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Picaflor I

Se escapó el fuego y fue llevado
por un movimiento de oro
que lo mantuvo suspendido,
fugaz, inmóvil, tembloroso:
vibración erectil, metal:
pétalo de los meteoros.
Siguió volando sin volar
concentrando el sol diminuto
en helicóptero de miel,
en sílaba de la esmeralda
que de flor a flor disemina
la identidad del arcoiris.
Al sol sacude el tornasol
la suntuaria seda suntuosa
de las dos alas invisibles
y el más minúsculo relámpago
arde en su pura incandescencia,
estático y vertiginoso.

Pablo Neruda

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Resumen

El Neotrópico presenta una de las avifaunas más ricas del mundo asociada a la gran variedad de ecosistemas producto de la heterogeneidad topográfica y climática. Como parte de esta zona megadiversa, los Andes colombianos presentan un escenario prometedor para la investigación en hemoparásitos aviares, poco estudiados en tierras altas en todo el mundo. El orden Haemosporida incluye especies causantes de malaria aviar y parásitos relacionados, los cuales han sido implicados con la mortalidad de aves en cautiverio así como en la disminución poblacional de especies endémicas en Hawaii. Este estudio evaluó la diversidad de los géneros *Haemoproteus* y *Plasmodium* presentes en aves de la región Andina de Colombia; utilizando en conjunto la determinación morfológica de las especies y la identificación de linajes de citocromo *b*. En total se determinaron 14 morfoespecies de las cuales seis son nuevas para el mundo y se da la descripción de una de ella: *Plasmodium* (*Novyella*) *unalis* sp. nov. También se da la redescripción de *Plasmodium* (*Haemamoeba*) *lutzi* un parásito que solo había sido reportado en tierras bajas. Las especie *Plasmodium* (*N*.) *unalis* y *P*. (*H*.) *lutzi* resultaron genéticamente relacionados con *Plasmodium* (*N*.) *vaughani* y *P*. (*H*.) *relictum* respectivamente, parásitos de amplia distribución geográfica y de hospederos. Se reportan también 25 linajes de citocromo *b* de los cuales 18 no han sido reportados previamente. En cuanto a la concordancia entre identificación morfológica y molecular, el marcador citocromo *b* mostró ser útil para la determinación de especies, a pesar de su baja resolución filogenética. El genero *Plasmodium* presentó diferencias en las especies entre tierras altas (este estudio) y las reportadas previamente en tierras bajas neotropicales. Finalmente se consideraron las diferencias entre los parásitos que infectan aves residentes y migratorias, encontrándose que existen linajes restringidos a residentes y otros a migratorias boreales; solo un linaje fue compartido entre estos tipos de aves, sin embargo este parásito no fue encontrado en aves boreales no-migratorias; indicando que el linaje compartido no cuenta con un ciclo de transmisión establecido en la región boreal. Las diferencias en fauna aviar y vectora pueden estar restringiendo la transmisión de linajes parásitos foráneos en Colombia.

Palabras clave: Colombia, Andes, Malaria aviar, *Haemoproteus*, Neotrópico.

Abstract

Diversity of blood parasites *Plasmodium* and *Haemoproteus* in some species of Colombian Andean birds

The Neotropical region is one of the richest in avifauna around the world, associated with a large variety of ecosystems because of heterogeneous topography and climatic conditions. As part of this mega-diverse zone, the Colombian Andes shows a promising scenario for research on avian blood parasites, which are scarcely studied in highlands around the world. The Haemosporida order includes species causing avian malaria and related parasites, which have been implicated in population decrease of endemic bird species in Hawaii and mortality in captive birds. The present study evaluated *Haemoproteus* and *Plasmodium* diversity on the highly diversified avifauna from the highland Andean region of Colombia, by using both morphological determination and cytochrome *b* lineages. In total 14 morphospecies were determined, six of them new science; the description of *Plasmodium* (*Novyella*) *unalis* sp. nov is given. In addition, a redescription of *Plasmodium* (*Haemamoeba*) *lutzi* a parasite previously reported only in low lands is provided. The species *Plasmodium* (*N*.) *unalis* and *P*. (*H*.) *lutzi* turned out genetically related to *Plasmodium* (*N*.) *vaughani* and *P*. (*H*.) *relictum* respectively; parasites with wide geographical distribution and host range. Twenty-five cytochrome *b* lineages were found, 18 of them not reported before. Regarding the concordance between morphological and molecular identification, cytochrome *b* gene showed to be useful for species determination in spite of low phylogenetic resolution. *Plasmodium* genus showed differences of species between lowlands (previously reported) and highland in the Neotropical region. Finally, the differences between parasites infecting resident and migratory birds were evaluated. Parasite lineages were found to be restricted to either Colombian resident or boreal migratory birds and a single lineage was shared, this lineage was not found in boreal non-migratory birds which could indicate that the lineage has not established a transmission cycle in the boreal region. The avian and vector differences between regions could be restricting the transmission of foreign parasites in Colombia.

