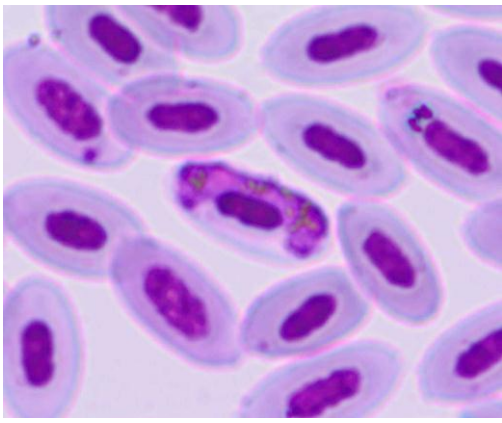

A comparison of avian haemosporidian parasite communities across the strait of Gibraltar

Vanessa Cristina Alves Mata
Mestrado em Biodiversidade, Genética e Evolução
FCUP-CIBIO
2012

Orientador
Serguei Drovetski, Senior Scientist, Assoc. Researcher, FCUP/CIBIO

Coorientador
Ricardo Jorge Lopes, Post-Doc, CIBIO

Acknowledgments

I would like to thank all the people who have somehow contributed to the existence of this thesis, either by cheering me up and giving me strength to continue or by helping me out with my countless questions and doubts.

A special thanks to:

My supervisors Sergei Drovetski and Ricardo Jorge Lopes for giving me this unique opportunity and for all their help, guidance and patience;

Sandra Reis for the precious help and orientation in the lab;

Luis Silva, not only for helping in the Morocco field work and collecting most of the blood samples from Portugal, but also for all the ideas and interest shown in the work, not to mention everything else;

All my colleagues in CIBIO/CTM for having filled my days with something more than work, turning them a little brighter; labwork, lunchtimes and Metro rides wouldn't have been the same without you;

João Leite and Fernando Seixas for your friendship, companionship, moments of humor and silliness, but also for your help, support and encouragement when I most needed;

Francisco Amorim and all the people in the GVC ringing group for giving me the opportunity (and the excuse) to get out of the lab/computer, keep my mental sanity and enjoy myself while working and learning;

My mum and brother for having endured another year without my presence, always compelling me to go on and to never give up; all I am and have accomplished is thanks to you.

I would also like to thank the University of Porto and Santander for partially funding this work through the project PP_IJUP2011 195.

CIBIO Centro de Investigação em Biodiversidade e Recursos Genéticos

U.PORTO FACULDADE DE CIÊNCIAS UNIVERSIDADE DO PORTO

Santander

Abstract

I used molecular tools to examine the diversity of haemosporidian parasites from the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in birds of Northwest Africa and Northwest Iberia. In total, 459 birds of 36 species from Portugal and 324 birds of 46 species from Morocco were tested using PCR for the presence of infections.We identified a total of 169 unique haemosporidian lineages. We found 127 parasite lineages in North Africa and 74 lineages in Iberia. Only 32 lineages were shared between the study areas. Overall prevalence was higher in Morocco, where 79% of the birds carried haemosporidian infections compared to only 44% in Iberia. The rate at which new parasite lineages were discovered with increasing sample size did not differ between the areas, however, the higher infection prevalence in Morocco translated into greater haemosporidian diversity compared with Portugal. The number of hosts from which a parasite lineage was recovered varied from one to sixteen. Parasite specificity varied among parasite genera. *Haemoproteus* was the most host-specific and *Plasmodium* was the most host-generalist. The composition of haemosporidian communities differed between Maghreb and Iberia. *Haemoproteus* was more common in Maghreb but *Plasmodium* dominated in Iberia. Infections with parasites found in both areas accounted for 63% of total infections. However, no correlation was found between the number of lineage observations in Iberia and Morocco for any parasite genus, suggesting that the parasite composition of both areas is different at both levels – the generic composition and prevalence of individual lineages.

Resumo

Foram utilizados métodos moleculares para avaliar a diversidade de parasitas haemosporidos do género *Plasmodium*, *Haemoproteus* e *Leucocytozoon*, em aves do Noroeste de África e Noroeste da Ibéria. Um total de 459 aves de 36 espécies foi amostrada em Portugal, e 324 aves de 46 espécies em Marrocos, e examinadas para a presência de infecções. Foram identificadas 127 linhagens de parasitas no Norte de África e 74 na Ibéria. A prevalência geral de infecção foi mais elevada em Marrocos com 79% das aves apresentando infecções, contra 44% de aves infectadas em Portugal. A taxa á qual foram encontradas novas linhagens de parasitas foi igual em ambas as áreas, no entanto, a maior prevalência em Marrocos traduziu-se numa maior diversidade de haemosporidos do que em Portugal. O nº de hospedeiros do qual a mesma linhagem de parasita foi recuperada variou entre 1 e 16. A especificidade dos parasitas variou de acordo com o género pertencente, sendo que *Haemoproteus* eram mais específicos e os *Plasmodium* mais generalistas. A prevalência de cada género variou entre as duas áreas de estudo, com *Haemoproteus* sendo mais comum em Marrocos e *Plasmodium* em Portugal. Infecções com parasitas encontrados em ambas as áreas de estudo contabilizaram 63% das infecções. No entanto, não foi encontrada qualquer correlação entre o número de observações de cada parasita entre a Ibéria e o Norte de África para nenhum dos géneros, sugerindo que a composição de parasitas em ambas as áreas é diferente a dois níveis - a estrutura geral da composição e prevalência individual das linhagens.

Contents

List of Tables and Figures

Chapter 1

Introduction

Parasitism has evolved several times independently in the history of life. It is even argued that it may be the most prevalent means of obtaining food (Price 1977) and that parasites make up most of the species on Earth (Windsor 1998). The most commonly accepted definition of a parasite is that it is an organism living in another organism (the host), feeding on it, showing some degree of structural adaptation to it, and causing it some harm (Poulin 2007).

Birds have long served as a model system to study infectious disease (Atkinson & Van Riper III 1991; Valkiūnas 2005). They provide much needed data on pathogen ecology, essential for understanding the impact of ongoing global changes (climate, biotic invasion, landscape modification) that affect the biology of hosts and their parasites and increase the risk of devastating disease outbreaks (Freed *et al.* 2005; Garamszegi 2011). Avian haemosporidians in particular are excellent models to study the effects of parasites on wild populations (Valkiūnas 2005).

1. Haemosporidians and birds

Haemosporidians (Sporozoa: Haemosporida) are one of the best-studied groups of parasitic protists (Valkiūnas 2005). They include agents of human malaria, one of the most common diseases in warm climate countries, which kills and causes serious illness in millions of people every year (WHO 2010). However, the few species responsible for such an impact are just a tiny part of the systematic and ecological diversity of haemosporidians. Systematic parasitologists have established 15 genera within the order Haemosporidia which contain over 500 species that infect reptiles, birds and mammals (Martinsen *et al.* 2008). They are found in every terrestrial habitat and use several families of dipteran vectors.

Avian haemosporidians, hereafter also referred to as avian malaria parasites (for the sake of simplicity), are comprised of three main genera – *Haemoproteus, Plasmodium and Leucocytozoon* – and constitute the most diverse group of haemosporidians with 206 species described from hundreds of avian species and from 16 genera of insect vectors (Valkiūnas 2005). They have long been the object of intensive research, since they were used as models to study human malaria. However, with the discovery of malaria parasites in rodents during the second half of the 20th century, investigation of this intriguing group of protists considerably declined. Nevertheless, the great body of knowledge remained, and when ecologists and evolutionary biologists searched for models to test their hypotheses, avian haematozoa provided some of the best existing databases. During the last decade, increased use of molecular tools, especially of PCR diagnostics brought attention back to avian haemosporidians and the number of studies focusing on biology of these parasites grew exponentially (LaPointe *et al.* 2012).

1.1 Haemosporidians Life Cycle

Haemosporidian are obligate heteroxenous parasites. They need two hosts to complete their life cycle. Parasites go through a series of asexual divisions in an intermediate vertebrate host until the development of sexual stages (gametocytes). Their sexual reproduction occurs in a definitive host (dipteran vector). The life cycle begins with a vector feeding on an infected intermediate host's blood (Figure 1). The three haemosporidian genera use different vectors. *Plasmodium* employs blood-

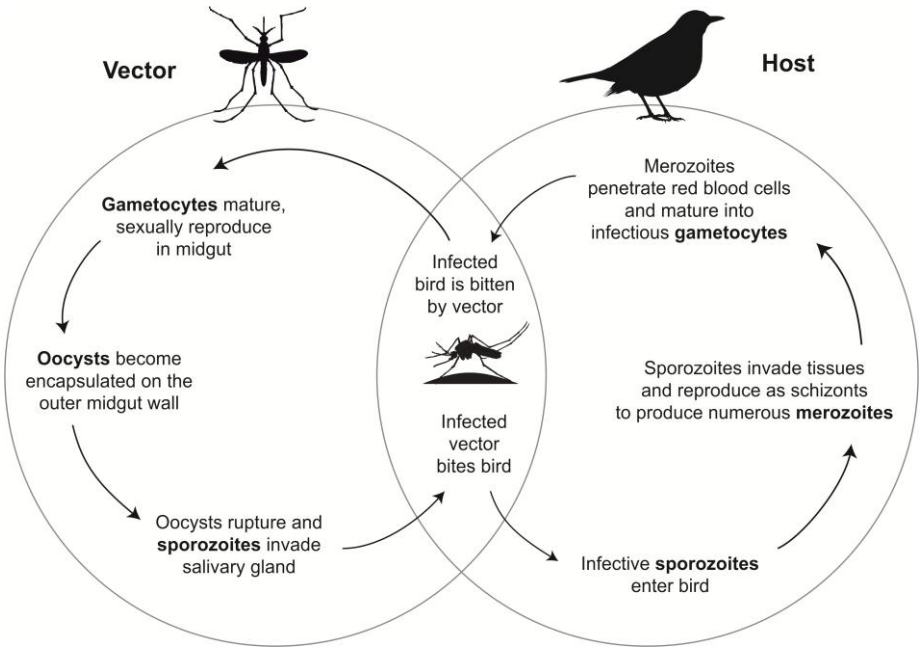

Figure 1 General life cycle of avian haemosporidian parasites (adapted from Atkinson 1999).

sucking mosquitoes (Diptera: Culicidae), *Haemoproteus* uses biting midges (Diptera: Ceratopogonidae) and louse flies (Diptera: Hippoboscidae), and *Leucocytozoon uses* blood-sucking black flies (Diptera: Simuliidae). Shortly after the insect vector acquires infectious gametocytes from an intermediate host during a blood meal, gametocytes go through gametogenesis and sexual reproduction in the insect's midgut. The gametogenesis produces a motile ookinete that penetrates the epithelial layer of the midgut and develops into an encapsulated oocyst. Numerous elongated, uninucleated sporozoites are formed in the oocyst during the process called sporogony. When oocyst fully matures, it bursts and sporozoites invade the salivary glands of the vector. This process may take from a couple of hours to days, depending on the species and ambient temperature. The insect vector may die if the number of ingested gametocytes is too high (Valkiūnas 2005).

Sporozoites infect birds when the vector injects them with saliva during feeding. The development steps inside the host largely depend on the genus of the parasite, but in general, one can consider five main stages of infection. The first phase – prepatent – occurs when the parasite is developing in the organ tissue cells and, it usually takes approximately 5 days for *Plasmodium* and *Leucocytozoon*, and 11 days to 3 weeks for *Haemoproteus* (Valkiūnas 2005). During this phase, sporozoites invade the host's organ tissue cells and produce exoerythrocytic meronts or schizonts, which then undergo a series of asexual divisions to form merozoites. Merozoites can induce a new cycle of merogony or invade the blood stream and develop into sexual stages (gametocytes) in the blood cells. The number of merogony cycles varies greatly among species, but *Plasmodium* has the unique ability of also using erythrocytes for this process after undergoing two cycles in the organ tissue cells. *Leucocytozoon* parasites, on the other hand, are able to use mononuclear leukocytes for the development of gametocytes (Valkiūnas 2005).

The first appearance of parasites in the host's blood marks the beginning of the second phase called the acute period. It is characterized by a sharp increase in the number of infected red-blood cells or parasitaemia. Crisis, the third phase, occurs when the parasitaemia reaches its peak. During the last two phases, chronic (4^{th}) and latent (5^{th}), the parasitaemia sharply decreases and even can be eliminated due to the host's immune response. However, once a bird is infected, it usually retains chronic or latent infection for many years or the rest of its life, serving as a source of infection for vectors. Relapses of parasitaemia may occur in many species especially in the spring and fall, before and after the reproduction period of hosts in temperate regions, facilitating the infection of vectors and transfer of parasites to offspring, but the

mechanisms responsible for the regulation of this process are poorly understood (Valkiūnas 2005).

