

A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporida): new insights from molecular data

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Abstract

Biogeographic patterns of parasite diversity are useful for determining how host-parasite interactions can influence speciation. However, variation in methodologies and sampling effort can skew diversity estimates. Avian haemosporidians are vector-transmitted blood parasites represented by over 1300 unique genetic lineages spread across over 40 countries. We used a global database of lineage distributions for two avian haemosporidian genera, *Plasmodium* and *Haemoproteus*, to test for congruence of diversity among haemosporidians and their avian hosts across 13 geographic regions. We demonstrate that avian haemosporidians exhibit similar diversity patterns to their avian hosts; however, specific patterns differ between genera. *Haemoproteus* spp. diversity estimates were significantly higher than *Plasmodium* spp. in all areas where the genera co-occurred, apart from the *Plasmodium* spp.-rich region of South America. The geographic distributions of parasite genera also differed, with *Haemoproteus* spp. absent from the majority of oceanic regions while *Plasmodium* spp. were cosmopolitan. These findings suggest

Note: Supplementary data associated with this article

fundamental differences in the way avian haemosporidians diverge and colonise new communities. Nevertheless, a review of the literature suggests that accurate estimates of avian haemosporidian diversity patterns are limited by (1) a concentration of sampling towards passerines from Europe and North America (2) a frequent failure to include microscopic techniques alongside molecular screening and (3) a paucity of studies investigating distributions across vector hosts.

Keywords: *Plasmodium*; *Haemoproteus*; avian malaria; parasite diversity; PCR; microscopy

1. Introduction

Global patterns of species diversity can yield important insights into the ecological and environmental mechanisms that promote diversification (Orme et al. 2005, Grenyer et al. 2006). For many taxa, high levels of diversity typically occur in biogeographic ‘hotspots’, which are generally centred on tropical regions in low latitudes (Gaston and Blackburn 2000). In addition to climate and latitude, landmass also appears to play a role in regulating species diversity, with island communities often exhibiting reduced diversity compared to continental regions (MacArthur 1967, Diamond 1975). However, these patterns are not always congruent across taxa, suggesting that different mechanisms may be important for maintaining different facets of biodiversity (Orme et al. 2005). For parasitic organisms, biogeographic patterns may be useful for understanding the evolutionary and ecological implications of host-parasite interactions and how they can influence parasite diversity (Krasnov et al. 2007, Ishtiaq et al. 2010, Jenkins and Owens 2011). For instance, protozoan parasites of primates present higher richness towards tropical

regions, which may be the result of higher abundance and diversity of biting arthropods that act as vectors (Nunn et al. 2005). This diversity of resources (i.e. hosts) may promote parasite diversification through co-speciation (Poulin 2011) and permit the coexistence of a larger range of parasite species (Krasnov et al. 2007). However, despite their utility for interpreting mechanisms of speciation, global analyses of pathogen diversity are scarce (Brooks and Hoberg 2001, Bordes et al. 2010). In this study we describe global diversity patterns for two commonly studied genera of avian blood parasites and investigate whether avian host diversity patterns can predict parasite diversity. We also conduct a methodical literature review in order to identify emerging methodological patterns from a rapidly expanding field. In doing so, we identify geographical sampling gaps and methodological points that need to be considered as empirical data is amassed to allow global perspectives on haemosporidian diversity to be refined and tested.

Descriptions of large-scale biogeographic patterns for wildlife pathogens require sensitive methodologies and adequate sampling regimes. However, due to their small size and often sparse infection rates, the diversity of pathogens is intrinsically difficult to determine, particularly across large geographic scales or in diverse host communities (Brooks and Hoberg 2001, Rohde 2002). Moreover, variations in methodologies and inadequate sampling in potentially diverse geographic areas can skew estimates of pathogen diversity (Poulin 2004). To put global patterns into context, we need to consider geographic and host-species sampling coverage as well as the contributions and limitations of different sampling

methodologies. Methodical literature reviews can be useful for highlighting such gaps in sampling (Pickering and Byrne 2012).

Avian malaria (*Plasmodium* spp.) and other haemosporidians (*Haemoproteus* and *Leucocytozoon* spp.) are a diverse group of vector-transmitted blood parasites that are abundant in most avian families (Valkiūnas 2005). Traditionally the life-history, morphology and classification of avian haemosporidians have been studied using light microscopy (Valkiūnas 2005). However, the recent adoption of molecular methods using polymerase chain reactions (PCR) to identify infections has led to a surge in research, revealing fascinating insights into the parasites' genetic diversity and host-specificity (Hellgren et al. 2009, Ventim et al. 2012). Additionally, the design of nested PCR primers to screen for infections has led to improved detection efficiency, particularly for chronic infections that typically have low levels of parasitaemia (Waldenstrom et al. 2004). Molecular studies have identified over 1300 unique avian haemosporidian lineages, prompting the creation of a coordinated database (MalAvi) to record the distributions of lineages and facilitate the investigation of global patterns (Bensch et al. 2009). For instance, MalAvi submissions have revealed that the dominant avian *Plasmodium* lineage found in Hawaii, GRW4, has been recorded in hosts spanning multiple avian families and across geographic regions from mainland USA to French Polynesia (Beadell et al. 2006, Ishtiaq et al. 2006, Marzal et al. 2011). While recent review papers of this rapidly emerging field have been conducted, they have focussed primarily on the advantages and disadvantages of molecular and microscopic methods (Braga et al. 2011, Marzal 2012). For example, recent findings indicate a vast underestimation of

mixed species haemosporidian infections using common PCR methods, highlighting limitations in the current methodologies used to identify and describe mixed infections (Valkiūnas et al. 2006, Martinez et al. 2009). However, since the advent of PCR detection protocols and the creation of the MalAvi database, no large-scale analyses of avian haemosporidian diversity have been carried out. The objective of this study is to systematically document the results from molecular-based avian haemosporidian research in order to (1) describe biogeographic patterns of diversity for avian haemosporidians (*Plasmodium* and *Haemoproteus* spp.), (2) determine how representative sampling for avian haemosporidians has been with respect to geography and host-species assemblages and (3) document trends in methodology to identify potential biases that may influence interpretations of avian haemosporidian biogeography.

