Loye & M. Zuk (eds.). Oxford University Press, New York.

Reid, J. M, Arcese, P. & Keller, L. F. 2003. Inbreeding depresses immune response in song sparrows (*Melospiza melodia*): direct and inter-generational effects. *Proceedings of the Royal Society London B*, **270**, 2151-2157.

Richard, F. A., Sehgal, R. N. M., Jones, H. I. & Smith, T. B. 2002. A comparative analysis of PCR-based detection methods for avian malaria. *Journal of Parasitology*, **88**, 819-822.

Richardson, D. S., Jury, F. L., Dawson, D. A., Salgueiro, P., Komdeur, J. & Burke, T. 2000. Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in Sylviidae species and their cross-species amplification in other passerine birds. *Molecular Ecology*, **9**, 2155-2234.

Richardson, D. S. & Westerdahl, H. 2003. MHC diversity in two *Acrocephalus* species: the outbred Great reed warbler and the inbred Seychelles warbler. *Molecular Ecology*, **12**, 3523-3529.

Ricklefs, R. E. & Fallon, S. M. 2002. Diversification and host switching in avian

malaria parasites. *Proceedings of the Royal Society London B*, **269:** 885-892.

Ricklefs, R. E., Fallon, S. M. & Bermingham, E. 2004 Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. *Systematic Biology*, **53**, 111-119.

Roelke, M. E., Martenson, J. S. & O'Brien, S. J. 1993. The consequences of demographic reduction and genetic depletion in the endangered Florida panther. *Current Biology*, **3**, 340-350.

Ronquist, F. & Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572-1574.

Scheuerlein, A. & Ricklefs, R. E. 2004. Prevalence of blood parasites in European passeriform birds. *Proceedings of the Royal Society, London B*, **271**, 1363-1370.

Schloegel, L. M., Hero, J.-M., Berger, L. Speare, R., McDonald, K. & Daszak, P. 2006. The decline of the sharp-snouted day frog (*Taudactylus acutirostris*): the first documented case of extinction by infection in a free-ranging wildlife species? *Ecohealth*, **3**, 35-40.

Schmid-Hempel, P. & Ebert, D. 2003. On the evolutionary ecology of specific immune defence. *Trends in Ecology and Evolution*, **18**, 27-32.

Schrenzel, M. D., Maalouf, G. A., Keener, L. L. & Gaffney, P. M. 2003. Molecular characterization of malarial parasites in captive passerine birds. *Journal of Parasitology*, **89**, 1025-1033.

Scrimshaw, N. S. 1991. Effect of infection on nutrient requirements. *Journal of Parenteral and Enteral Nutrition*, **15**, 589-600.

Sefc, K. M., Payne, R. B. & Sorenson, M. D. 2001. Characterization of microsatellite loci in village indigobirds *Vidua chalybeata* and cross-species amplification in estrildid and ploceid finches. *Molecular Ecology Notes*, **1**, 252-254.

Sibley, C.G. & Ahlquist, J. E. 1985. The phylogeny and classification of the Australo-Papuan passerine birds. *Emu*, **85**, 1-14.

Siikamaki, P., Ratti, O., Hovi, M. & Bennett, G. F. 1997 Association between haematozoan infections and reproduction in the Pied Flycatcher. *Functional Ecology*, **11**, 176-183.

Smith, K. F., Sax, D. F. & Lafferty, K. D. 2006. Evidence for the role of infectious disease in species extinction and endangerment. *Conservation Biology*, **20**, 1349-1357.

Smits, J. E., Bortolotti, G. R. & Tella, J. L. 1999. Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. *Functional Ecology*, **13**, 567-572.

Smits, J. E., Tella, J. L., Carrete, M., Serrano, D. & Lopez, G. 2005. An epizootic of avian pox in endemic short-toed larks (*Calandrella rufescens*) and Berthelot's pipits (*Anthus berthelotti*) in the Canary Islands, Spain. *Veterinary Pathology*, **42**, 59-65.

Speilman, D., Brook, B. W., Briscoe, D. A. & Frankham, R. 2004. Does inbreeding and loss of genetic diversity decrease disease resistance? *Conservation Genetics*, **5**, 439-448.

Steadman, D. 1997. The historic biogeography and community ecology of Polynesian pigeons and doves. *Journal of Biogeography*, **24**, 737-753.

Swofford, D. L. 1999 PAUP*: Phylogenetic analysis using parsimony (*and other methods). Sinauer, Sunderland, MA.

Tarr, C. L., Conant, S. & Fleischer, R. C. 1998. Founder events and variation at microsatellite loci in an insular passerine bird, the Laysan finch (*Telespiza cantans*). *Molecular Ecology*, **7**, 719:731.

Taylor, L. H., Latham, S. M. & Woolhouse, M. E. 2001. Risk factors for human disease emergence. *Philosophical Transactions of the Royal Society London B*, **356**, 983-989.

Thompson, J. R., Marcelino, L. A. & Polz, M. F. 2002. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. *Nucleic Acids Research*, **30**, 2083-2088.