1.2 Diversity and Ecology

For a century, haemosporidians were classified based on morphology, life cycle, and vertebrate and insect host taxa. The traditional taxonomy was contradicted by the findings of pioneering molecular systematic studies. These initial studies were based on single genes and, although several important nodes were poorly supported, they created a great controversy in the definition of the term “malaria parasite” (Pérez-Tris *et*

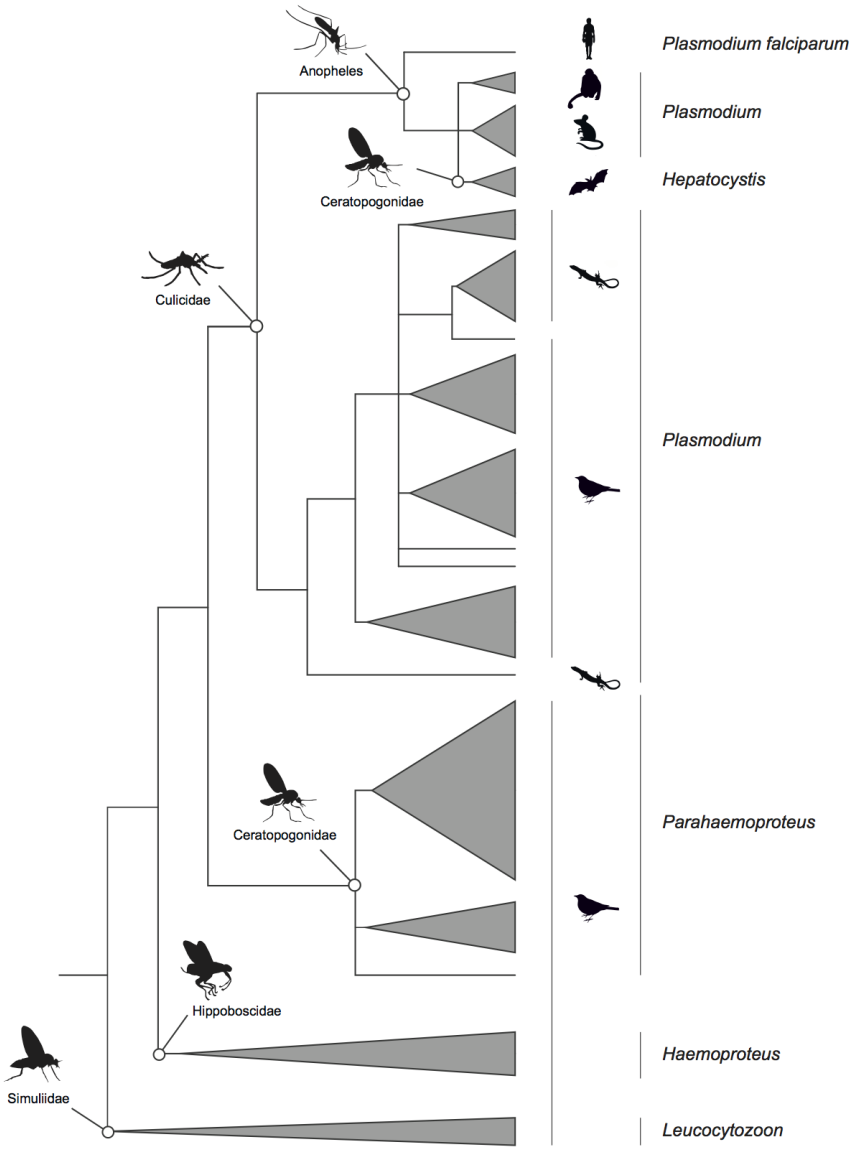

Figure 2 Phylogenetic relationships among the major haemosporidian genera. Arrows indicate major vector shifts and triangle size indicates the number of sampled host species (adapted from Martinsen et al. 2008).

al. 2005; Valkiūnas 2005). That is because the genus *Plasmodium* was found to be paraphyletic in respect to *Haemoproteus*, with avian and reptilian *Plasmodium* species appearing more closely related to *Haemoproteus* than to mammalian *Plasmodium* parasites (*i.e.* Duval *et al.*, 2007). More recently however, Martinsen *et al.* (2008) proposed a phylogenetic hypothesis for *Plasmodium* and related haemosporidian parasites using four genes. They suggested that mammalian and avian *Plasmodium* is paraphyletic to *Hepatocystis*, a group with very different life-history and morphology, specific to bats. Also, *Haemoproteus* appears to be divided into two divergent clades corresponding to *Parahaemoproteus* and *Haemoproteus* that were previously considered subgenera (Figure 2). Major haemosporidian clades were found to be associated with vector shifts to different dipteran families, while other characters used in traditional parasitological studies had low phylogenetic signal.

The use of PCR-based techniques resulted in the discovery of a high genetic diversity of haemosporidian parasites, which has been compiled in GenBank nucleotide database or, more recently, in a specialized database, the MalAvi database (Bensch *et al.* 2009), specially designed to compile cytochrome-*b* (cyt-*b*) haplotypes of avian haemosporidian parasites. Some recent studies reported findings of almost as many mtDNA cyt-*b* parasite haplotypes as the number of hosts used in these studies (Bensch *et al.* 2000; Ricklefs & Fallon 2002; Merino *et al.* 2008; Belo *et al.* 2011). New haplotypes of haemosporidians are being discovered in practically every published survey. It is not yet clear whether this great diversity of genetic haplotypes results from intraspecific variation within parasite species, speciation within a single host population, or reinvasion of a former host following species formation in an alternative host species (Ricklefs *et al.* 2005). However, some studies using mitochondrial and nuclear markers, indicate that mtDNA cyt-*b* haplotypes represent evolutionarily independent lineages, populations or species as they are associated with different nuclear haplotypes (Bensch *et al.* 2004; Martinsen *et al.* 2006; Hellgren *et al.* 2007). In this thesis we will adopt the terminology of lineages rather than haplotypes, following this reasoning and the widely adoption of this terminology in avian haemosporidian parasites publications.

Plasmodium lineages appear to be predominantly host generalists. They are often found in hosts from different avian families. In contrast, *Haemoproteus* and *Leucocytozoon* exhibit more narrow host preferences, usually infecting a single avian family (Ricklefs & Fallon 2002; Waldenström *et al.* 2002; Beadell *et al.* 2004; Hellgren *et al.* 2008; Dimitrov *et al.* 2010; la Puente *et al.* 2011). Although host shifts within families are common, as well as vector sharing (Kimura *et al.* 2010; Njabo *et al.* 2011), some cases of high host-specificity have been observed, especially in *Haemoproteus* parasites (Bensch *et al.* 2000; Ricklefs & Fallon 2002; Beadell *et al.* 2004; Fallon *et al.*

2005). Host-specificity is an important characteristic of a parasite that may affect its virulence and prevalence in different host species, its genetic variability and response to selection within each host (Hellgren *et al.* 2009). Various studies have found a negative correlation between host-specificity and the number of individuals infected (Ricklefs *et al.* 2005; Arriero & Møller 2008; Hellgren *et al.* 2009; Szöllosi *et al.* 2011), *i.e.*, generalist parasites reaching higher prevalence within host-species (or population). The ecological reason behind this pattern is not clear, but the ability of a parasite to infect and complete its development in many different host species may increase transmission success due to the increase in both the number of potential and infected hosts (Hellgren *et al.* 2009). This leads to an increase in the overall parasite prevalence in the host community.

In addition to the host specificity, parasite prevalence is also affected by the transmission rate of arthropod vectors, their abundance and ecological requirements, as well as the immunological capacity of the host to either prevent parasite infection or to clear established infections (Atkinson & Van Riper III 1991). Finally, associations of host age, body-mass, and latitude with parasite prevalence have also been reported. Older and heavier birds usually have higher prevalence of haemosporidian parasites than younger and lighter birds, and tropical areas have higher prevalence than temperate regions (Scheuerlein & Ricklefs 2004; Merino *et al.* 2008).

1.3 Effects on wild bird populations

Although parasitic organisms have a worldwide distribution, great diversity, and high prevalence, ecologists frequently ignore them while considering processes that occur in the wild, especially in ornithology (Valkiūnas 2005). The pathogenic impact of malaria parasites on birds is extremely heterogeneous due to the complexity of haemosporidian life cycles and disease epidemiology. This complexity is responsible for our poor understanding of their dynamics in wild populations. In immunologically *naïve* birds of Hawaii, malaria parasites have been shown to radically increase host mortality, reduce population sizes, and limit host species distributions (Atkinson & Van Riper III 1991; Atkinson & Samuel 2010). In areas of endemic transmission, however, malaria parasites are thought to have little impact on wild populations. Rare cases of haemosporidian caused mortality have been reported, but studies addressing haemosporidian effects provided inconclusive results or failed to find significant fitness costs of malaria infections to their avian hosts (*e.g.* Weatherhead and Bennett 1992; Bennett *et al.* 1993; Davidar and Morton 1993; Stjernman *et al.* 2004).

This difficulty in assessing the impact of haemosporidians on wild birds could be related to several methodological issues. First, there might be differences in the capture rate of uninfected and infected individuals. Pathogen-induced changes in behavior and/or activity levels may lead to variation in capture probability, biasing the patterns of prevalence and survival rates (Jennelle *et al.* 2007). Seriously ill individuals will rarely be caught and sampled, as they are weak, less agile, and more prone to predation (Møller & Nielsen 2007). Rare exceptions to this bias happen near human-inhabited areas (Valkiūnas 2005). Furthermore, the acute stage of malaria infection is very brief and can involve high mortality, therefore most infected individuals sampled in natural populations are likely to be survivors harboring chronic or latent infections (Lachish *et al.* 2011). If the effect of chronic infections is small, then studies with large sample sizes and long-term data, or direct experimentation, will be needed to detect and quantify it. The latter are more common, and some studies have been able to clearly show, by either the use of anti-malaria drugs or brood manipulation, that even chronic infections can exert a significant selection pressure on their hosts and decrease their survival (Marzal *et al.* 2008; la Puente *et al.* 2010; Lachish *et al.* 2011) or reproductive success (Marzal *et al.* 2005; Asghar *et al.* 2011).

Another problem regarding the assessment of malaria impacts on wild birds is the considerable diversity of malaria species. Haemosporidian species differ in their distribution (Wood *et al.* 2007) and have different effects on different avian species (Palinauskas *et al.* 2008) obscuring their individual fitness effects. Due to this heterogeneity, few studies have considered that host-parasite interactions and infection dynamics may vary with malaria parasites, and those that did, indeed, found differences among different parasite infections and host fitness costs (Ortego *et al.* 2008; Marzal *et al.* 2008; Lachish *et al.* 2011).

2. Host-parasite biogeography

Host-parasite relationships can be complex and unpredictable. For example, strong parasite virulence increases its prevalence and insures its persistence in a host population. However, when the parasite's virulence reaches a certain threshold and becomes too strong, it leads to a decrease in its fitness. In addition, the presence of other parasites can modify the compatibility, either by crossed vaccinating effects, which reduces fitness of a new parasite, or by immunosuppressive effects, which has the opposite effect (Combes 2000). Finally, changes in environmental variables may easily disrupt the equilibrium between parasites and their hosts. This is the reason why

studies of host–parasite relationships in the spatial and temporal context become of special importance during current rapid environmental change.

One of the major anticipated consequences of global climate change is the disruption of ecologic communities due to significant changes in species abundance (Hurrell & Trenberth 2010). Poleward and upslope range shifts in mountain areas are the expected reactions to temperature increase and have been observed in a number of different species (Parmesan 2006). However, climate change is a complex process and the simple move of species latitudinally and altitudinally is not the only factor affecting ecological communities. Differences in species' physiological tolerance, life-history strategies, and dispersal abilities, are responsible for the high variability in response to the climate change among species exposed to similar climatic trends (Parmesan 2006).

For many species, the first impact of climate change is caused by an asynchrony between species' food and habitat resources due to changes in resources' distribution and availability (Pimm 2009). This might lead to a cascade of disruption events in the timing between the life cycles of predators and their prey, herbivorous insects and host plants, insect pollinator and flowering plants, and parasitoids and their host insects (Parmesan 2006). The outcome of this restructuring of ecological communities is currently unpredictable because interactions among species are extremely complex and poorly understood (Pimm 2009).

Of particular concern is our inability to incorporate complex biological factors in our models predicting parasites' ability to shift distributions, hosts, and to increase in virulence with the progression of the climate change. This concern is rooted in the exposure of immunologically *naïve* potential hosts to novel pathogens as a consequence of the changes in the composition of ecological communities, which might have negative effects on biodiversity (Dobson & Foufopoulos 2001). In fact, there are several studies demonstrating the devastating consequences of invasive or emerging parasites on animal and plant populations (*e.g.* Daszak 2000; Anderson *et al.* 2004; Smith *et al.* 2009). The global climate change is expected to especially favor the pathogens employing arthropod vectors for transmission. A number of studies have confirmed the importance of climate as a limiting factor in the distribution of many insect and tick vectors (Kovats *et al.* 2001), therefore, changes in climatic patterns and in seasonal conditions may affect disease behavior in terms of spread, survival, transmission rate, and persistence in novel habitats (Patz & Reisen 2001; Harvell *et al.* 2002; de la Roque *et al.* 2008; Dukes *et al.* 2009).

2.1 The Mediterranean Basin as a study area

The Mediterranean Basin is one of the world's greatest centers of biodiversity (Dernegi 2010) and it is considered a hotspot for conservation priorities (Myers *et al.* 2000). Climate model projections suggest that it might be an especially vulnerable region to global change (Giorgi & Lionello 2008) with a high risk of endemic species extinction (Malcolm *et al.* 2006). If climate warming allows vector-transmitted pathogens to spread from the tropics into higher latitudes, Mediterranean countries will probably be the first to feel the impact. Furthermore, when these pathogens are restricted to tropical areas, their impact may be buffered by high levels of species diversity, but with their expansion to northern, less diverse regions, the disease impact may be greater. However, most models of infectious diseases are focused on human pathogen systems, where a single pathogen infects a single host species. They seldom consider multiple host systems infected with multiple pathogens (Dobson 2009). This is probably due to our poor knowledge of the parasite dynamics at the community level (Ricklefs *et al.* 2005).

The first important step for modeling or predicting a pathogen distribution in a climate change scenario is, therefore, to understand the evolution and dynamics of host-parasite communities. This is best accomplished by understanding their structure, as well as the geographic scales of the interactions, by analyzing the genetic structure of parasites and their hosts (Thompson 2005). In theory, coevolution between hosts and their parasites is influenced by the relative rates of gene flow among the parasite and host populations (Lively 1999; Gandon & Michalakis 2002). According to these theoretical models, parasites are more likely to adapt to their local host population if the migration rate of the parasites is higher than that of their hosts.

The Iberian Peninsula and Maghreb are located at the southwestern edge of the Palearctic, and share similar forest and scrubland bird communities (Covas & Blondel 1998). Although most Iberian species also occur in Maghreb, some Maghreb species are not found in Iberia. This has been attributed to the weak exchange of fauna between the two regions or, in other words, to the apparent constraint of gene flow imposed by the Mediterranean Sea (Garcia *et al.* 2008; Dietzen *et al.* 2008; Valera *et al.* 2011). This lack of gene flow between Iberia and Maghreb also seems to be true for other groups of vertebrates (*e.g.* Carranza *et al.* 2006) and invertebrates (*e.g.* Wahlberg & Saccheri 2007), but not for dipteran insects (Esseghir *et al.* 1997; Porretta *et al.* 2011), which are the vectors of haemosporidian parasites and can facilitate their transmission between the two areas.