2: Materials and methods

2.1: Observed and estimated lineage richness

To describe patterns in the diversity and distribution of avian haemosporidians, we extracted observed lineage diversity information from the MalAvi database (Bensch et al. 2009). In this database, unique haemosporidian lineages are identified based on sequence data from a fragment of the cytochrome-*b* gene (*cyt-b*) that is commonly targeted in molecular studies of avian haemosporidians (Waldenstrom et al. 2004), reducing the risk of using multiple names for identical lineages. We recorded lineage occurrence information using the 'Hosts and Sites' query in the MalAvi database to record the country where sampling was conducted and the academic reference for each lineage occurrence (Bensch et al. 2009). To maximise

the dataset, we included *cyt-b* lineages that were amplified from a different region of the parasite *cyt-b* gene (Fallon et al. 2003, Fallon et al. 2004, Fallon et al. 2005, Belo et al. 2011, Belo et al. 2012, Fecchio et al. 2013, Svensson-Coelho et al. 2013) . Finally, we gathered unpublished lineage occurrence information directly from authors (A. Marzal, S. Olsson-Pons, F. Ishtiaq). Due to the lack of lineages recorded within a number of countries, we grouped sampling locations into broader geographic regions in order to describe large-scale patterns in lineage diversity (Table 1). We used observed lineage recordings to generate an estimate of undiscovered lineage diversity for *Plasmodium* and *Haemoproteus* spp. in each geographic region using the non-parametric Chao2 estimator, which takes into account the frequency of each observed lineage (Chao et al. 2005). Chao2 diversity estimates were generated using 1000 randomizations in EstimateS v. 8.0 (Colwell 2001).

For avian haemosporidians, global diversity patterns could reflect higher diversity of potential avian and vector hosts, both of which exhibit increased richness in biogeographical hotspot regions around low latitudes (Grenyer et al. 2006, Foley et al. 2007). An ideal test to examine whether avian haemosporidians follow a similar pattern would be to map the latitude and longitude of infection occurrences for each parasite lineage. Unfortunately, adequate location records are not available for the majority of haemosporidian lineages. Therefore, to facilitate statistical comparisons among biogeographic regions, we utilised a global database of the distributions of breeding avian species (sourced from Orme *et al.* 2005) to delineate geographic regions into three categories of avian diversity (“continental avian hotspot”,

“continental avian non-hotspot”, and “oceanic”; Table 1). The “continental avian hotspot” and “continental avian non-hotspot” regions naturally fell into categories of 300 - 1000+ avian species and 60 - 299 avian species, respectively. We then used one-way analyses of variance (ANOVAs) to test for differences in the total observed lineage diversity among the three avian diversity categories. We also conducted ANOVAs to test for differences in estimated lineage diversity among categories using mean Chao2 diversity estimates for each sampling region. Significant differences among were determined using Tukey’s post-hoc HSD tests. Each ANOVA was carried out for *Plasmodium* and *Haemoproteus* spp. separately in Statistica 10.0 (www.statsoft.com).

2.2: Literature review

To assess bias and knowledge gaps arising from research methodologies, we identified and examined peer-reviewed research articles that used PCR to investigate avian haemosporidians. First, we searched the electronic databases Web of Science, ProQuest, Science Direct, PubMed and Google Scholar for articles published between 1995 and November 2012 using combinations of the following key words: ‘avian’, ‘bird*’, ‘malaria’, ‘*Haemoproteus*’, ‘*Plasmodium*’, ‘*Leucocytozoon*’, ‘disease’, ‘vector*’, ‘phylogenetic*’, ‘haematozoa*’, ‘parasite*’, ‘PCR’ and ‘blood’. The asterisk (*) operator was used as a wildcard to search for all possible variations of keywords. We then checked the reference lists of all articles for additional references that were not found in the primary searches. Studies using experimentally infected or captive birds were excluded, as we were interested in patterns of natural infections in wild hosts. A flowchart showing the article selection process is presented in Appendix 1a.

For each article, we recorded the following information: year of publication, host group investigated (i.e. avian, vector or both), haemosporidian genera screened and the methods used to describe infections. Methods were categorized based on the type of PCR carried out, the molecular marker(s) targeted and whether microscopy of blood films was used in addition to PCR. We also recorded whether mixed infections were separately characterised and the methods utilised. Although double peaks on chromatograms have been interpreted as evidence of mixed infections (Ricklefs et al. 2005, Kimura et al. 2006), such observations do not specifically describe the infection and these cases were therefore not recorded as an investigation of mixed infection. Finally, we recorded the country, latitude and longitude of each sampling site and for avian host studies, the taxonomic orders and the total number of avian species sampled in each case.

3: Results

3.1: Biogeography of *Plasmodium* and *Haemoproteus*

We recorded the geographic occurrences for 599 unique *cyt-b* lineages of avian *Haemoproteus* and 488 lineages of avian *Plasmodium*. Lineages of *Plasmodium* have been recorded in every region analysed (Table 1; Figure 1). In contrast, *Haemoproteus* lineages have not been detected from three of the six “oceanic” regions (New Zealand, Hawaii and F. Polynesia). It should be noted that *Haemoproteus columbae* and *H. iwa* have been previously described from blood smears in taken from Hawaiian birds (Valkiūnas 2005); however, molecular screening of Hawaiian passerines has not established the presence or identity of any *Haemoproteus* lineages (Beadell et al. 2006, Bensch et al. 2009). For *Haemoproteus*,

total observed lineages ranged from zero to 122 across regions and varied significantly among geographic categories of avian diversity (ANOVA; $df = 2, 10$; $F = 10.86$; $p < 0.01$; Table 1). Tukey's post-hoc tests revealed that "continental avian hotspots" and "continental avian non-hotspots" did not differ significantly; however, "oceanic" regions exhibited significantly fewer observed lineages than the other two categories (Figure 2a). In contrast, Chao2 diversity estimates for *Haemoproteus* spp. varied significantly among all three categories (ANOVA; $df = 2, 10$; $F = 91.49$; $p < 0.001$), with "continental avian hotspot" regions exhibiting the highest *Haemoproteus* spp. estimates while "oceanic" regions exhibited the lowest estimates (Figure 2b).

For *Plasmodium* spp., total observed lineages ranged from one to 159 across regions and varied significantly among geographic categories of avian diversity (ANOVA; $df = 2, 10$; $F = 6.01$; $p = 0.02$; Table 1), with significantly fewer observed lineages in "oceanic" regions than in "continental avian hotspot" and "continental avian non-hotspot" regions (Figure 2c). Chao2 diversity estimates for *Plasmodium* spp. exhibited the same pattern as observed lineages, with significantly lower estimates in "oceanic" regions than in "continental avian hotspot" and "continental avian non-hotspot" regions (ANOVA; $df = 2, 9$; $F = 4.53$; $p < 0.04$; Figure 2d).