Keywords: Colombia, Andes, avian malaria, *Haemoproteus*, Neotropic

Objectives

Main objective

To evaluate the diversity of molecular lineages and morphospecies of the genera *Plasmodium* and *Haemoproteus* present in some bird species from the highlands of Colombia

Specific objectives

- To determine the *Plasmodium* and *Haemoproteus* morphospecies, in some bird species from the Colombian Central and Eastern mountain range.

- To determine the *Plasmodium* and *Haemoproteus* cytochrome *b* lineages in some bird species from the Colombian Central and Eastern mountain range.

- To analyze the correlation between molecular lineages and morphospecies of the parasite genera under study.

Introduction

Birds have been widely used in the study of human malaria as a model to characterize the transmission cycle and to develop antimalarial drugs. In the last decade avian malaria research has increased in topics such as speciation, coevolution, sexual selection, association between ecological traits and parasite prevalence, infection in endemic fauna and its implications on conservation, experimental infection and treatment (Marzal 2012).

Traditionally, the identification of *Haemoproteus* and *Plasmodium* has been obtained by using morphological features and measurements obtained on blood smears. Currently there are over 40 recognized species of *Plasmodium*, and over 130 of *Haemoproteus* (Valkiūnas 2005). Molecular data show large genetic diversity of these parasites, which indicates that the number of species and their taxonomic diversity may be greater than that recognized by traditional taxonomy (Bensch et al. 2004, Sehgal et al. 2006).

Current research on avian malaria and related parasites examine mainly identitity of these parasites by amplifying a fragment of cytochrome *b* (Cyt *b*) gene. In a few cases, other markers from mitochondrial or the apicoplast have been used (Martinsen et al. 2008, Santiago-Alarcon et al. 2010). Recently, a 200 bp fragment of nuclear gene dihydrofolate reductase- thymidylate synthase (DHFR-ST) has been used to infer recombination events and explore concordance with results obtained using mitochondrial sequences (Bensch et al. 2004, Beadell et al. 2006).

Research in Latin American countries like Cuba, Brazil, Colombia, Costa Rica, Chile, Ecuador, Guyama, Uruguay and Peru have mostly used molecular methods for identification of blood parasite biodiversity (Fallon et al. 2005, Durrant et al. 2006, Merino et al. 2008, Levin et al. 2009, Santiago-Alarcon et al. 2010, Belo et al. 2011, Levin et al. 2011, Svensson-Coelho and Ricklefs 2011, Belo et al. 2012, Levin et al. 2012, Mijares et al. 2012, Fecchio et al. 2013, Levin et al. 2013, Svensson-Coelho et al. 2013); however, several studies have determined some haemoparasite taxa by microscopy, including *Haemoproteus* and *Plasmodium* (Ribeiro et al. 2005, Munro et al. 2009, Rodríguez et al. 2009, Valkiūnas et al. 2010, Levin et al. 2011, Levin et al. 2012).

In Colombia the presence of avian blood parasites in several locations (Renjifo et al. 1952, Bennett and Borrero 1976, Rodríguez and Matta 2001, Valkiūnas et al. 2003, Matta et al. 2004, Basto et al. 2006, Londoño et al. 2007, Rodríguez et al. 2009, Lotta et al. 2013) has been explored. However, these studies have only indicated the prevalence of infection according to host/geographical area with parasite identification at the genus level; these studies have shown high prevalence of infection by *Plasmodium* and *Haemoproteus*.