Unfortunately, there is a lack of information about host-parasite interactions in these areas. Community-wide relationships among avian blood parasites and their hosts have never been described in forests of the Iberian Peninsula or North Africa using PCR-based methods, which are much more sensitive and informative than the standard microscopy screening.

3. Objectives

This study aims to provide a description of the diversity and prevalence of the forest avian haemosporidian communities of northwest Iberia and northwest Africa, which can be used as baseline data for future analysis of the impact of environmental changes on the dynamics of these communities and their host-parasite interactions. More specifically, the goals of this study are to:

1. ***describe the diversity of haemosporidians in northwest Africa and northwest Iberia***. Due to inverse relationship between latitude and prevalence and diversity of haemosporidian parasites (Merino *et al.* 2008), we expect that Morocco will have more haemosporidian lineages and higher proportion of infected birds than in Portugal.
2. ***characterize host-specificity in both parasite communities***. Existing data suggests that *Plasmodium* lineages are often generalists in their host preferences whereas *Leucocytozoon* and, especially, *Haemoproteus* are usually more host-specialized (Ricklefs & Fallon 2002; Waldenström *et al.* 2002; Beadell *et al.* 2004; Hellgren *et al.* 2008; Dimitrov *et al.* 2010; la Puente *et al.* 2011). If this is a general pattern, we expect that in both of our study areas the number of host taxa parasitized by a *Plasmodium* lineage will be correlated with the frequency of this lineage detection in our samples, but no such correlation is expected for *Leucocytozoon* or *Haemoproteus*.
3. ***compare haemosporidian community structure between the two areas***. Existing data suggest a great degree of spatial and temporal variation in prevalence of individual haemosporidian lineages found in a single of a few closely related avian species (Reullier *et al.* 2006; Bensch *et al.* 2007; Durrant *et al.* 2008). Much less is known about the entire parasite community spatial and temporal dynamics. This is why we combined data from multiple years, localities, and host taxa in this study. If the parasite communities of Moroccan and Portuguese forest birds are similar, we expect similar proportions of infections caused by different

haemosporidian genera and similar prevalence of individual lineages within parasite genera.

These aims will be pursued using PCR screening of blood parasites, using a novel approach with several primers, to better resolve multiple infections and increase the sensitivity of the screening.

Chapter 2

Methods

1. Study area and sampling procedure

A total of 459 breeding or resident birds of 36 species were sampled in Portugal, and 324 birds of 46 species were sampled in Morocco in 2009-2011 (Figure 3). In both areas, sites were selected to include forest habitats. Birds were captured using mist-nets. Each bird was ringed, measured, weighed, and when possible, aged and sexed.

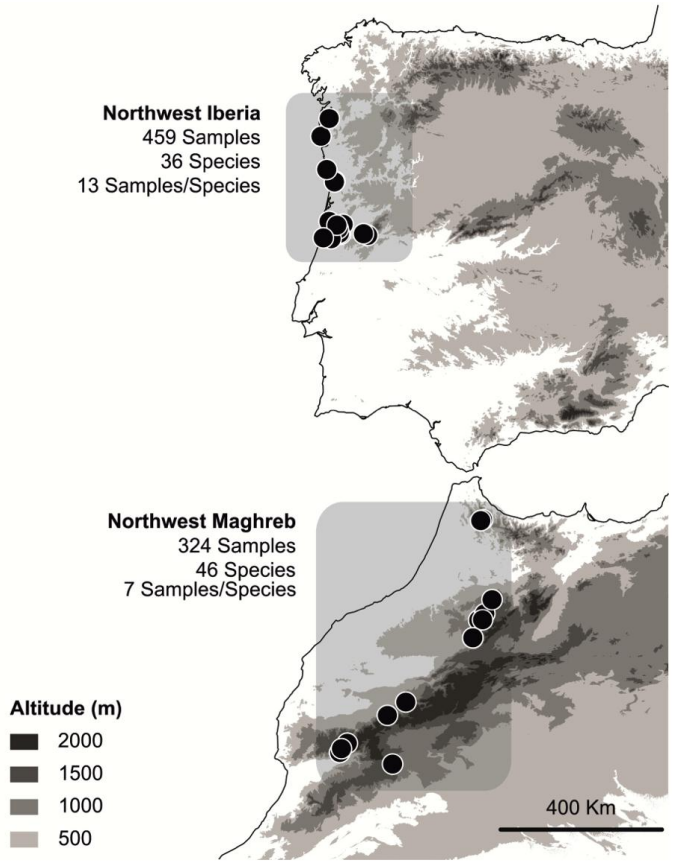

Figure 3 Study areas and sampling locations in the West Mediterranean. Each location comprises a different number of samples.

From each bird, a blood sample was obtained by brachial venipuncture with a sterile needle. Blood was collected into a heparin-free capillary tube and immediately transferred into a vial with 96% ethanol. Samples were kept at room temperature until DNA extraction. Nomenclature of host species followed The Howard & Moore Complete Checklist of the Birds (Dickinson 2003).

2. Parasite screening

Total DNA was extracted from avian blood samples using the JETQUICK Tissue DNA Spin Kit (Genomed) according to the manufacturer's protocol.

For parasite detection new primers were designed by Sergei Drovetski, using all the sequences available in GenBank for avian malaria (*Plasmodium, Haemoproteus* and *Leucocytozoon*) mtDNA cyt-*b* gene which covered the 479 bp fragment of MalAvi's database (the database of avian haemosporidian cyt-b sequences; Bensch *et al.* 2009). A total of three primer pairs were designed by modifying previously published primers (Bensch *et al.* 2009) in order to amplify as many known haplotypes as possible. These pairs share the same forward primer but have different reverse primers (Figure 4).

Each sample was screened two times with each primer pair and was considered parasite free when negative for all 6 PCR runs. PCRs were run in 12.5µl volumes that contained 1x GoTaq Flexi buffer, 2mM $MgCl_2$, 0.2mM of each dNTP, 0.3mM of each primer, and 0.313u of GoTaq Flexi DNA polymerase (Promega), and 2µl of DNA extract. The thermal profile for amplification with the different primer pairs was the same and started with 3 min of denaturation at 94^o C, followed by 41 cycles at 94^o C for 30 s., 52^o C for 30 s., and 72^o C for 45 s., ending with an elongation step at 72^o C for 10 min. All reactions were accompanied by negative and positive controls to control for contamination and PCR success.

PCR products were purified using ExoSAP according to the manufacturer's instructions (United States Biochemical Corporation, Cleveland, Ohio) and sequenced directly on the Applied Biosystems 3730xl DNA Analyzer at Macrogen's sequencing facility (Macrogen Inc., Netherlands). PCR fragments were sequenced in both directions when positive for UNIVF-UNIVR1 primer pair and only with UNIVR2 and UNIVR3 for their respective pairs to ensure complete coverage of the 505 bp region between primers UNIVF and UNIVR1.

Sequences were aligned using Sequencher 5.0.1 (Gene Codes, Ann Arbor, Michigan) and trimmed to 505bp – the sequence length between primers UNIVF and UNIVR1 covered by all three primer pairs.

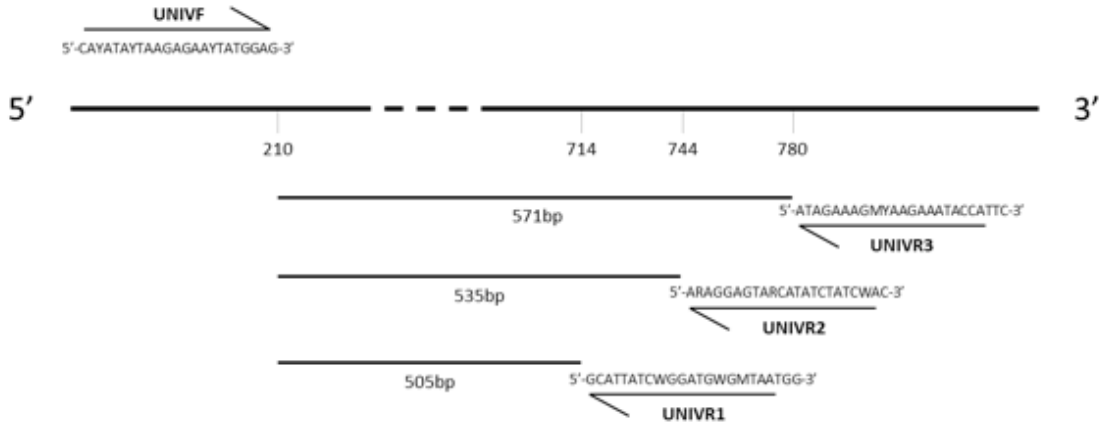

Figure 4 Schematic illustration of the direction and position of each primer in the haemosporidian mtDNA cytochrome-*b* gene. M, R, W and Y stand for nucleotide combinations of A/C, A/G, A/T, and C/T, respectively.

Multiple infections present in a single PCR fragment were resolved employing several approaches. If the other primer pair(s) produced an unambiguous sequence identical to the one present in the multiple-infection PCR fragment (MIF) of the same sample, the unambiguous sequence was subtracted to reveal the remaining sequence. In some cases, the height of the peaks in MIF's chromatogram was consistently and significantly different along the entire sequence length that also allowed us to resolve multiple infections. If the peaks were the same height and there were no unambiguous sequences available for a particular sample with the MIF, we aligned the MIF with all unambiguous sequences and eliminated, one by one, sequences that had differences with the MIF in unambiguous sites (positions that did not contain double-peaks). After this consecutive elimination, we were left with fragments whose consensus produced the same pattern of double peaks as we observed in the MIF. Therefore, we considered the MIF as being composed of these lineages. In all but two samples with MIFs we were able to resolve all infections using combination of these approaches, including a few cases when a primer pair amplified three different haplotypes. All the sequences were checked for codon structure and in all cases no stop codons were found. All new haplotypes found only in multiple infections were double-checked in order to assure that they were indeed new lineages and not misreads of the chromatograms.

Unique haplotypes were identified from the individual sequences in DnaSP 5.10.00 (Librado & Rozas 2009) and compared with GenBank sequences and MalAvi database (Bensch *et al.* 2009) in order to identify known parasite lineages, morphospecies, and lineage distribution. Additionally, we also compared our data to that from an unpublished survey of haemosporidian parasites in the Caucasus that used the same primers (Drovetski and Aghayan, unpublished data) for a better understanding of lineage distributions.

3. Phylogenetic analysis

Phylogenetic relationships among parasite lineages were estimated using bayesian and maximum likelihood (ML) methods. We used a GTR+G+I model of DNA substitution, that was selected using AIC in jModelTest (Posada 2008). For Bayesian inference we used BEAST v1.7.4 (Drummond *et al.* 2012) with a strict molecular clock and Yule speciation priors. Two independent runs of 10 million generations were conducted, with trees being sampled every 1000 generations. Tracer (Drummond *et al.* 2012) was used to assess convergence, which was visually determined by examination of the plots and estimates of effective sample size (ESS>200 indicated that the run had converged). The initial 1000 trees of each run were deleted as burn-in, and the remaining 18000 trees were combined and used to calculate the maximum credibility tree. We also evaluated the lognormal relaxed clock model, but it produced the same tree topology and several model parameters failed to converge. For ML inference we used MEGA5 (Tamura *et al.* 2011) and 1000 bootstrap replicates, using the same model of DNA substitution. For the tree branches that were congruent with the bayesian results, we added the ML bootstrap support value to the maximum credibility tree.

4. Statistical analysis

Relative diversity of parasites was calculated for each genus in both areas as the number of lineages found belonging to a certain genus, divided by the total number of lineages found in that area. Prevalence of infection was calculated as the number of infected individuals, divided by the total number of birds. G-test was used to test for differences in relative diversity and parasite prevalence while Fisher's exact test was used to test the differences in the proportion of species, genera, and families infected. Correlations between variables were tested using Pearson test while relations between variables were quantified using linear regressions. All tests and regressions were done using the add-in software XLSTAT 7.5.2 (Addinsoft) for Microsoft Excel.

Chapter 3

Results

1. Parasite diversity and prevalence

A total of 169 different parasite lineages were found in this study, of which less than a half (74) are already known from earlier studies and are deposited to MalAvi and/or GenBank. However, because our cyt-*b* fragment is 26 bp longer than MalAvi fragment, on several occasions two of our different haplotypes matched the same MalAvi sequence.

The parasites grouped into four major clades (Figure 5) that correspond to the genera *Leucocytozoon*, and *Plasmodium, and* sub-genera *Parahaemoproteus* and *Haemoproteus* of the genus *Haemoproteus*.

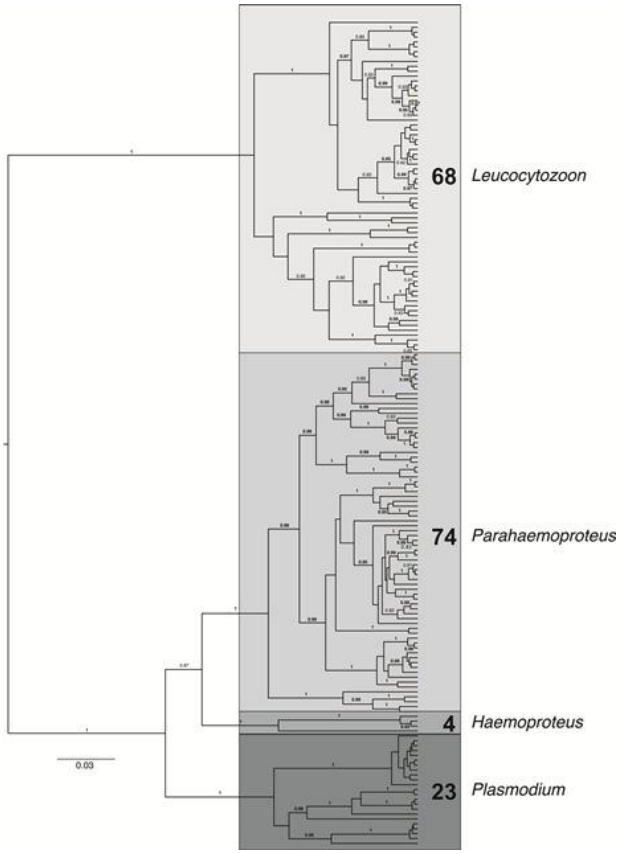

Figure 5 Bayesian tree of all parasite lineages found in this study. The numbers show the number of lineages found in each genus. Scale refers to the number of substitutions per site.