Toft, C. A. 1991 Current theory of host-parasite interactions. *In* Host-parasite interactions, J. E. Loye & M. Zuk (eds.). Oxford University Press, New York.

Tompkins, D. M. & Gleeson, D. M. 2006. Relationship between avian malaria distribution and an exotic invasive mosquito in New Zealand. *Journal of the Royal Society of New Zealand*, **36**, 51-62.

Torchin, M. E., Lafferty, K. D., Dobson, A. P., McKenzie, V. J. & Kuris, A. M. 2003 Introduced species and their missing parasites. *Nature*, **421**, 628-630.

Valkiunas, G. 2005. Avian malaria parasites and other haemosporidia. CRC Press, Boca Raton.

Valkiunas, G., Bensch, S., Iezhova, T. A., Krizanauskiene, A., Hellgren, O. &

Bolshakov, C. V. 2006. Nested cytochrome B polymerase chain reaction diagnostics underestimate mixed infections of avian blood haemosporidian parasites: Microscopy is still essential. *Journal of Parasitology*, **92**, 418-422.

van Riper III, C. 1991. The impact of introduced vectors and avian malaria on insular passeriform bird populations in Hawaii. *Bull. Soc. Vect. Ecol.*, **16**, 59-83.

van Riper, C., S. G. van Riper, M. L. Goff & Laird, M. 1986. The epizootiology and ecological significance of malaria on the birds of Hawaii. *Ecological Monographs*, **56**, 327-344.

Viney, M. E., Riley, E. M. & Buchanan, K. L. 2005. Optimal immune responses: immunocompetence revisited. *Trends in Ecology and Evolution*, **20**, 665-669.

Waldenström, J., Bensch, S., Hasselquist, D. & Ostman, O. 2004. A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *Journal of Parasitology*, **90**, 191-194.

Waldenström, J., Bensch, S., Kiboi, S., Hasselquist, D. & Ottosson, U. 2002. Cross-species infection of blood parasites between resident and migratory songbirds in Africa. *Molecular Ecology*, **11**, 1545-1554.

Warner, R. E. 1968. The role of introduced diseases in the extinction of the endemic Hawaiian avifauna. *The Condor*, **70**, 101-120.

Webster, M. S., Marra, P. P., Haig, S. M., Bensch, S. & Holmes, R. T. 2002. Links between worlds: unraveling migratory connectivity. *Trends in Ecology and Evolution*, **17**, 76-83.

White, E. M., Greiner, E. C., Bennett, G. F. & Herman, C. M. 1978. Distribution of hematozoa of Neotropical birds. *Revista de Biologia Tropical*, **26**, 43-102.

Whiteman, N. K., Matson, K. D., Bollmer, J. L. & Parker, P. G. 2006. Disease ecology in the Galapagos Hawk (*Buteo galapagoensis*): host genetic diversity, parasite load and natural antibodies. *Proceedings of the Royal Society London B*, **273**, 797-804.

Wiggins, D. A., Moller, A. P., Sorensen, M. F. L. & Brand, L. A. 1998. Island biogeography and the reproductive ecology of great tits *Parus major*. *Oecologia*, **115**, 478-482.

Wikelski, M., Foufopoulos, J., Vargas, H. & Snell, H. 2004. Galapagos birds and diseases: invasive pathogens as threats for island species. *Ecology and Society*, **9**, [online]. Available at www.ecologyandsociety.org/vol19/iss1/art5.

Wilkinson, H. 1950. Spanish intentions for Bermuda 1603-1615. *Bermuda Historical Quarterly*, **7**, 50-89.

Woodworth, B., Atkinson, C. T., LaPointe, D. A., Hart, P. J., Spiegel, C. S., Tweed, E. J., Henneman, C., LeBrun, J., Denette, T., DeMots, R., Kozar, K. L., Triglia, D., Lease, D., Gregor, A., Smith, T. & Duffy, D. 2005. Host population persistence in the face of introduced vector-borne diseases: Hawaii amakihi and avian malaria. *Proceedings of the National Academy of Sciences U. S. A.*, **102**, 1531-1536.

Woolhouse, M. E. J. & Gowtage-Sequeria, S. 2005. Host range and emerging and reemerging pathogens. *Emerging Infectious Disease*, **11**, 1842-1847.

Wright, S. 1931. Evolution in Mendelian populations. *Genetics*, **16**, 97-159.

Yorinks, N. & Atkinson, C. T. 2000. Effects of malaria (*Plasmodium relictum*) on activity budgets of experimentally infected juvenile Apapane (*Himatione sanguinea*). *Auk*, **117**, 731-738.

Young, B. E., Garvin, M. C. & McDonald, D. B. 1993. Blood parasites in birds from Monteverde, Costa Rica. *Journal of Wildlife Diseases*, **29**, 555-560.

Zuk, M. & Stoehr, A. M. 2002. Immune defense and host life history. *The American Naturalist*, **160**, S9-S22.