For regions where *Haemoproteus* lineages were recorded, Chao2 diversity estimates tended to be higher for *Haemoproteus* spp. than for *Plasmodium* spp., apart from South America (Figure 1), although this trend was not statistically significant (t-test, $t = -1.13$, $df = 8$, $p = 0.28$). However, when excluding South America,

estimates for *Haemoproteus* spp. diversity were significantly higher than *Plasmodium* spp. ($df = 7$, $t = 2.23$, $p = 0.04$).

3.2: Adequacy of geographic and host species sampling

We identified a total of 162 papers that used PCR to investigate avian haemosporidian infections. There has been a rapid rise in molecular avian malaria studies since the first publications in the mid-1990's, with most (98%) published after the year 2000 when *cyt-b* primers for avian *Plasmodium* and *Haemoproteus* spp. were designed. The majority of these publications (86.4%) examined avian host infection, while far fewer publications (16%) examined vector host infection (Table 2). Sampling locations for molecular studies of avian haemosporidians represent every continent and over 100 countries (Figure 3). However, there is a substantial geographical bias in the distribution of field collection sites, with half of all sampling sites located in "continental avian non-hotspot" regions in Europe and North America (170 out of 340 total; Figure 3). Conversely, "continental avian hotspot" regions had a total of 88 sampling sites (22.9% of total; Figure 3). A wide range of avian hosts have been studied, however, the number of publications screening passerines (Order Passeriformes) was significantly higher than those screening other avian orders (χ^2 test; $df = 19$, $\chi^2 = 1033$, $p < 0.01$; Table 1). The number of avian host species ranged from one to over 900 (mean 22.1 ± 7.5), with 41.5% of avian studies investigating a single host species and 18.5% of avian publications investigating 25 or more host species.

3.3: Trends in methodology

Nested PCR targeting *cyt-b* has been the dominant molecular screening method since 2004, used in 61.7% of all publications (Table 2). In contrast to mitochondrial screening, only 8.9% of publications used nuclear markers (Table 2). A total of six different nuclear markers have been used, however, only two of these markers (DHFR-TS and SSU-rRNA) have been targeted by more than one avian host publication. To date, a single vector host study has used a nuclear marker (Table 2). Specific studies that targeted parasite genes other than *cyt-b* are highlighted in Appendix 1b. For both avian and vector hosts, far fewer studies screened for *Leucocytozoon* spp. than for either *Plasmodium* or *Haemoproteus* spp. (Table 2).

Less than half of all avian host publications (47.4%) included microscopy of blood films in conjunction with PCR (Table 2). A total of 16.3% of avian host publications have used multiple genetic markers to characterize infections while no vector host publications have used multiple markers (Table 2). Mixed species infections were specifically investigated in 30.7% of avian host publications, with microscopy the most frequently used method (Table 2). The single vector host publication that identified mixed infections used restriction enzyme digestion (Table 2).

4: Discussion

4.1: Diversity and biogeography of lineages

Our review of studies of avian haemosporidian *cyt-b* lineages indicated a high diversity of parasite lineages that are heterogeneously dispersed across biogeographic regions. Moreover, global patterns of observed and estimated lineage

diversity varied between the two parasite genera, *Plasmodium* and *Haemoproteus*. There is a tendency for continental avian diversity 'hotspots' to have higher estimates of lineage richness compared to avian 'non-hotspots' and oceanic areas, both for *Haemoproteus* spp. and, to a lesser extent, *Plasmodium* spp. Our findings therefore reflect a biogeographic pattern of higher diversity in low latitude tropical areas, as has been widely recognised across a range of taxa, including parasites (Sherratt and Wilkinson 2009). A number of theories have been proposed to explain the phenomenon of a latitudinal diversity gradient, most of which are probably not mutually exclusive (Pianka 2011). For example, the relatively stable year-round climate in tropical areas has been suggested to promote ecosystem productivity (Phillips et al. 1994). For avian haemosporidians, temperature and precipitation are important abiotic variables that may be conducive to parasite diversification by promoting parasite development and vector breeding opportunities (Beier 1998, Santiago-Alarcon et al. 2012b). However, tropical archipelagos appear to be limited in avian haemosporidian diversity, suggesting that climatic conditions alone do not drive diversity. Rather, haemosporidian diversity may be a function of avian and / or vector host diversity, both of which increase in tropical continental regions (Grenyer et al. 2006, Foley et al. 2007).

The tropics have also been suggested to have stronger biotic interactions than temperate habitats, with higher rates of parasitism or predation driving increased rates of speciation (Pitts and Roberts 1997). This hypothesis of 'diversity begets diversity' is reliant on the principle that if new species evolve in an already hyper-diverse ecosystem, they are likely to remain in that ecosystem and maintain or

even accelerate the diversity gradient (MacArthur and MacArthur 1961, Hechinger and Lafferty 2005, Sherratt and Wilkinson 2009). For avian haemosporidians, many vectors feed on a variety of avian host species (Ejiri et al. 2011, Santiago-Alarcon et al. 2012a) suggesting that opportunities for a parasite to infect a naïve host (i.e. host-switching) should be higher in species rich vector-host systems than in species poor systems. Phylogenetic and molecular studies suggest that host switching is common in avian haemosporidians (Ricklefs et al. 2004, Krizanauskiene et al. 2006), a process that may be facilitated when potential hosts are phylogenetically close to the original host (Poulin 2011). Therefore, high diversities of potential hosts could provide more opportunities for host switching and subsequent diversification (Hayakawa et al. 2008). Finally, the tropics may encourage faster rates of evolution either through shorter generation times or through the facilitation of sympatric speciation due to increased niche complexity (Mittelbach et al. 2007). Time-calibrated phylogenies can be useful for determining whether parasites in the tropics have diverged faster than their temperate counterparts (Currie et al. 2004, Bordes et al. 2010). However, while molecular clocks for avian haemosporidians have been estimated (Ricklefs and Outlaw 2010), the frequent host-shifts evident in the evolutionary history of these parasites make estimations of divergence rates particularly problematic (Bensch et al. 2013).