Table 1 The sample size of each avian species with the parasite prevalence, number of parasite sequences retrieved from each avian species with the mean per infected bird, and the number of lineages found in each avian species.

Family	Species (Code)	Sample Size (Prevalence %)		Nº of sequences (Mean±SD)		Nº of lineages	
		NW Africa	NW Iberia	NW Africa	NW Iberia	NW Africa	NW Iberia
Accipitridae	*Accipiter nisus* (Anis)	1 (0.0)	1 (0.0)	---	---	---	---
Aegithalidae	*Aegithalos caudatus* (Acau)	---	15 (0.0)	---	---	---	---
Alaudidae	*Galerida cristata* (Gcri)	1 (100.0)	---	1 (1.0±0.0)	---	1	---
Certhiidae	*Certhia brachydactyla* (Cbrac)	9 (22.2)	16 (18.8)	2 (1.0±0.0)	4 (1.3±0.5)	2	4
Cettiidae	*Cettia cetti* (Ccet)	---	6 (66.7)	---	4 (1.0±0.0)	---	1
Columbidae	*Columba palumbus* (Cpal)	1 (100.0)	2 (100.0)	1 (1.0±0.0)	4 (2.0±1.0)	1	3
	Streptopelia decaoto (Sdec)	---	1 (0.0)	---	---	---	---
	Streptopelia turtur (Stur)	4 (100.0)	2 (50.0)	5 (1.3±0.4)	1 (1.0±0.0)	3	1
Corvidae	*Garrulus glandarius* (Ggla)	3 (100.0)	6 (83.3)	3 (1.0±0.0)	10 (2.0±0.6)	1	5
Emberizidae	*Emberiza cirlus* (Ecir)	4 (100.0)	2 (100.0)	9 (2.3±0.4)	4 (2.0±1.0)	4	4
Falconidae	*Falco tinnunculus* (Ftin)	---	1 (0.0)	---	---	---	---
Fringillidae	*Carduelis carduelis* (Ccar)	5 (20.0)	15 (20.0)	1 (1.0±0.0)	3 (1.0±0.0)	1	3
	Carduelis chloris (Cchl)	8 (75.0)	25 (36.0)	7 (1.2±0.4)	13 (1.4±0.5)	5	5
	Cocothraustes cocothraustes (Ccoc)	6 (100.0)	---	13 (2.0±0.8)	---	7	---
	Fringilla coelebs (Fcoe)	75 (96.0)	7 (57.1)	131 (1.8±0.9)	6 (1.5±0.5)	28	6
	Serinus serinus (Sser)	11 (63.6)	22 (50.0)	9 (1.3±0.5)	12 (1.1±0.3)	7	5
Laniidae	*Lanius senator* (Lsen)	2 (0.0)	---	---	---	---	---
Muscicapidae	*Cercotrichas galactotes* (Cgal)	1 (100.0)	---	1 (1.0±0.0)	---	1	---
	Ficedula hypoleuca (Fhyp)	2 (50.0)	---	1 (1.0±0.0)	---	1	---
	Muscicapa striata (Mstr)	9 (100.0)	---	17 (1.9±0.7)	---	8	---
	Oenanthe deserti (Odes)	1 (100.0)	---	1 (1.0±0.0)	---	1	---
	Oenanthe leucura (Oleu)	1 (100.0)	---	1 (1.0±0.0)	---	1	---
	Oenanthe seebohmi (Osee)	1 (100.0)	---	1 (1.0±0.0)	---	1	---
	Phoenicurus moussieri (Pmou)	8 (87.5)	---	8 (1.1±0.3)	---	4	---
	Phoenicurus ochruros (Poch)	---	12 (16.7)	---	2 (1.0±0.0)	---	1
Paridae	*Parus ater* (Pate)	21 (90.5)	16 (50.0)	28 (1.6±0.6)	9 (1.3±0.7)	8	4
	Parus caeruleus (Pcae)	21 (90.5)	14 (71.4)	43 (2.3±1.2)	18 (1.7±0.6)	15	10
	Parus cristatus (Pcri)	---	4 (50.0)	---	2 (1.0±0.0)	---	2
	Parus major (Pmaj)	18 (94.4)	29 (72.4)	51 (2.9±1.3)	45 (2.1±0.8)	13	11
Passeridae	*Passer domesticus* (Pdom)	8 (87.5)	31 (19.4)	9 (1.3±0.5)	6 (1.0±0.0)	5	2
	Passer hispaniolensis (Phis)	7 (71.4)	---	7 (1.4±0.5)	---	5	---
	Passer montanus (Pmon)	---	6 (0.0)	---	---	---	---
Picidae	*Dendrocopos major* (Dmaj)	4 (0.0)	5 (0.0)	---	---	---	---
	Picus viridis (Pvir)	2 (50.0)	1 (0.0)	1 (1.0±0.0)	---	1	---
Pycnonotidae	*Pycnonotus barbatus* (Pbar)	3 (100.0)	---	3 (1.0±0.0)	---	1	---
Regulidae	*Regulus ignicapillus* (Rign)	4 (0.0)	17 (0.0)	---	---	---	---
Sittidae	*Sitta europaea* (Seur)	3 (33.3)	4 (0.0)	1 (1.0±0.0)	---	1	---
Strigidae	*Athene noctua* (Anoc)	1 (100.0)	1 (100.0)	2 (2.0±0.0)	2 (2.0±0.0)	2	2
	Otus scops (Osco)	2 (100.0)	---	2 (1.0±0.0)	-	2	---
	Strix aluco (Salu)	1 (100.0)	1 (100.0)	1 (1.0±0.0)	1 (1.0±0.0)	1	1
Sylviidae	*Hippolais pallida* (Hpal)	2 (100.0)	---	3 (1.5±0.5)	---	3	---
	Hippolais polyglotta (Hpol)	4 (75.0)	12 (16.7)	3 (1.0±0.0)	3 (1.5±0.5)	2	2
	Phylloscopus bonelli (Pbon)	1 (0.0)	---	---	---	---	---
	Sylvia atricapilla (Satr)	3 (100.0)	61 (72.1)	9 (3.0±0.8)	77 (1.7±0.8)	6	15
	Sylvia borin (Sbor)	1 (100.0)	---	1 (1.0±0.0)	---	1	---
	Sylvia cantillans (Scan)	6 (50.0)	2 (100.0)	4 (1.3±0.5)	2 (1.0±0.0)	3	1
	Sylvia deserticola (Sdes)	4 (25.0)	---	3 (3.0±0.0)	---	3	---
	Sylvia hortensis (Shor)	2 (50.0)	---	1 (1.0±0.0)	---	1	---
	Sylvia melanocephala (Smel)	21 (76.2)	8 (25.0)	23 (1.4±0.6)	2 (1.0±0.0)	9	1
Troglodytidae	*Troglodytes troglodytes* (Ttro)	6 (50.0)	20 (20.0)	3 (1.0±0.0)	4 (1.0±0.0)	2	3
Turdidae	*Erithacus rubecula* (Erub)	9 (11.1)	51 (23.5)	1 (1.0±0.0)	17 (1.4±0.6)	1	8
	Turdus merula (Tmer)	13 (100.0)	40 (97.5)	22 (1.7±0.6)	52 (1.3±0.6)	8	8
	Turdus philomelos (Tphi)	---	2 (100.0)	---	4 (2.0±0.0)	---	4
	Turdus viscivorus (Tvis)	4 (100.0)	---	6 (1.5±0.5)	---	5	---
Upupidae	*Upupa epops* (Uepo)	---	1 (100.0)	---	1 (1.0±0.0)	---	1
Total		324 (78.7)	459 (44.2)	439 (1.7±0.9)	308 (1.5±0.7)	127	74

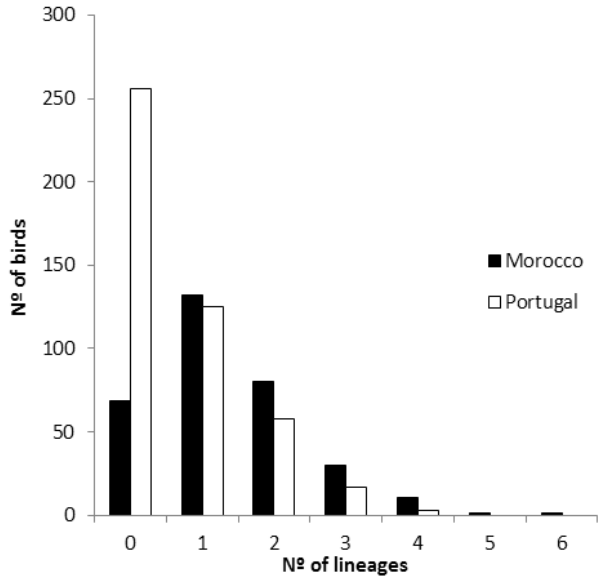

Figure 6 Distribution of the cumulative number of parasite lineages discriminated by number of birds and study area.

In Northwest Africa, we found 127 unique lineages among 439 parasite mtDNA cyt-*b* sequences from 324 birds of 46 avian species. In Northwest Iberia, we found 74 lineages among 308 sequences from 459 birds of 36 avian species. The majority of the infections in Morocco were found in the common chaffinch (*Fringilla coelebs*), great tit (*Parus major*), and blue tit (*Parus caeruleus*); and in Portugal most of them were found in the blackcap (*Sylvia atricapilla*), blackbird (*Turdus merula*), and great tit (Table 1). Despite the difference in the number of parasite lineages found in Morocco and Portugal, we found no differences in the relative diversity of each genus between the two areas ($G = 0.484$, $df = 2$, $p = 0.785$).

Although in most avian species with sample sizes > 2 we detected haemosporidian parasites, there were several exceptions worth notice. We failed to detect haemosporidian pathogens in the Eurasian tree sparrow (*Passer montanus*; n = 6 all from Portugal), greater spotted woodpecker (*Dendrocopos major*; Morocco n = 4, Portugal n = 5), long-tailed tit (*Aegithalos caudatus*; Portugal n = 15), and firecrest (*Regulus ignicapillus*; Morocco n = 4, Portugal = 17). Our failure to detect parasites in these species suggests either that these species have unusually low prevalence of haemosporidian parasites or our primers failed to amplify lineages infecting these species.

Overall parasite prevalence was higher in NW Africa with 79% of the birds being infected by haemosporidians, while in Iberia only 44% of the tested birds carried infections. Multiple infections affected 40% and 16% of birds in Morocco and Portugal, respectively. The number of parasite lineages per bird with multiple infections varied from two to six (Figure 6), with most birds carrying only two.

Table 2 Distribution of the different combinations of infection types, discriminated by area and genera: *Plasmodium* (P), *Haemoproteus* (H) and *Leucocytozoon* (L).

Lineages (N)	Type	NW Africa	NW Iberia
0	-	69	256
1	1H	70	34
	1L	29	24
	1P	33	67
2	1H1L	10	6
	1H1P	14	4
	2H	22	16
	1L1P	17	12
	2L	14	11
	2P	3	9
3	1H1L1P	2	0
	1H2L	4	0
	1H2P	0	1
	2H1L	2	3
	2H1P	4	1
	3H	2	2
	1L2P	0	3
	2L1P	13	7
	3L	3	0
4	1H1L2P	0	1
	2H1L1P	2	0
	2H2L	1	2
	3H1L	1	0
	3H1P	1	0
	3L1P	6	0
5	3L2P	1	0
6	5L1P	1	0

The most common combinations of parasites were double infections of *Haemoproteus* (2H: n = 22 Morocco, n = 16 Portugal), and of *Leucocytozoon* with *Plasmodium* (1L1P: n = 17 Morocco, n = 12 Portugal). Double infections of *Leucocytozoon* were also fairly common (2L: n = 15 Morocco, n = 11 Portugal) (Table 2). Double infections of *Plasmodium* lineages were much less frequent. Double *Plasmodium* infections were found in 3 Moroccan and in 9 Portuguese birds, in addition, there were 6 cases of mixed double infections of *Plasmodium* with *Haemoproteus* and *Leucocytozoon infection(s)* (one 1H2P, three 1L2P, one 1H1L2P, and one 3L2P). The reason is that although *Plasmodium* infections were common, the number of *Plasmodium* lineages was much lower than the number of lineages in other genera. Therefore, the probability of a bird infected with a *Plasmodium* haplotype to get a second *Plasmodium* infection is much lower than the probability to get an infection from a different parasite genus, which are much more diverse.

The number of parasite sequences retrieved per host species increased with the host sample size in both study areas (Figure 7a). However, the slope of this relationship was significantly higher in Morocco (1.862) than in Portugal (1.103, slope difference $p < 0.0001$) confirming that the prevalence of haemosporidian parasites in

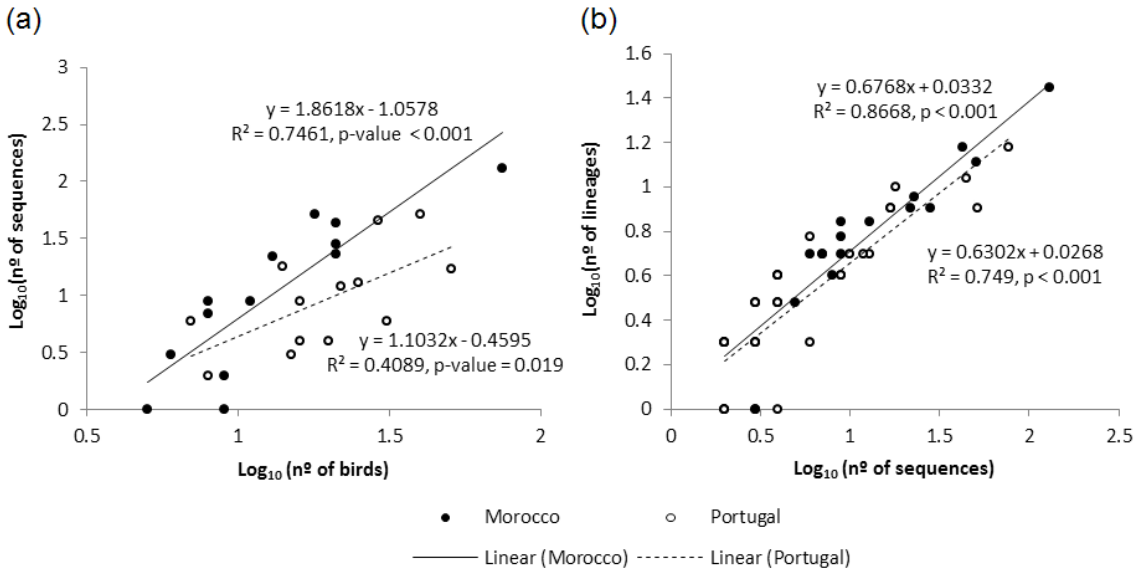

Figure 7 The relationship between the: (a) number of parasites found per host species and the number of sampled birds (Only infected avian species with n ≥ 5 in both areas were used in this regression); (b) the number of lineages found per host species and to the number of parasite sequences retrieved from it (Only species with n ≥ 2 were used in this regression).

Morocco was higher than in Portugal. The number of parasite lineages found in a host species increased with the number of parasite sequences found in that host (Figure 7b). The slopes of this relationship did not differ between Morocco (0.677) and Portugal (0.630, slope difference p = 0.295). This suggests that the parasite lineages were discovered at a similar rate in both areas and the higher prevalence of parasites in Morocco is responsible for the higher haemosporidian lineage diversity found in that area compared to Portugal.

2. Parasite specificity

In our study, the number of hosts from which a parasite lineage was recovered varied between one and sixteen in a single area. The proportion of species, genera, and families infected by each parasite genus did not differ between North Africa and Iberia (Table 3).

The relationship between the number of avian species parasitized by a haemosporidian lineage and the number of parasite lineage observations did not follow the same pattern among the three parasite genera, but did not differ significantly between our study areas (Figure 8). For *Haemoproteus* lineages, an increase in the number of recoveries was not associated with an increase in number of host species, genera, or families. This suggests that *Haemoproteus* lineages are fairly host-specific, and infect only a few avian species and rarely from different families.

Table 3 Number of host species, genera and family, infected in both study areas, and probability values (p) from Fisher's exact test.

	Species			Genera			Families		
	Infected	Not-infected	p	Infected	Not-infected	p	Infected	Not-infected	p
Haemoproteus									
NW Africa	29	17	0.119	22	11	0.295	13	6	0.325
NW Iberia	16	20		14	13		9	10	
Leucocytozoon									
NW Africa	19	27	0.505	14	19	0.999	10	9	0.999
NW Iberia	18	18		12	15		10	9	
Plasmodium									
W Africa	26	20	0.999	17	16	0.609	11	8	0.999
NW Iberia	21	15		16	11		12	7	
Total									
NW Africa	41	5	0.138	28	5	0.345	16	3	0.693
NW Iberia	27	9		20	7		14	5	

For *Leucocytozoon* parasites, there was a positive correlation between number of recoveries and number of host-species. However, at the host-genus and family levels, the correlation was weak. Therefore, *Leucocytozoon* is less specific at the host species level than *Haemoproteus*, but it is specific at the host genus and family levels. Nevertheless, some generalist lineages were observed in both parasite genera, especially in *Leucocytozoon*. Therefore, there is a considerable degree of heterogeneity in the host specificity of some lineages within both genera.

Plasmodium parasites had a strong positive correlation between the numbers of lineage recoveries and avian host species parasitized, with abundant lineages infecting a larger number of host-species, genera and families. This was expected, as *Plasmodium* is known to be more host-generalist then other haemosporidian genera.

3. Parasite community structure

The prevalence of each parasite genus differed between both study areas ($G = 13.920$, $df = 2$, $p = 0.001$). *Plasmodium* was predominant in Portugal, and *Haemoproteus* was the most common among infections in Morocco (Figure 9). The most common lineage in both areas was SGS1 (n = 50 in NW Africa, n = 60 in NW Iberia), which is a cosmopolitan and abundant *Plasmodium relictum* strain. It was found in 24 different species in our study. This was one of the cases when two of our haplotypes matched a single sequence in the MalAvi database (H3 n = 109, H55 n = 1). The second most common lineage was H102 matching SYAT05 *Plasmodium vaughani.* It parasitized five avian species and was found in both study areas (Morocco n = 11, Portugal n = 35). The dominance of *Plasmodium* parasites among individual lineages was not surprising given the lack of the host specificity and low diversity of lineages in this genus.

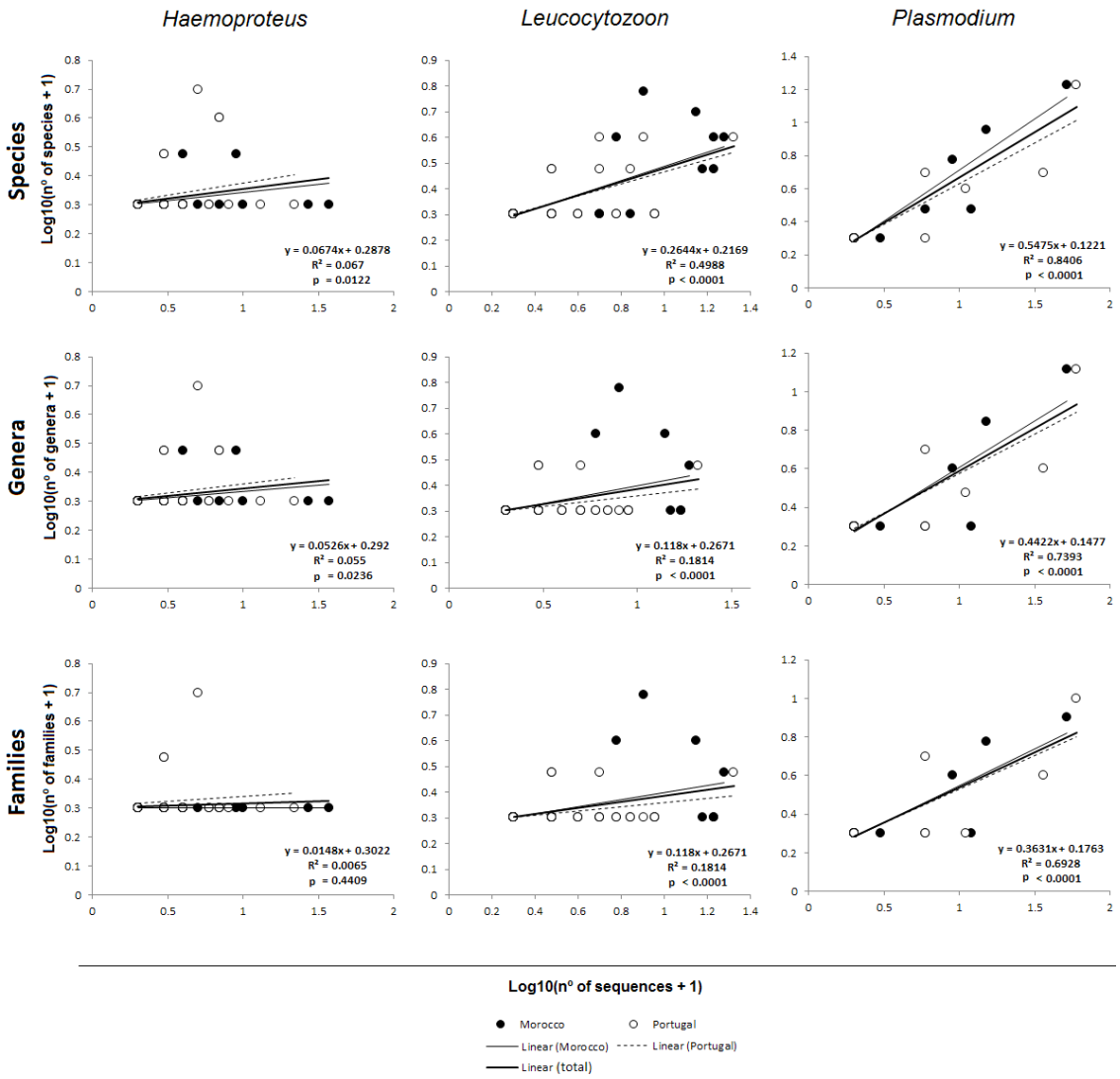

Figure 8 Relationship between the number of species, genera and families infected by a parasite, and the number of times it was found.

Of the 169 haplotypes found in this study, 95 were only observed in North Africa, 42 only in Iberia, and 32 were observed in both areas (shared lineages). However, when the information on the distribution of lineages from previously published studies is taken into account, the number of shared lineages approached the number of unshared ones. When previously published data for Iberia is used, the number of shared lineages increases to 40, but when data for the whole Europe is considered, the number of shared lineages increases to 61, leaving Morocco with only 66 unique haplotypes.

This means that, approximately one third of the parasite lineages we found only in Morocco, have also been found in Europe, and could therefore potentially occur in Iberia as well. We would need to sample many more birds in Iberia than in Morocco to obtain the same number of infections and, consequently, diversity of parasites lineages.

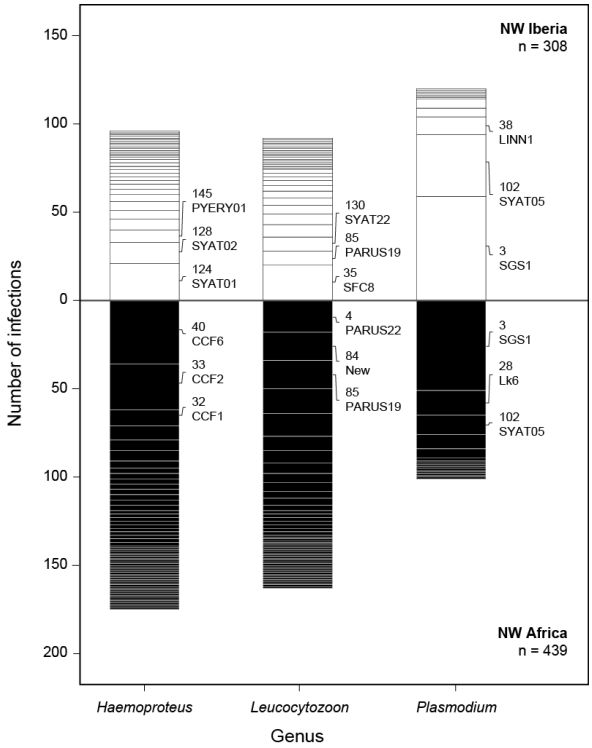

Figure 9 Distribution of parasite infections among the three malaria genera in both areas. Each division in the stacked columns corresponds to a different lineage. The three most abundant lineages are labeled for each genus in each study area.

Although infections by shared lineages (according to our data) accounted for 63% of all parasite observations, there was no correlation between number of lineage observations in Iberia and Morocco for any parasite genus (Figure 10). The lack of this correlation, which would be expected if the parasite communities were similar, results from the presence of lineages observed only in one study area, from differences in prevalence of lineages found in both study areas, and from differences in host community composition.

The regressions remained non-significant even when only shared haplotypes were used in analyses (*Haemoproteus*: y = -0.1636x + 0.7063, R^2 = 0.0508, p = 0.419;

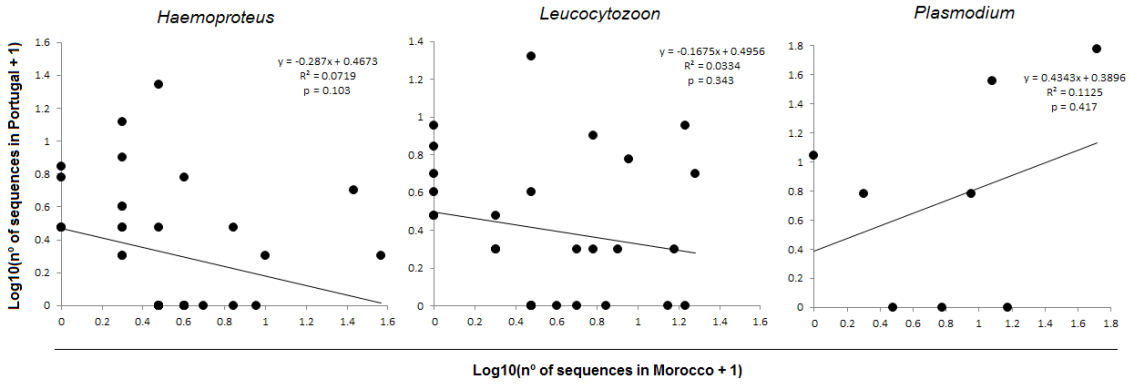

Figure 10 Relationship between parasite recoveries in Portugal and Morocco. Only lineages with n ≥ 2 were used.

Leucocytozoon: y = 0.1487x + 0.4697, R^2 = 0.0253, p = 0.604; *Plasmodium*: y = 0.7485x + 0.4648, R^2 = 0.6935, p = 0.167). Therefore, parasite communities in our study areas differ not only at the level of relative frequencies of different parasite genera, but at the level of individual parasite lineages as well.

We found little spatial structure in the phylogeny of the parasites (Figure 11, Figure 12, and Figure 13, respectively for *Haemoproteus* and *Plasmodium*, *Parahaemoproteus*, and *Leucocytozoon*). Although some clades appear to occur only in Morocco according to our data, some lineages in those clades have been previously described in Iberia and/or in Europe. Nevertheless, we found one *Haemoproteus* clade recovered from spotted flycatcher (Muscicapa striata) in Morocco, which did not contain lineages that have been found elsewhere (haplotypes 81, 76 and 80). Another *Haemoproteus* clade (haplotypes 63, 9 and 15) was recovered from fringillids in Portugal.