The higher lineage richness exhibited by *Haemoproteus* spp. compared to *Plasmodium* spp. and the different biogeographic patterns exhibited by the two genera suggest fundamental differences in the way lineages from the two genera diverge. Lineage diversity may result from high degrees of host specialization, a life-

history strategy that promotes the partitioning of resources (i.e. avian and vector hosts) and facilitates species coexistence (Lewinsohn and Roslin 2008). Host occurrence information from a number of studies suggests that *Haemoproteus* spp. tend to be more host-specific when compared to *Plasmodium* spp. (Ishtiaq et al. 2007, Beadell et al. 2009, Dimitrov et al. 2010, Ishtiaq et al. 2010), which may explain why estimates of *Haemoproteus* spp. diversity were consistently higher than *Plasmodium* spp. estimates. Greater partitioning of resources could also explain why *Haemoproteus* species diversity significantly increased in continental regions with higher avian host diversity while *Plasmodium* species diversity did not. With more potential avian hosts available in the tropics, the relative abundance of hosts may promote niche-specialisation, particularly for parasites that demonstrate a proclivity for host specialisation (Norton and De Lange 1999). Alternatively, *Haemoproteus* spp. infections may simply be easier to detect using molecular techniques. For example, the duration of relapses (i.e. secondary parasitaemia in the host blood), tends to longer in *Haemoproteus* spp. (up to several months) compared to generally shorter relapse durations in *Plasmodium* spp. (Valkiūnas 2005). Because PCR can fail to amplify when parasite intensity is very low (Waldenstrom et al. 2004), it is possible that the higher richness of *Haemoproteus* lineages found is partially an artefact of the longer window of opportunity to detect secondary parasitaemia.

An exception to the pattern of higher diversity for *Haemoproteus* lineages was displayed in South America. This region exhibited considerably more lineage recordings and higher lineage diversity estimates for *Plasmodium* spp. than any other region. This high richness of *Plasmodium* lineages in South America is well

exemplified by two particular studies, one in southeast Brazil (Lacorte et al. 2013), and the other in the western Amazon of Ecuador (Svensson-Coelho et al. 2013). *Plasmodium* lineages sampled from the western Amazon of Ecuador were more specialized than *Haemoproteus* lineages (Svensson-Coelho et al. 2013), while in southeast Brazil, over 55 *Plasmodium* lineages exhibited host ranges of only one species (Lacorte et al. 2013). This suggests that *Plasmodium* spp. may have experienced an exceptional radiation alongside avian hosts in South America's ecologically diverse tropical habitats. However, even though South America contains the most speciose avian and mosquito communities in the world (Grenyer et al. 2006, Rueda 2008), this does not explain why *Haemoproteus* lineage diversity was not similarly escalated in this region. It may be possible that *Plasmodium* lineages in South America are phylogenetically distinct and contain particular *cyt-b* sequences that increase the rate of detection over *Haemoproteus* lineages during mixed infections (see Valkiūnas et al. 2006). However, more sampling is needed in South America's diverse habitats to improve assessments of avian haemosporidian diversity and to certify the existence of these unique biogeographical patterns.

4.2: Absence of *Haemoproteus* on certain islands and archipelagoes

The lack of *Haemoproteus* lineages on some archipelagoes (e.g. Hawaii, French Polynesia) may be due to the absence of appropriate vectors or to founder events where low numbers of colonizers are less likely to carry infections (Peirce and Adlard 2004, Krasnov et al. 2007, Hellgren et al. 2011). For *Plasmodium* spp., the occurrence of lineages on south Pacific islands such as Hawaii, French Polynesia and New Zealand are thought to be in large part due to anthropogenic introductions of

infected hosts and suitable vectors (Beadell et al. 2006, Tompkins and Gleeson 2006, Ewen et al. 2012). However for *Haemoproteus* spp., the complete life cycles, specifically transmission dynamics between avian and vector hosts, have been poorly studied (but see Ishtiaq et al. 2008, Valkiūnas et al. 2010, Martinez-de la Puente et al. 2011). Further investigation into vector transmission will help to determine whether a lack of suitable vectors has led to the absence of *Haemoproteus* spp. on certain islands.

Another hindrance to colonisation of *Haemoproteus* species could be higher degrees of avian host specialization for *Haemoproteus* lineages when compared to *Plasmodium* lineages and, therefore, *Haemoproteus* spp. may have difficulty switching to new avian hosts in isolated island communities. Nevertheless, there are numerous cases of generalist *Haemoproteus* lineages recorded from avian hosts across broad geographic ranges including islands (Fallon et al. 2005, Beadell et al. 2009). It is more plausible that *Haemoproteus* spp. are absent in islands that are simply too isolated for natural colonisation of these parasites to occur without anthropogenic introductions of infected hosts and / or suitable vectors (e.g. Perez-Rodriguez et al. 2013). Regardless of the mechanisms limiting *Haemoproteus* spp. distributions on islands, continued monitoring for introduced haemosporidian parasites will be valuable as the threat of emerging wildlife disease accelerates with the increasing mobility of humans (Huijben et al. 2007).

Diversity patterns from molecular studies are supported by studies using only microscopic data, with higher *Haemoproteus* species richness for the majority of zoogeographical regions (Valkiūnas 2005). For example, Bennett *et al.* (1992) found

sub-Saharan birds have higher *Haemoproteus* species richness (63 species) when compared with *Plasmodium* (16 species). The same pattern was found in Western Europe, with the occurrence of 28 *Haemoproteus* species and 12 *Plasmodium* species (Peirce 1981). In addition, some microscopic evidence from the Neotropics suggests that avian hosts in both the Amazon basin and Central America have higher prevalence of *Plasmodium* spp. than *Haemoproteus* spp. (White et al. 1978).

However, our analysis of *cyt-b* lineages suggests variations across biogeographic regions that were not previously noted using microscopy alone (Valkiūnas 2005), such as the high diversity of *Plasmodium* spp. in the neotropical region and the higher diversity of haemosporidians in the tropics. These differences may therefore have arisen from overlooked infections due to the often low parasitaemia in natural infections.