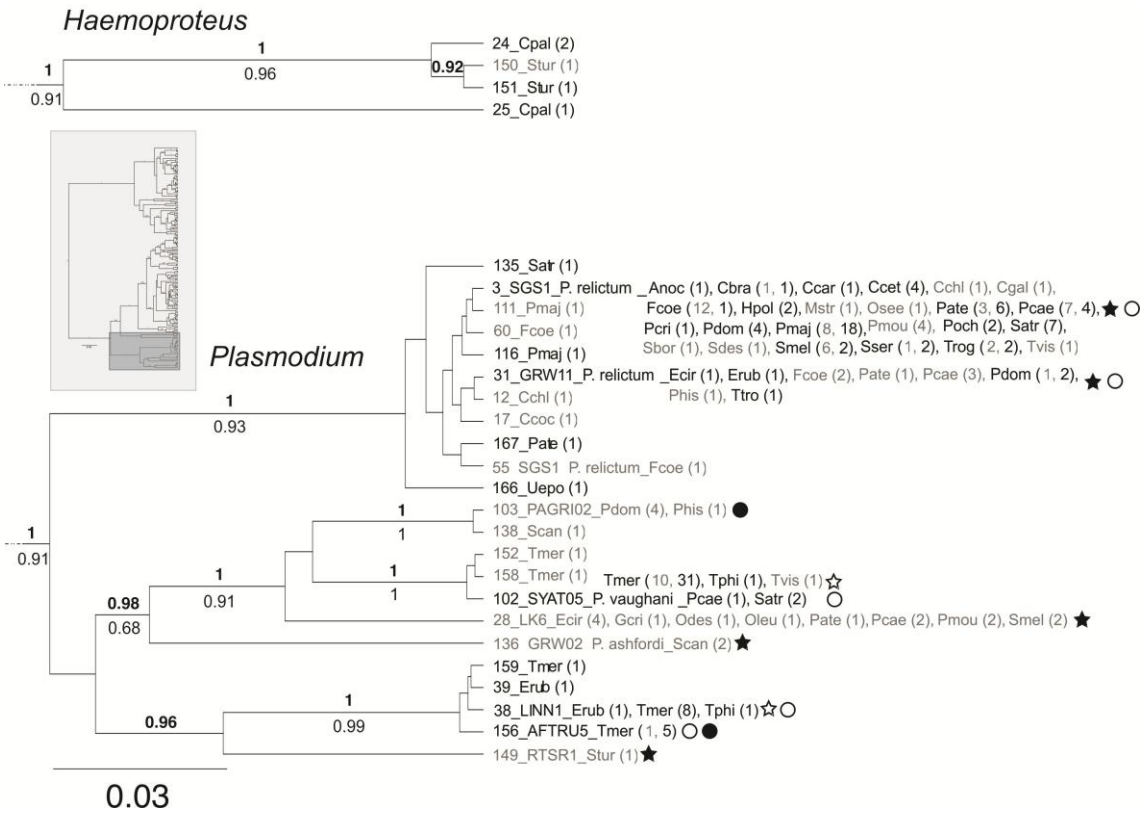

Figure 11 Sub-trees of *Haemoproteus* and *Plasmodium* parasites. Values above branches indicate bayesian posterior probabilities (only values ≥0.85 are shown), and below ML bootstrap support (only values ≥0.5 are shown). Tips labels consist in the number of the haplotype, MalAvi's name and the parasite's morphospecies (whenever available), and the host-species (abbreviated as in Table 1) with number of individuals in which the lineage was found in parentheses. Grey, black and bold labels represent haplotypes found in NW Africa, NW Iberia, and in both areas, respectively. Symbols represent the closest areas of occurrence in other studies: black-filled starts - Iberia, white-filled stars - Europe, white-filled circles - Caucasus, and black-filled circles – continents other than Europe. Scale refers to the number of substitutions per site.

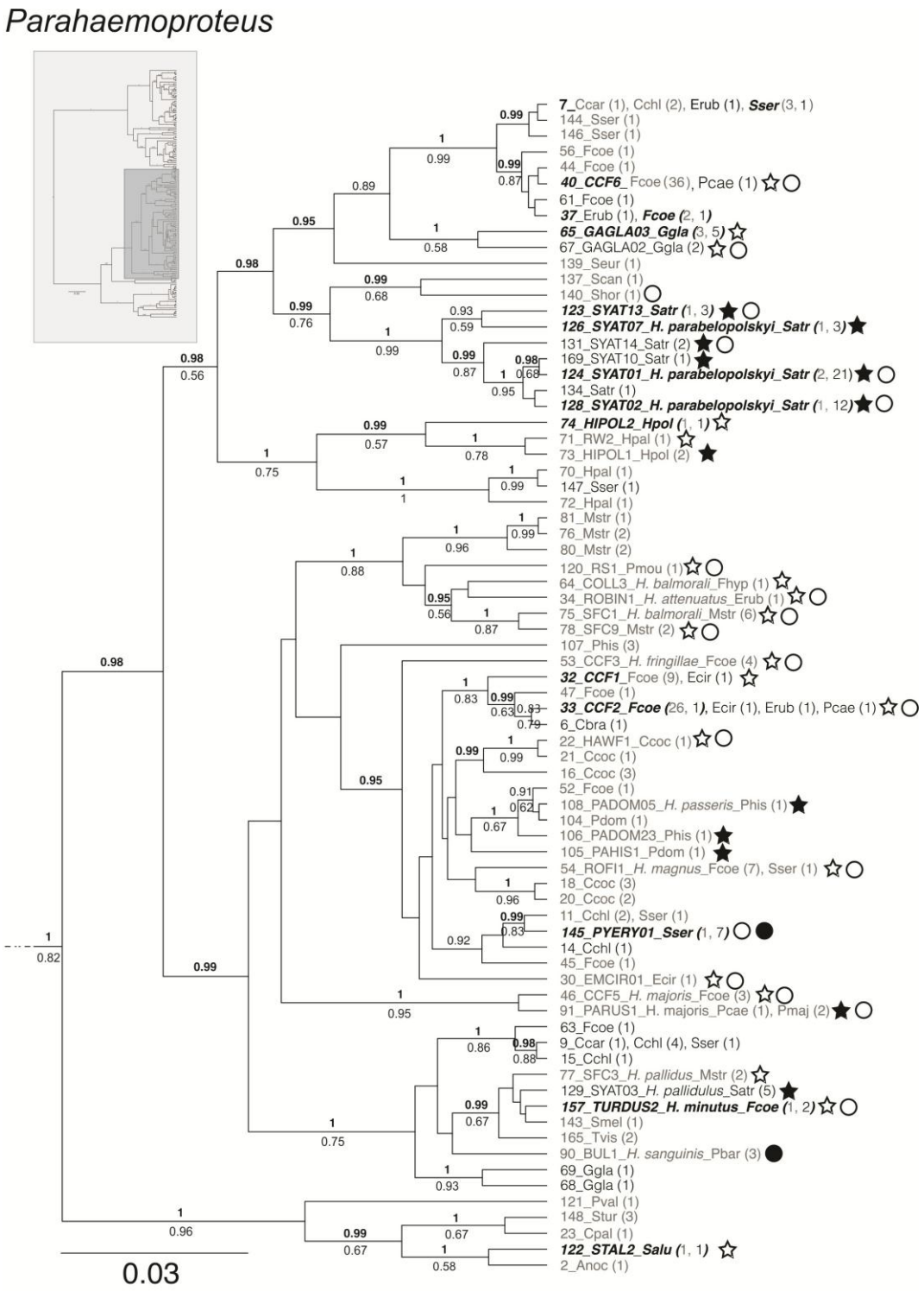

Figure 12 Sub-tree of *Parahaemoproteus* parasites. Values above branches indicate bayesian posterior probabilities (only values ≥0.85 are shown), and below ML bootstrap support (only values ≥0.5 are shown). Tips labels consist in the number of the haplotype, MalAvi's name and the parasite's morphospecies (whenever available), and the host-species (abbreviated as in Table 1) with number of individuals in which the lineage was found in parentheses. Grey, black and bold labels represent haplotypes found in NW Africa, NW Iberia, and in both areas, respectively. Symbols represent the closest areas of occurrence in other studies: black-filled starts - Iberia, white-filled stars - Europe, white-filled circles - Caucasus, and black-filled circles – continents other than Europe. Scale refers to the number of substitutions per site.

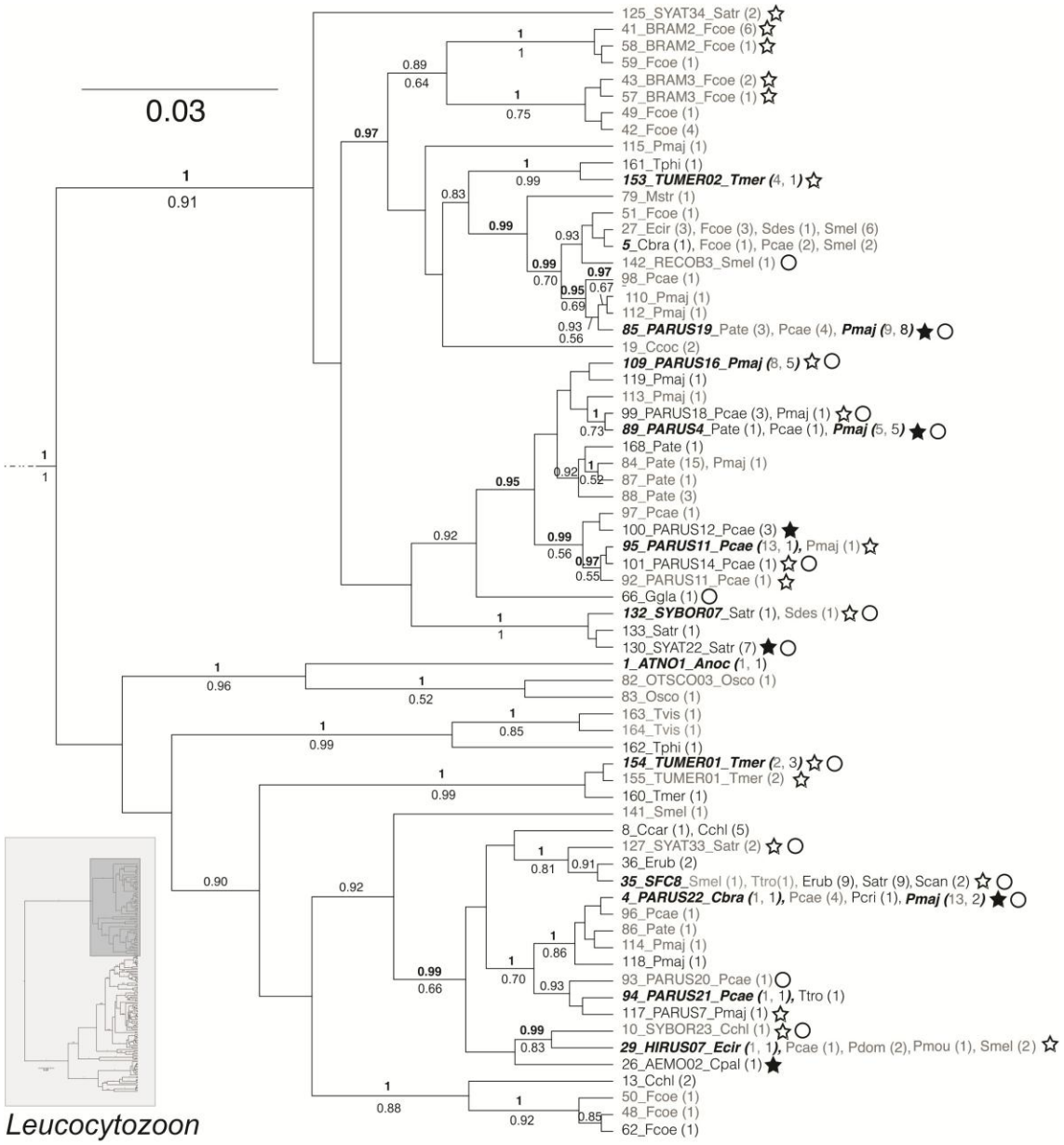

Figure 13 Sub-tree of *Leucocytozoon* parasites. Values above branches indicate bayesian posterior probabilities (only values ≥0.85 are shown), and below ML bootstrap support (only values ≥0.5 are shown). Tips labels consist in the number of the haplotype, MalAvi's name and the parasite's morphospecies (whenever available), and the host-species (abbreviated as in Table 1) with number of individuals in which the lineage was found in parentheses. Grey, black and bold labels represent haplotypes found in NW Africa, NW Iberia, and in both areas, respectively. Symbols represent the closest areas of occurrence in other studies: black-filled starts - Iberia, white-filled stars - Europe, white-filled circles - Caucasus, and black-filled circles – continents other than Europe. Scale refers to the number of substitutions per site.

Chapter 4

Discussion

1. Parasite diversity and prevalence

We presented here the first extensive molecular survey of haemosporidian parasites in forest bird communities of southwestern continental Palearctic. Although other studies have focused on Iberian malaria parasites using molecular methods (*i.e.* Spain: Bensch *et al.* 2004; Marzal *et al.* 2008; Martínez-de la Puente *et al.* 2011 and Casanueva *et al.* 2012; Portugal: Ventim 2011), these have usually focused on a restricted number of species or on long-distance migrants.

We found an overall high number of haemosporidian lineages - 169. More than half of these lineages were recorded for the first time. Such a high diversity of parasites can only be compared, as far as we are aware, to an extensive survey done by Pérez-Tris *et al.* (2007) in which 4513 birds of 47 avian species were sampled from Spain to Sweden and where 137 haplotypes (45 *Plasmodium* and 92 *Haemoproteus*) were found. Nevertheless, the high diversity found in our study, with a much smaller sample size, can potentially be explained by three factors. First, we used new primers that were specifically designed to amplify as much known diversity of haemosporidians as possible and allowed us to resolve multiple infections, greatly increasing our number of parasite observations. Second, compared to most studies in the western Palearctic, we sampled a high number of different species (56 in total), most of which are resident. Finally, the fact that we sampled two different areas geographically divided by the Mediterranean Sea instead of one continuous area might have contributed to the overall parasite diversity we discovered. These factors, plus the scarce knowledge of haemosporidian parasite communities in North Africa, have also contributed to the high proportion of new mtDNA cyt-*b* lineages found in this study.

Among the species for which we sampled more than two individuals, we did not obtain any haemosporidians from only four: the long-tailed tit, great spotted woodpecker, Eurasian tree sparrow, and firecrest. These species have rarely been sampled in the literature, except tree sparrows, that were well sampled in reedbeds of central and south Portugal by Ventim (2011), and for which *Plasmodium* infections of SGS1 were found. The lack of infected individuals of this species in our study cannot be attributed to the inability of our primers to detect this lineage - the SGS1 was the most common lineage found in our study. Perhaps, tree sparrows have a lower prevalence of haemosporidians in forest than in reed bed habitats, or the number of birds we sampled was not sufficient to detect infections. Nevertheless, for the remaining species, most studies reported no haemosporidian infections. Valkiūnas (2005) has described the great spotted woodpecker and long-tailed tit as usually being free from haemosporidians. A recent survey in the Caucasus (Drovestki and Aghayan, unpublished data), has also found no infections in the long-tailed tit (n = 8) and only one infected woodpecker (of 7) with a new *Haemoproteus* lineage. Other studies, regardless whether employing molecular methods or not, also failed to find malaria parasites in long-tailed tits (Peirce 1981; Ishtiaq *et al.* 2010). Interestingly, they are known to frequently carry other blood parasites, *e.g.* trypanosome (Valkiūnas 2005). The only study we found that had information about blood parasites in common firecrests also reported the presence of trypanosoma, but of no haemosporidian infections (Peirce 1981). The fact that these species do not seem to carry any haemosporidians, or at least not frequently, is rather intriguing. Such cases have been described in birds with unique life histories - the swift (*Apus apus*) and cuckoo (*Cuculus canorus*) (Valkiūnas & Iezhova 2001), but usually attention is given to birds that have high diversity of parasites (*i.e.* blackcaps: Pérez-Tris and Bensch 2005; Pérez-Tris *et al.* 2007; Santiago-Alarcon *et al.* 2011; among many others). Perhaps the fact that both species are quite small could make them less attractive for Haemosporidian vectors than large species. However, other causes would be needed to explain the low prevalence of these parasites in the great spotted woodpecker.

As expected, the overall haemosporidian prevalence was higher in North Africa than in Iberia, confirming the inverse latitudinal trend found in other studies (Merino *et al.* 2008). This difference in prevalence was even more striking when the proportion of multiple infections is compared between our study areas. In Morocco, it was more than twice that observed in Portugal. It was also much higher than reported in other studies. Pérez-Tris and Bensch (2005b), for example, reported 20% of infected blackcaps to carry multiple infections, while Marzal *et al.* (2008) reported 22.5% of the infected common house martin (*Delichon urbicum*). In our study, these proportions varied

greatly among species. In blackcaps, 39% of the sampled birds (53% of the infected birds) carried multiple infections, whereas in great tits these proportions were even higher with 64% of the sampled birds (79% of the infected individuals) carrying multiple infections. This overall pattern remained in the parasite genus specific comparisons. Jenkins and Owens (2011) reported only 5% of their blue and great tits to harbor mixed infections of *Leucocytozoon*, whereas we found 34% and 45% of these birds, respectively, to carry more than one *Leucocytozoon* parasite. This suggests that multiple infections affect a much larger number of birds than previously thought. Although difficult to deal with, demanding more time and effort, mixed infections deserve much attention as they seem to be the rule rather than exception. Studies of multiple infections can provide crucial information about host-parasite interactions, and help to better understand the dynamics of intra-host competition, and the evolution of parasite virulence and transmission (Rigaud *et al.* 2010). The reason we had unusually high success in detecting multiple infections in our study is likely related to the generalist nature of the primers we used and to the use of several primer pairs. The use of multiple primer pairs was essential to the successful phasing of haplotypes in multiple infections.

We found a substantially higher number of haemosporidian lineages in North Africa than in Iberia (n = 127, n = 74, respectively). This richness of the Moroccan community seems to be related to the higher overall parasite prevalence. Although we did find a higher number of infections in the Maghreb, the rate of lineage recovery per infection was the same in both areas. The fact that we sampled a higher number of different species in North Africa might have also elevated richness of the sampled parasite community in that area. The presence or absence of certain host species is likely the most important factor influencing the presence of parasite lineages (Ricklefs *et al.* 2004, 2005).

2. Parasite specificity

Parasites from the same haemosporidian genera had a similar degree of host specificity in both our study regions. However, the specificity of the parasites varied among the three haemosporidian genera. *Haemoproteus* lineages were the most host specific at all host taxonomic levels – species, genus, family. *Leucocytozoon* lineages were much less specific to host species than *Haemoproteus*, but were specific to host genera and families. *Plasmodium* lineages were host-generalist at all host taxonomic levels, so the increase in the sample size of lineage strongly correlated with increase in number of host species, genera, and families parasitized by it. This is consistent with

other observations for haemosporidian parasites (Ricklefs and Fallon 2002; Waldenström *et al.* 2002; Beadell *et al.* 2004; Hellgren *et al.* 2008; Dimitrov *et al.* 2010; Martínez-de la Puente *et al.* 2011).

Many of the previously discovered lineages we found in this study infect multiple species in other regions in addition to those we found infected with them in our study areas. However, one lineage, the *Plasmodium sp.* LK6, that until our study was known only from the Lesser Krestel (*Falco naumanni*) in Spain, appears to be able to infect 8 passerine species from 4 families in Morocco. Interestingly, although we sampled 4 of those 8 species in Portugal, we failed to detect LK6 there. Another interesting lineage, *Parahaemoproteus sp.* PYERY01, was known before from a greyheaded-bullfinch (*Pyrrhula erythaca*) from the Hymalaias. In our study it was found in 8 serins (*Serinus serinus*), one from Morocco and 7 from Portugal. This clearly shows that there is still much to learn about haemosporidians and that each lineage may have very different histories and specificity patterns from those we identified so far. The rarity of community level studies and the lack of informations from large portion of the globe are likely result in erraneous conclusions about haemosporidian life history traits.

3. Parasite community structure

Our data suggest that the structure of the parasite communities differed between Portugal and Morocco. Not only the prevalence of each parasite genus differed between these areas, probably reflecting differences in vector abundance and activity, but also individual lineage composition and abundance were different as well. However, we failed to find any clear spatial structure in the phylogenetic relationships of the parasites from both communities. This means that although the communities are structurally different they are not evolving in isolation. Parasites from Morocco can invade Iberia and vice versa.

These findings are not surprising. Both study areas are used by a large number of migratory or partially-migratory species (Cramp 1998). By the time the migrants arrive, most vectors are likely still active, and host-generalist parasites can probably use those birds to jump from one area to the other. However, host-specialists of resident species should not be able to cross between the areas unless either the hosts or the vectors can move across the strait of Gibraltar. A recent study has found a fit between host and parasite phylogenies in *Leucocytozoon* parasites and showed that this pattern was due only to associations between non-migratory hosts and their parasites (Jenkins *et al.* 2012).

The most apparently host-specific parasites (or clades) that we were able to sample in reasonable numbers were family-specific parasites found in fringilids and tits. Of particular interest was a clade of *Leucocytozoon* parasites that occurred only in tits (from haplotype 109 to 92, following their order in the tree, Figure 13). This clade was divided in 3 sub-clades, each infecting mostly blue tits, coal tits, or great tits, respectively. The primarily great tit clade contained less specific lineages that also infected blue tits. However, the coal tit clade did not infect blue tits, and vice versa, but both were able to infect great tits. Coal and blue tits of Europe and North Africa are known to be genetically different, with no gene flow occurring between them (Martens *et al.* 2005; Dietzen *et al.* 2008), while great tits are genetically similar across their European and African range (Kvist *et al.* 2003). This suggests that great tits could function as a bridge for tit *Leucocytozoon* parasites between Iberia and North Africa. However, a better sampling of this group of species and their vectors would be needed in order to understand if the parasites use great tits to cross the strait, the vector, or both.

Chapter 5

Conclusions

This work established the first extensive molecular survey of haemosporidian parasites in forest bird communities of southwestern continental Palearctic. Overall we found a very diverse fauna of haemosporidians with complex relationships with their avian hosts. One thing that became clear with this work is that the world of relationships between haemosporidian parasites and their hosts is of enormous dimensions. Sample size seems to play a crucial role in the observed patterns, and often limits us from attempting to resolve complex interactions. Common host-switches and the lack of any clear spatial structure in the distribution of these parasites, make them a challenging group of organisms to work with.

Future studies should focus on the community-wide analysis of host-parasite interactions rather than on a single or few host species and parasite lineages, as this seems to be the only way to start understanding the general patterns of spatial distribution of avian haemosporidian parasites and their host specificity.

Another urgent challenge is to include multiple infections in the analysis. Currently, most studies simply discard them despite their apparently high frequency in avian populations. Only multiple infections can elucidate the relationships among different members of the parasite community and their joint effects on the host community. Nevertheless, few studies have addressed the effects of multiple infections on birds, and none have tried to understand the interactions among different parasite lineages, particularly whether the presence of one parasite lineage can facilitate or inhibit the development of a second infection.

Temporal variation of the parasite community should also be a priority for further research. Many studies have shown a strong variation in the prevalence of individual parasite lineages throughout the year, so differences in timing of our sampling could be partially responsible for the differences observed in the parasite community structure between our study areas. Standardized sampling of birds should also be a goal in future studies in order to have representative samples of both the bird and the parasite communities.

Finally, the incorporation of data about the distribution and abundance of dipteran vectors, as well as their host specificity and of the haemosporidian parasites they carry (for both the vector and the host), will also help elucidating the complex network of interactions among birds and their insect and haemosporidian parasites. To ignore either the vector or the bird communities, their ecology and their evolution, is to ignore an important part of the equation of haemosporidians ecology and evolution.

References

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P (2004) Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology & Evolution*, **19**, 535–544.

Arriero E, Møller AP (2008) Host ecology and life-history traits associated with blood parasite species richness in birds. *Journal of Evolutionary Biology*, **21**, 1504–1513.

Asghar M, Hasselquist D, Bensch S (2011) Are chronic avian haemosporidian infections costly in wild birds? *Journal of Avian Biology*, **42**, 530–537.

Atkinson CT (1999) Hemosporidiosis. In: *Field Manual of Wildlife Diseases: General Field Procedures and Diseases of Birds* (eds Friend M, Franson JC), pp. 193–200. US Department of the Interior, US Geological Survey, Washington DC.

Atkinson CT, Van Riper III C (1991) Pathogenicity and epizootiology of avian haematozoa: Plasmodium, Leucocytozoon, and Haemoproteus. In: *Bird-parasite interactions: ecology, evolution, and behaviour* (eds Loye JE, Zuk M), p. 406. Oxford University Press, New York.

Atkinson CT, Samuel MD (2010) Avian malaria Plasmodium relictum in native Hawaiian forest birds: epizootiology and demographic impacts on àpapane *Himatione sanguinea*. *Journal of Avian Biology*, **41**, 357–366.

Beadell JS, Gering E, Austin J, Dumbacher JP, Peirce MA, Pratt TK, Atkinson CT, Fleischer RC (2004) Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. *Molecular Ecology*, **13**, 3829–44.

Belo NO, Pinheiro RT, Reis ES, Ricklefs RE, Braga ÉM (2011) Prevalence and Lineage Diversity of Avian Haemosporidians from Three Distinct Cerrado Habitats in Brazil. *PLoS ONE*, **6**, e17654.

Bennett GF, Peirce MA, Ashford RW (1993) Avian Haematozoa: mortality and pathogenicity. *Journal of Natural History*, **27**, 993–1001.

Bensch S, Hellgren O, Pérez-Tris J (2009) MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. *Molecular Ecology Resources*, **9**, 1353–1358.

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**, 1617–21.

Bensch S, Stjernman M, Hasselquist D, Ostman O, Hansson B, Westerdahl H, Pinheiro RT (2000) Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proceedings of the Royal Society of London B: Biological Sciences*, **267**, 1583–9.

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**, 112–22.

Carranza S, Harris DJ, Arnold EN, Batista V, Vega JPG de la (2006) Phylogeography of the lacertid lizard, *Psammodromus algirus*, in Iberia and across the Strait of Gibraltar. *Journal of Biogeography*, **33**, 1279–1288.

Casanueva P, Fernández M, Rojo MÁ, Campos F (2012) High prevalence of haemosporidian parasites infection in southern grey shrike Lanius meridionalis (Laniidae, Aves) from agricultural areas. *Italian Journal of Zoology*, **79**, 315–318.

Combes C (2000) Introduction: Parasites, hosts, questions. In: *Evolutionary Biology of Host-Parasite Relationships: Theory Meets Reality* (eds Poulin R, Morand S, Skorping A), pp. 1–8. Elsevier, Amsterdam.

Covas R, Blondel J (1998) Biogeography and history of the Mediterranean bird fauna. *Ibis*, **140**, 395–407.

Cramp S (Ed.) (1998) *The Complete Birds of the Western Palearctic on CD-ROM*. Oxford University Press.

Daszak P (2000) Emerging Infectious Diseases of Wildlife - Threats to Biodiversity and Human Health. *Science*, **287**, 443–449.

Davidar P, Morton ES (1993) Living with parasites: Prevalence of a blood parasite and its effect on survivorship in the Purple Martin. *The Auk*, **110**, 109–116.

Dernegi D (Ed.) (2010) *Ecosystem Profile - Mediterranean Basin Biodiversity Hotspot*. Critical Ecosystem Partnership Fund.

Dickinson EC (Ed.) (2003) *The Howard & Moore Complete Checklist of the Birds*. Christopher Helm, London.

Dietzen C, Garcia-del-Rey E, Castro G, Wink M (2008) Phylogeography of the blue tit (*Parus* teneriffae-group) on the Canary Islands based on mitochondrial DNA sequence data and morphometrics. *Journal of Ornithology*, **149**, 1–12.

Dimitrov D, Zehtindjiev P, Bensch S (2010) Genetic diversity of avian blood parasites in SE Europe: Cytochrome b lineages of the genera *Plasmodium* and *Haemoproteus* (Haemosporida) from Bulgaria. *Acta Parasitologica*, **55**, 201–209.

Dobson AP (2009) Climate variability, global change, immunity, and the dynamics of infectious diseases. *Ecology*, **90**, 920–927.

Dobson AP, Foufopoulos J (2001) Emerging infectious pathogens of wildlife. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **356**, 1001–12.