4.3: Current limitations to estimates of diversity

The high estimates of undiscovered richness coupled with relatively limited sampling in many regions with high avian diversity indicate a need for further molecular studies, which should ideally be complimented with microscopic methods. However, the spread of current research suggests a bias towards European and North American Passeriform communities. While a wealth of information regarding the prevalence, seasonality, diversity and host distribution of avian haemosporidians has been generated in these host-parasite communities (Krizanauskiene et al. 2006, Bensch et al. 2007, Shurulinkov and Ilieva 2009), such knowledge is currently limited elsewhere. In Australia, for example, morphological descriptions previously indicated a paucity of haemosporidian species, interpreted as a result of limited colonisation

and restricted diversification as well as the limited number of microscopic studies in the region (Valkiūnas 2005). However, it is becoming increasingly apparent that Australia harbours high haemosporidian lineage diversity, particularly for *Haemoproteus* spp., even though Australian studies have been restricted to two major community studies in the north (Beadell et al. 2004, Zamora-Vilchis et al. 2012) and one study in the south (Balasubramaniam et al. 2013). Additionally, while Passeriformes is the most diverse of the avian orders (Hackett et al. 2008) and likely contains the most diverse haemosporidian fauna (Valkiūnas 2005), sampling from other orders, such as Columbiformes, Falconiformes and Strigiformes, has revealed high diversity and cryptic speciation of haemosporidians (Sehgal et al. 2006, Santiago-Alarcon et al. 2010, Valkiūnas et al. 2010, Yildirim et al. 2013). The limited sampling in species-rich habitats and among a range of avian host orders likely hampers descriptions of the overall diversity and the phylogenetic relationships of haemosporidians.

The creation of the MalAvi database has encouraged the widespread use of a single nested PCR protocol, leading to methodological conformity among the majority of molecular studies and aiding our understanding of haemosporidian diversity. While it may be suggested that the dearth of nuclear investigations presents a hindrance to true estimates of parasite diversity, evidence from simultaneous nuclear and mitochondrial studies suggests that most *cyt-b* lineages probably represent reproductively isolated entities (Bensch et al. 2004, Beadell et al. 2009). However, this rapid increase in molecular studies may be leading a shift away from more traditional parasitology methods, such as rigorous microscopic

examinations (Braga et al. 2011). It is therefore important to emphasize that our conclusions are based on lineage detection using PCR methods. This method has a very low detection threshold and is capable of amplifying DNA from different life stages of haemosporidians (Valkiūnas et al. 2009), even when parasites are present in non-competent hosts. This can be a problem because sporozoites, which are injected by dipteran vectors into the avian hosts' blood stream, can persist for some time in the peripheral blood stream without developing into a full infection (Valkiūnas 2005). Although such abortive infections can limit our understanding of parasite-host transmission dynamics, inference of biogeographical patterns is less affected as these types of infections still inform the number of existing lineages per region.

In addition to confirming vector and host competence, microscopy alongside PCR can link genetic and morphological descriptions of parasite diversity, which is crucial for phylogeographic studies and should lead to a better understanding of the evolutionary histories of avian haemosporidians (Martinsen et al. 2006, Palinauskas et al. 2007). However, such studies are lacking in many host-parasite communities, leading to a poor understanding of the true diversity of these parasites (Braga et al. 2011). The dynamics and frequency of mixed infections may also be overlooked without microscopic analysis (Valkiūnas et al. 2006, Valkiūnas et al. 2008), which may also limit our knowledge of haemosporidian diversity. Examination of blood smears alongside PCR remains the ideal approach when characterising infections from avian hosts.

The paucity of studies analysing haemosporidian infections in vectors may lead to a limited understanding of the true diversity of lineages as well as the dynamics of haemosporidian transmission (Santiago-Alarcon et al. 2012b). For instance, recent molecular surveys in southern Melanesia recorded numerous lineages of *Plasmodium* and *Haemoproteus* spp. in vectors that have not yet been recovered from avian hosts in the region (Ishtiaq et al. 2008, Ishtiaq et al. 2010). Because sexual reproduction occurs in the vector host (Valkiūnas 2005), vectors are vital for the reproductive isolation of haemosporidian species (Gager et al. 2008). Therefore, it is fundamental to establish vector competence, which at the moment is only possible with the use of microscopy that allows the observation of sporozoites. Studies of vector host-specificity may therefore help to determine the role of vectors in driving lineage distributions among avian hosts (Medeiros et al. 2013). However, vector feeding patterns can be complex, particularly since some species seem to adjust their feeding preferences according to host availability (Santiago-Alarcon et al. 2012b). Some studies have recorded a tendency for vector host-specialisation among lineages, suggesting that vectors can act as ecological barriers by restricting lineages to avian hosts that belong to the vector's diet (Ejiri et al. 2008, Hellgren et al. 2008). This suggests that differences in parasite prevalence among different habitats may be strongly related to the presence or absence of suitable vectors (Mendes et al. 2005, Svensson and Ricklefs 2009, Yohannes et al. 2009). It is clear that a great deal more remains to be discovered about vector host-specialisation and its role in driving parasite diversification (Santiago-Alarcon et al. 2012b).

4.4: Future directions

While nested PCR targeting of cyt-b has been instrumental to uncovering avian haemosporidian diversity across the globe, future studies may benefit from the use of variable nuclear markers that are already widely used in human malaria studies, such as merozoite surface protein-1 (Silva et al. 2000, Poulin 2011, Hellgren et al. 2013). The development of such markers for avian haemosporidian studies could lead to a better understanding of host-specificity and geographic limitations for parasite lineages as well as more sophisticated taxonomic revisions (e.g. Pick et al. 2011, Tachibana et al. 2012). However, no complete genomes for avian haemosporidians are currently available, (but see incomplete genome for *Plasmodium gallinaceum*; Wellcome Trust Sanger Institute, UK), owing primarily to difficulty obtaining adequate concentrations of parasite DNA from avian blood samples (Palinauskas et al. 2010). As methods for collection of pure parasite template improve, we can expect avian haemosporidian studies to branch into more fine-scale population genetics as well as more informative phylogenetic analyses of relatedness among haemosporidians of vertebrates (Bensch et al. 2013). However, the identification of competent vector and avian hosts relies on widespread utilisation of traditional parasitological methods, particularly as PCR detection of haemosporidian DNA can occur despite abortive development in the avian host. Moreover, because remnants of infected blood cells and the presence of oocysts and ookinetes in the thorax of vectors can also provide parasite DNA template, positive parasite detection from the thorax of vectors species is inconclusive evidence for parasite transmission (Valkiūnas et al. 2013). Therefore, microscopic methods are still an essential tool, both for determining the vector species responsible for

parasite transmission and for generating a more complete understanding of haemosporidian diversity.

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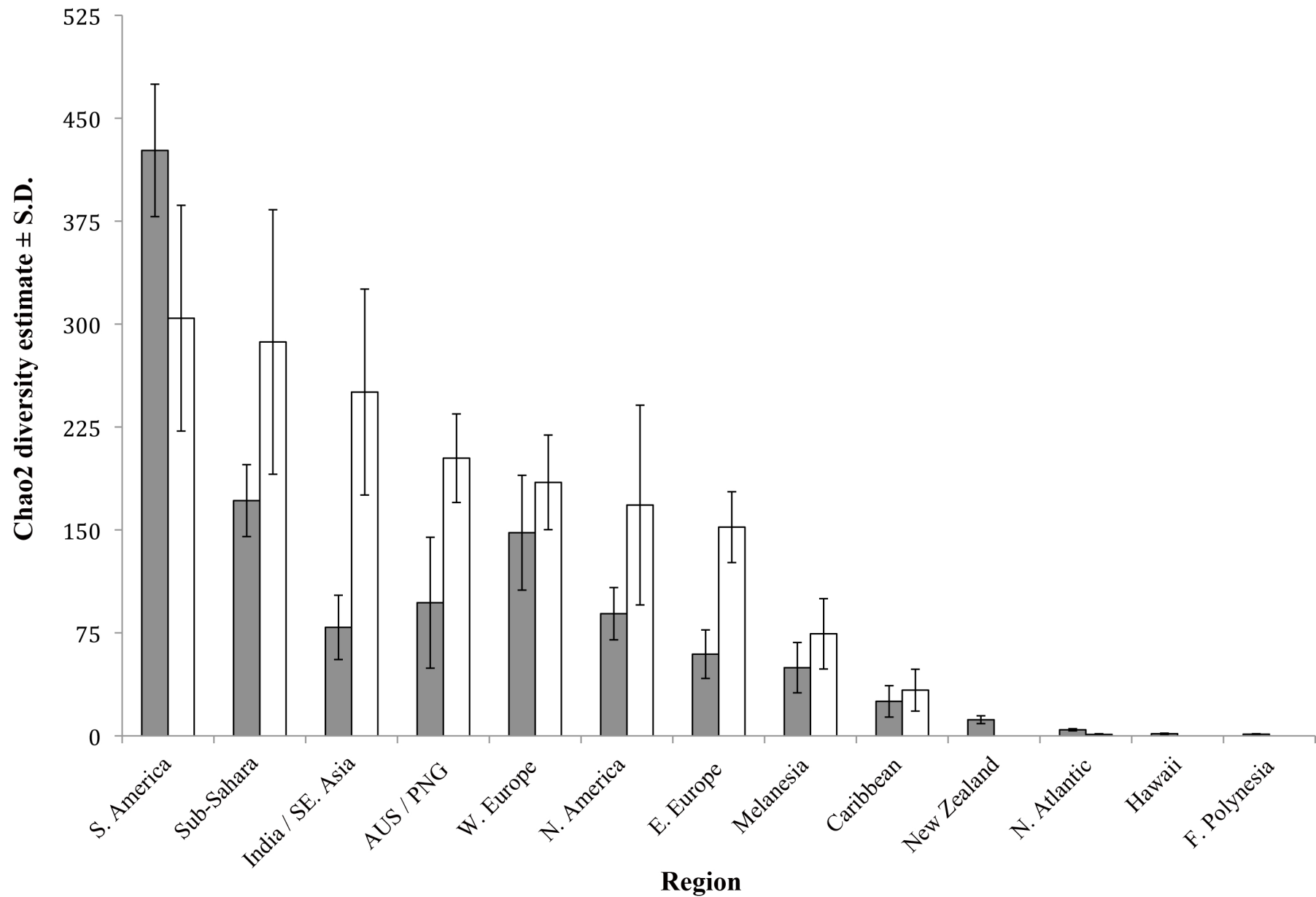
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Legends to figures:

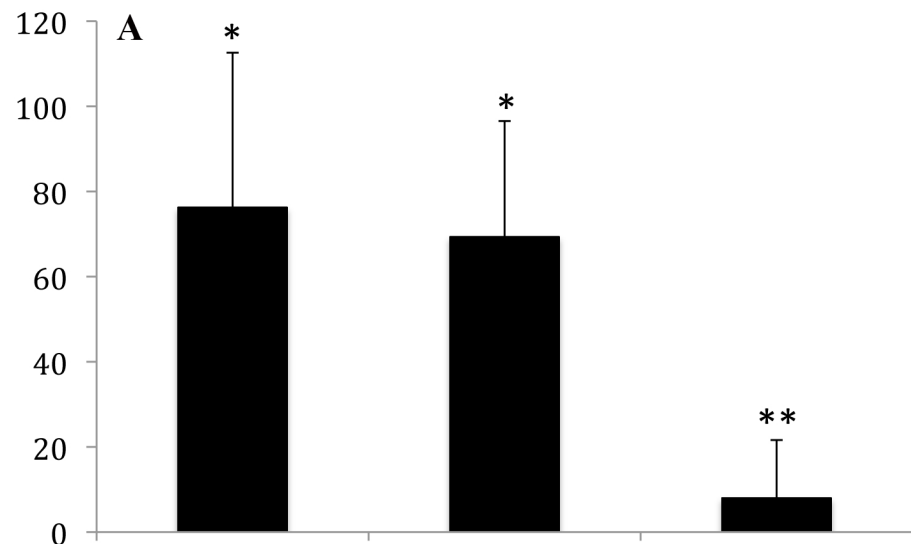
Figure 1: Chao2 estimates of lineage diversity among sampling regions for avian *Plasmodium* (grey bars) and *Haemoproteus* (white bars). Error bars indicate standard deviation. Note *Haemoproteus columbae* and *H. iwa* have been previously described from blood smears in Hawaiian birds (Valkiūnas 2005).

Figure 2: Mean observed and estimated lineage diversity among avian diversity categories. a) observed *Haemoproteus* diversity; b) estimated *Haemoproteus* diversity; c) observed *Plasmodium* diversity; d) estimated *Plasmodium* diversity. Asterisks represent significantly different groups determined from Tukey's post-hoc HSD tests.