Drummond A, Suchard M, Xie D, Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, **29**, 1969–1973.

Dukes JS, Pontius J, Orwig D, Garnas JR, Rodgers VL, Brazee N, Cooke B, Theoharides K a., Stange EE, Harrington R, Ehrenfeld J, Gurevitch J, Lerdau M, Stinson K, Wick R, Ayres M (2009) Responses of insect pests, pathogens, and invasive plant species to climate change in the forests of northeastern North America: What can we predict? *Canadian Journal of Forest Research*, **39**, 231–248.

Durrant KL, Marra PP, Fallon SM, Colbeck GJ, Gibbs HL, Hobson KA, Norris DR, Bernik B, Lloyd VL, Fleischer RC (2008) Parasite assemblages distinguish populations of a migratory passerine on its breeding grounds. *Journal of Zoology*, **274**, 318–326.

Duval L, Robert V, Csorba G, Hassanin A, Randrianarivelojosia M, Walston J, Nhim T, Goodman SM, Ariey F (2007) Multiple host-switching of Haemosporidia parasites in bats. *Malaria Journal*, **6**, 157.

Esseghir S, Ready PD, Killick-Kendrick R, Ben-Ismail R (1997) Mitochondrial haplotypes and phylogeography of Phlebotomus vectors of *Leishmania major*. *Insect Molecular Biology*, **6**, 211–225.

Fallon SM, Bermingham E, Ricklefs RE (2005) Host specialization and geographic localization of avian malaria parasites: a regional analysis in the Lesser Antilles. *The American Naturalist*, **165**, 466–80.

Freed LA, Cann RL, Goff ML, Kuntz WA, Bodner GR (2005) Increase in avian malaria at upper elevation in Hawai'i. *Condor*, **107**, 753–764.

Gandon S, Michalakis Y (2002) Local adaptation, evolutionary potential and host-parasite coevolution: interactions between migration, mutation, population size and generation time. *Journal of Evolutionary Biology*, **15**, 451–462.

Garamszegi L (2011) Climate change increases the risk of malaria in birds. *Global Change Biology*, **17**, 1751–1759.

Garcia JT, Suárez F, Garza V, Calero-Riestra M, Hernández J, Pérez-Tris J (2008) Genetic and phenotypic variation among geographically isolated populations of the globally threatened Dupont's lark *Chersophilus duponti*. *Molecular Phylogenetics and Evolution*, **46**, 237–251.

Giorgi F, Lionello P (2008) Climate change projections for the Mediterranean region. *Global and Planetary Change*, **63**, 90–104.

Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP, Ostfeld RS, Samuel MD (2002) Climate warming and disease risks for terrestrial and marine biota. *Science*, **296**, 2158–62.

Hellgren O, Bensch S, Malmqvist B (2008) Bird hosts, blood parasites and their vectors - associations uncovered by molecular analyses of blackfly blood meals. *Molecular Ecology*, **17**, 1605–13.

Hellgren O, Krizanauskiene A, Valkiūnas G, Bensch S (2007) Diversity and phylogeny of mitochondrial cytochrome B lineages from six morphospecies of avian Haemoproteus (Haemosporida: Haemoproteidae). *Journal of Parasitology*, **93**, 889–96.

Hellgren O, Pérez-Tris J, Bensch S (2009) A jack-of-all-trades and still a master of some: prevalence and host range in avian malaria and related blood parasites. *Ecology*, **90**, 2840–2849.

Hurrell JW, Trenberth KE (2010) Climate Change. In: *Effects of Climate Change on Birds* (eds Moller AP, Fiedler W, Berthold P), pp. 9–29. Oxford University Press, New York.

Ishtiaq F, Clegg SM, Phillimore AB, Black R a., Owens IPF, Sheldon BC (2010) Biogeographical patterns of blood parasite lineage diversity in avian hosts from southern Melanesian islands. *Journal of Biogeography*, **37**, 120–132.

Jenkins T, Owens IPF (2011) Biogeography of avian blood parasites (*Leucocytozoon* spp.) in two resident hosts across Europe: phylogeographic structuring or the abundance-occupancy relationship? *Molecular Ecology*, **20**, 3910–3920.

Jenkins T, Thomas G, Hellgren O (2012) Migratory behavior of birds affects their coevolutionary relationship with blood parasites. *Evolution*, **66**, 740–751.

Jennelle CS, Cooch EG, Conroy MJ, Senar JC (2007) State-specific detection probabilities and disease prevalence. *Ecological Applications*, **17**, 154–167.

Kimura M, Darbro JM, Harrington L (2010) Avian malaria parasites share congeneric mosquito vectors. *Journal of Parasitology*, **96**, 144–51.

Kovats RS, Campbell-Lendrum DH, McMichel AJ, Woodworth BL, Cox JSH (2001) Early effects of climate change: do they include changes in vector-borne disease? *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **356**, 1057–1068.

Kvist L, Martens J, Higuchi H, Nazarenko AA, Valchuk OP, Orell M (2003) Evolution and genetic structure of the great tit (Parus major) complex. *Proceedings of the Royal Society of London B: Biological Sciences*, **270**, 1447–54.

De la Roque S, Rioux JA, Slingenbergh J (2008) Climate change: effects on animal disease systems and implications for surveillance and control. *Revue scientifique et technique - Office international des épizooties*, **27**, 339–354.

Lachish S, Knowles SCL, Alves R, Wood MJ, Sheldon BC (2011) Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure. *Journal of Animal Ecology*, **80**, 1196–206.

LaPointe DA, Atkinson CT, Samuel MD (2012) Ecology and conservation biology of avian malaria. *Annals of the New York Academy of Sciences*, **1249**, 211–26.

Librado P, Rozas J (2009) DnaSP v5: A Software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452.

Lively CM (1999) Migration, Virulence, and the Geographic Mosaic of Adaptation by Parasites. *The American Naturalist*, **153**, S34–S47.

Malcolm JR, Liu C, Neilson RP, Hansen L, Hannah L (2006) Global warming and extinctions of endemic species from biodiversity hotspots. *Conservation Biology*, **20**, 538–548.

Martens J, Tietze D, Sun Y (2005) Molecular phylogeny of *Parus* (*Periparus*), a eurasian radiation of tits (Aves: Passeriformes: Paridae). *Zoologische Abhandlungen*, **55**, 103–120.

Martinsen ES, Paperna I, Schall JJ (2006) Morphological versus molecular identification of avian Haemosporidia: an exploration of three species concepts. *Parasitology*, **133**, 279–88.

Martinsen ES, Perkins SL, Schall JJ (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. *Molecular Phylogenetics and Evolution*, **47**, 261–73.

Marzal A, Bensch S, Reviriego M, Balbontin J, De Lope F (2008) Effects of malaria double infection in birds: one plus one is not two. *Journal of Evolutionary Biology*, **21**, 979–87.

Marzal A, Lope F de, Navarro C, Møller AP (2005) Malarial parasites decrease reproductive success : an experimental study in a passerine bird. *Oikos*, **142**, 541–545.

Merino S, Moreno J, Vásquez RA, Martínez J, Sánchez-Monsálvez I, Estades CF, Ippi S, Sabat P, Rozzi R, Mcgehee S (2008) Haematozoa in forest birds from southern Chile: Latitudinal gradients in prevalence and parasite lineage richness. *Austral Ecology*, **33**, 329–340.

Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853–8.

Møller AP, Nielsen JT (2007) Malaria and risk of predation: a comparative study of birds. *Ecology*, **88**, 871–81.

Njabo KY, Cornel AJ, Bonneaud C, Toffelmier E, Sehgal RNM, Valkiūnas G, Russell a F, Smith TB (2011) Nonspecific patterns of vector, host and avian malaria parasite associations in a central African rainforest. *Molecular Ecology*, **20**, 1049–61.

Ortego J, Cordero PJ, Aparicio JM, Calabuig G (2008) Consequences of chronic infections with three different avian malaria lineages on reproductive performance of Lesser Kestrels (*Falco naumanni*). *Journal of Ornithology*, **149**, 337–343.

Palinauskas V, Valkiūnas G, Bolshakov C V, Bensch S (2008) *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. *Experimental Parasitology*, **120**, 372–80.

Parmesan C (2006) Ecological and Evolutionary Responses to Recent Climate Change. *Annual Review of Ecology, Evolution, and Systematics*, **37**, 637–669.

Patz JA, Reisen WK (2001) Immunology, climate change and vector-borne diseases. *Trends in Immunology*, **22**, 171–2.

Peirce MA (1981) Distribution and host-parasite check-list of the haematozoa of birds in Western Europe. *Journal of Natural History*, **15**, 419–458.

Pimm SL (2009) Climate disruption and biodiversity. *Current Biology*, **19**, R595–601.

Porretta D, Canestrelli D, Urbanelli S, Bellini R, Schaffner F, Petric D, Nascetti G (2011) Southern crossroads of the Western Palaearctic during the Late Pleistocene and their imprints on current patterns of genetic diversity: insights from the mosquito *Aedes caspius*. *Journal of Biogeography*, **38**, 20–30.

Posada D (2008) jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, **25**, 1253–6.

Poulin R (2007) *Evolutionary ecology of parasites*. Princeton University Press, New Jersey.

Price P (1977) General concepts on the evolutionary biology of parasites. *Evolution*, **31**, 405–420.

La Puente JM, Martínez J, Aguilar J-D, Herrero J, Merino S (2011) On the specificity of avian blood parasites: revealing specific and generalist relationships between haemosporidians and biting midges. *Molecular Ecology*, **20**, 3275–3287.

La Puente JM, Merino S, Tomás G, Moreno J, Morales FJ, Lobato E, García-Fraile S, Belda EJ (2010) The blood parasite *Haemoproteus* reduces survival in a wild bird: a medication experiment. *Biology Letters*, **6**, 663–5.

Pérez-Tris J, Bensch S (2005a) Dispersal increases local transmission of avian malarial parasites. *Ecology Letters*, **8**, 838–845.

Pérez-Tris J, Bensch S (2005b) Diagnosing genetically diverse avian malarial infections using mixed-sequence analysis and TA-cloning. *Parasitology*, **131**, 15–23.

Pérez-Tris J, Hellgren O, Krizanauskiene A, Waldenström J, Secondi J, Bonneaud C, Fjeldså J, Hasselquist D, Bensch S (2007) Within-host speciation of malaria parasites. *PLoS ONE*, **2**, e235.

Reullier J, Pérez-Tris J, Bensch S, Secondi J (2006) Diversity, distribution and exchange of blood parasites meeting at an avian moving contact zone. *Molecular Ecology*, **15**, 753–63.

Ricklefs RE, Fallon SM (2002) Diversification and host switching in avian malaria parasites. *Proceedings of the Royal Society of London B: Biological Sciences*, **269**, 885–92.

Ricklefs RE, Fallon SM, Bermingham E (2004) Evolutionary Relationships, Cospeciation, and Host Switching in Avian Malaria Parasites. *Systematic Biology*, **53**, 111–119.

Ricklefs RE, Swanson BL, Fallon SM, Martínez-Abraín A, Scheuerlein A, Gray J, Latta SC (2005) Community relationships of avian malaria parasites in southern Missouri. *Ecological Monographs*, **75**, 543–559.

Rigaud T, Perrot-Minnot MJ, Brown MJF (2010) Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. *Proceedings of the Royal Society of London B: Biological Sciences*, **277**, 3693–702.

Santiago-Alarcon D, Bloch R, Rolshausen G, Schaefer HM, Segelbacher G (2011) Prevalence, diversity, and interaction patterns of avian haemosporidians in a four-year study of blackcaps in a migratory divide. *Parasitology*, **138**, 824–835.

Scheuerlein A, Ricklefs RE (2004) Prevalence of blood parasites in European passeriform birds. *Proceedings of the Royal Society of London B: Biological Sciences*, **271**, 1363–70.

Smith KF, Acevedo-Whitehouse K, Pedersen AB (2009) The role of infectious diseases in biological conservation. *Animal Conservation*, **12**, 1–12.

Stjernman M, Råberg L, Nilsson J-Å (2004) Survival costs of reproduction in the blue tit (Parus caeruleus): a role for blood parasites? *Proceedings of the Royal Society of London B: Biological Sciences*, **271**, 2387–94.

Szöllosi E, Cichoń M, Eens M, Hasselquist D, Kempenaers B, Merino S, Nilsson J-Å, Rosivall B, Rytkönen S, Török J, Wood MJ, Garamszegi L (2011) Determinants of distribution and prevalence of avian malaria in blue tit populations across Europe: separating host and parasite effects. *Journal of Evolutionary Biology*, **24**, 2014–2024.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution*, **28**, 2731–9.

Thompson J (2005) Coevolution: the geographic mosaic of coevolutionary arms races. *Current Biology*, **15**, R989–92.

Valera F, Díaz-Paniagua C, Garrido-García JA, Manrique J, Pleguezuelos JM, Suárez F (2011) History and adaptation stories of the vertebrate fauna of southern Spain's semi-arid habitats. *Journal of Arid Environments*, **75**, 1342–1351.

Valkiūnas G (2005) *Avian malaria parasites and other haemosporidia*. CRC Press, Florida.

Valkiūnas G, Iezhova T (2001) Parasites in hosts with special life histories. *Acta Zoologica Lituanica*, **11**, 391–394.

Ventim R (2011) *Haemosporidian parasites in communities of southwestern European reedbeds: their passerine hosts and their vectors. PhD Thesis*. Universidade de Coimbra, Coimbra.

Wahlberg N, Saccheri I (2007) The effects of Pleistocene glaciations on the phylogeography of *Melitaea cinxia* (Lepidoptera: Nymphalidae). *European Journal of Entomology*, **104**, 675–684.

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**, 1545–54.

Weatherhead PJ, Bennett GF (1992) Ecology of parasitism of Brown-headed Cowbirds by haematozoa. *Canadian Journal of Zoology*, **70**, 1–7.

WHO (2010) *World Malaria Report 2010*. World Health Organization. WHO Press.

Windsor D (1998) Most of the species on Earth are parasites. *International Journal for Parasitology*, **28**, 1939–41.

Wood MJ, Cosgrove CL, Wilkin TA, Knowles SCL, Day KP, Sheldon BC (2007) Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. *Molecular Ecology*, **16**, 3263–73.