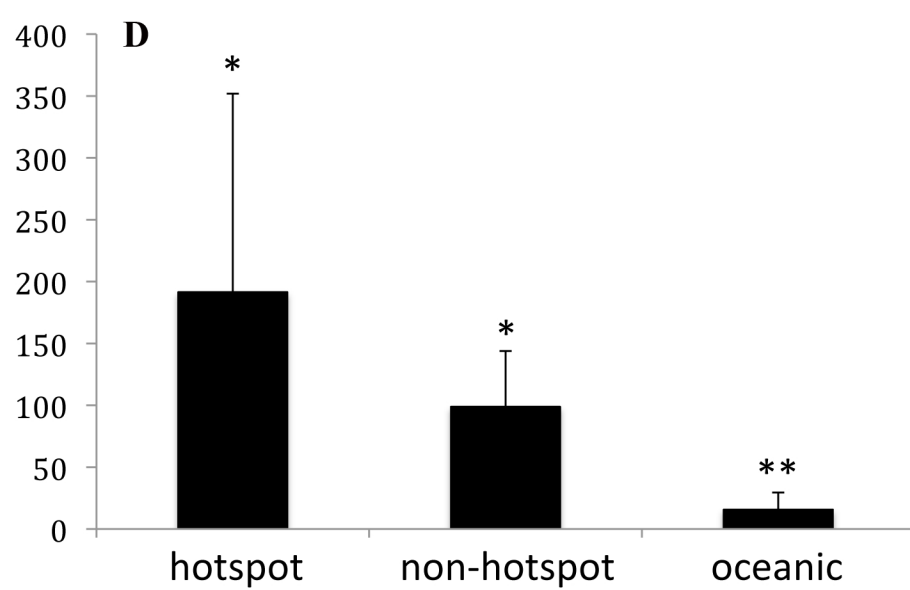
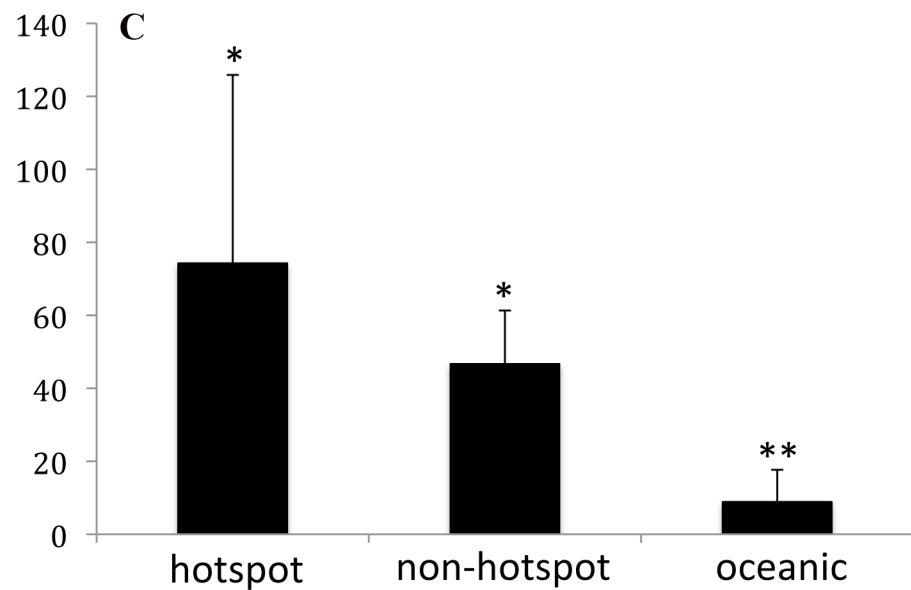
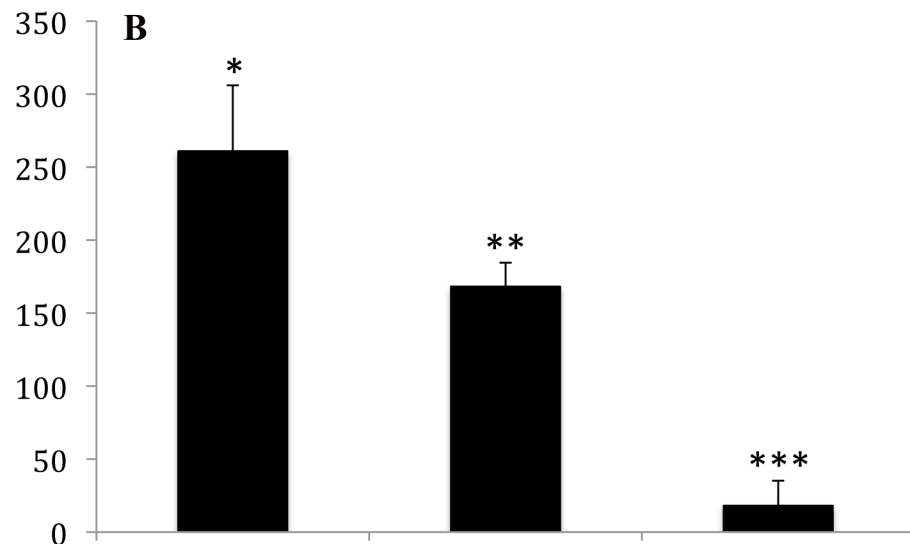
Figure 3: Sampling locations used in published molecular avian haemosporidian studies from 1995 to April 2012. GIS layers for avian species richness data were sourced from Orme *et al.* 2005.

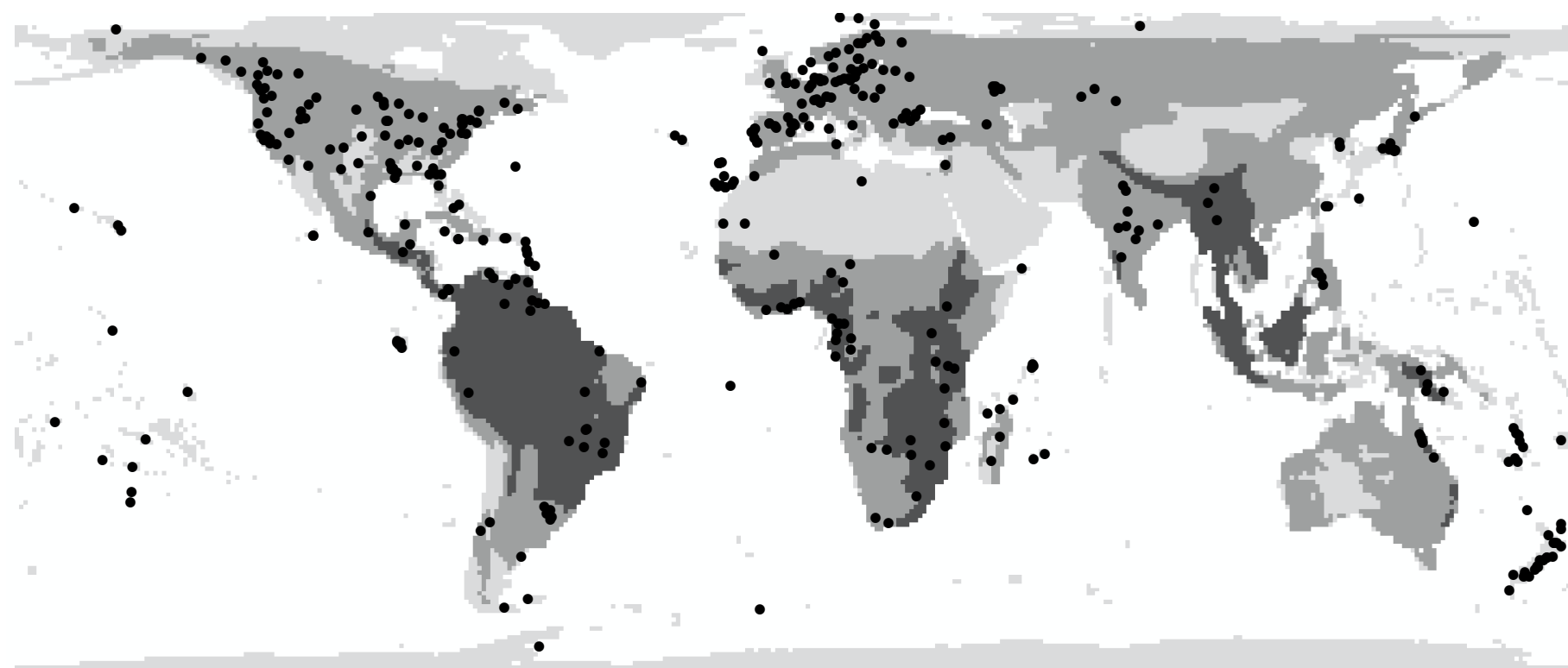


Mean # observed lineages \pm S.D.



Mean Chao2 diversity estimate \pm S.D.





avian spp. richness

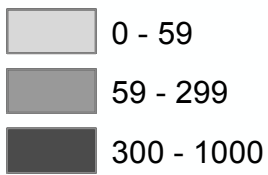


Table 1: Avian sample sizes and lineage diversity estimates for *Plasmodium* and *Haemoproteus* spp. among geographic regions. Regions were pooled into biogeographic groups based on avian richness hotspots: “continental avian hotspot” (300 – 1000+ spp.), “continental avian non-hotspot” (60 – 299 spp.) and “oceanic” (Orme *et al.* 2005). The total number of avian samples screened and the number of observed lineages in each region were recorded from the MalAvi database and a review of the literature. Estimates of lineage diversity were generated using the Chao2 diversity estimator. *Some samples were screened for only one genus of parasite. ** Some lineages were shared among regions. Note *Haemoproteus columbae* and *H. iwa* have been previously described from blood smears in Hawaii (Valkiūnas 2005).

region	avian richness	countries / territories sampled	# samples*	lineage diversity			
				observed**		estimated (SD)	
				<i>Plas</i>	<i>Haem</i>	<i>Plas</i>	<i>Haem</i>
Sub-Sahara	hotspot	Nigeria, Cameroon, Gabon, Kenya, Zimbabwe, Ghana, South Africa	4501	96	122	171.36 (26.15)	286.95 (96.37)
India / SE Asia	hotspot	India, Burma (Myanmar), Phillipines	1468	34	39	79.00 (23.41)	250.47 (75.01)
S. America	hotspot	Brazil, Ecuador, Guyana, Venezuela, Uruguay, Peru	8672	159	86	426.54 (48.24)	304.26 (82.25)
AUS / PNG	hotspot	Australia, Papua New Guinea	857	30	87	97.00 (47.70)	202.30 (32.27)

Note: Supplementary data associated with this article

E. Europe	non-hotspot	Russia, Lithuania, Ukraine, Bulgaria, Romania, Poland, Czech Republic, Hungary	4651	31	83	59.50 (17.67)	152.03 (25.81)
W. Europe	non-hotspot	UK, Spain, France, Germany, Italy, Norway, Sweden, Belgium	11193	60	87	147.99 (41.83)	184.65 (34.47)
N. America	non-hotspot	Mainland USA, Canada, Mexico	3245	49	38	89.00 (19.03)	168.15 (72.80)
Melanesia	oceanic	New Caledonia, Vanuatu	2314	24	33	49.65 (18.36)	74.34 (25.63)
Caribbean	oceanic	Lesser Antilles, Bermuda, Puerto Rico	2780	14	15	25.09 (11.42)	33.25 (15.27)
NZ	oceanic	New Zealand	1102	8	0	11.72 (2.82)	0
N. Atlantic	oceanic	Bioko, Madeira, Azores Islands	1661	4	1	4.37 (0.76)	1.27 (0.39)
Hawaii	oceanic	Hawaiian Islands	320	2	0	1.48 (0.45)	0
French Polynesia	oceanic	Society Islands, Marquesas	174	1	0	1.14 (0.38)	0

Table 2: Molecular screening methods and the host-parasite systems examined in avian haemosporidian publications (1995-2012). *Some studies analysed avian and vector hosts simultaneously. Specific studies that targeted parasite genes other than *cyt-b* are highlighted in Appendix 1b.

	total*	avian	vector
	162	140	26
Genus targeted			
<i>Plasmodium</i>	146	127	22
<i>Haemoproteus</i>	126	115	15
<i>Leucocytozoon</i>	43	39	4
Mitochondrial marker(s)	154	133	25
cyt - <i>b</i>	153	132	25
cytochrome oxidase 3	5	5	0
Nuclear marker(s)	14	13	1
DHFR-TS	4	4	0
SSU-rRNA	9	8	1
DGAT	1	1	0
LSU-rRNA	1	1	0
TRAP	1	1	0
ClpC	1	1	0
Reaction methods			
nested PCR	100	85	17
qPCR	9	7	2
PCR+1	1	1	0
Mixed infections identified	44	43	1
microscopy	19	19	0
restriction enzyme digestion	11	10	1
TA cloning	14	14	0
genus-specific primers	2	2	0
Avian orders screened			
Passeriformes	114	-	-
Columbiformes	21	-	-
Falconiformes	14	-	-
Strigiformes	11	-	-
Piciformes	10	-	-
Galliformes	9	-	-
Charadriiformes	9	-	-
Coraciiformes	5	-	-
Cuculiformes	5	-	-
Apodiformes	5	-	-
Other orders	22	-	-

Note: Supplementary data associated with this article