The great diversity of birds and ecosystems in Colombia contribute to generate host-parasite-vector relationships potentially different from those previously described for other areas of the world. These relationships for the highland birds are of particular interest, since they have been scarcely studied. In Colombia the research group *Caracterización genética*

e inmunología has studied the diversity of blood parasites in wild birds in several localities in the country. An interesting finding is the high prevalence of infection in some bird families and a differential distribution with respect to elevation for *Leucocytozoon* and microfilariae; however there are no studies characterizing the molecular diversity of these parasites.

The aim of this work is to solve the following questions: (1) What are the molecular lineages and morphospecies from *Plasmodium* and *Haemoproteus* infecting some species of birds in Colombian highlands?, (2) Which morphological species correspond to the molecular lineages found? This study also pretends to test the following hypotheses: (1) If there is intraspecific variation in some species of *Plasmodium* and *Haemoproteus*, it is expected to find different lineages associated with the same morphospecies. (2) Since there are no previous studies in this Neotropical area, it is expected to find new host-parasite-vector relationships, which could be reflected in not yet described parasites lineages.

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Literature review

Since last century, birds have been a model for studing vector-borne diseases in humans such as malaria. There are about 450 species of blood parasites infecting more than 4000 bird species (Bishop and Bennett 1992) which can be found in plasma, erythrocytes and leukocytes. The taxa most frequently reported in literarute are *Haemoproteus*, *Leucocytozoon*, *Plasmodium*, *Trypanosoma*, and to a lesser extent microfilariae *Atoxoplasma*, *Babesia*, *Hepatozoon*, *Lankesterella* and *Toxoplasma*.

The genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* belong to the order Haemosporida (Phylum: Apicomplexa) and include species that are etiologic agents of avian malaria. There is great diversity of hematophagous dipterans implied as vectors of these parasites in birds: Ceratopogonidae and Hipoboscidae families transmit *Haemoproteus*, whereas members of the Culicidae family transmit *Plasmodium* (Santiago-Alarcon et al. 2012). These parasites are distributed worldwide except in the Antarctica where it is likely not to find susceptible vectors (Atkinson 2008a, Atkinson 2008b).

Life cycle

Plasmodium and *Haemoproteus* species have an obligated heteroxenous life cycle involving two hosts: a vertebrate and a blood-sucking insect. All stages of the life cycle are haploid, except the zygote as meiosis occurs rapidly after fertilization. The cycle begins when a vector ingests infected blood with parasite gametocytes, which mature into gametes. Fertilization occurs inside the insect gut; then zygotes become mobile ookinetes which pass through the insect gut to the hemocoel and become oocysts (Fig. 1A). By mitototical division the oocyst forms the sporozoites which are released into the hemocoel and migrate to salivary glands (Fig. 1B). Sporozoites are the infective forms for birds and are transmitted by bite of the insect to a vertebrate host.

There are some differences in the cycle of *Haemoproteus* and *Plasmodium* (Valkiūnas 2005).In *Haemoproteus* once sporozoites have entered, they develop tropism for the endothelial cells where they undergo merogony (formation of meronts, including megalomeronts) where hundreds and even thousands of merozoites are released (Fig. 1C). These infect spleen cells and in some parasite species the skeletal muscle as well. In this tissues merogony occurs again (Fig. 1D,E) and merozoites realeased infect red blood cells where they develop into gametocytes (Fig. 1F) (Valkiūnas 2005).

For *Plasmodium,* when sporozoites have infected the bird they go to several organs such as liver, spleen and lungs where they undergo merogony and release the merozoites (Fig. 1G). These infect macrophages where another merogony cycle occurs (Fig. 1H), these merozoites are capable to invade capillary endothelial cells where they produce more merozoites (Fig. 1J). Merozoites from both macrophages and endothelial cells also infect erythrocytes where they may undergo merogony or develop gametocytes (Fig. 1F,I). (Valkiūnas 2005). The main difference between these cycles of infection is that only *Plasmodium* is able to develop in red blood cells both merozoites and gametocytes.

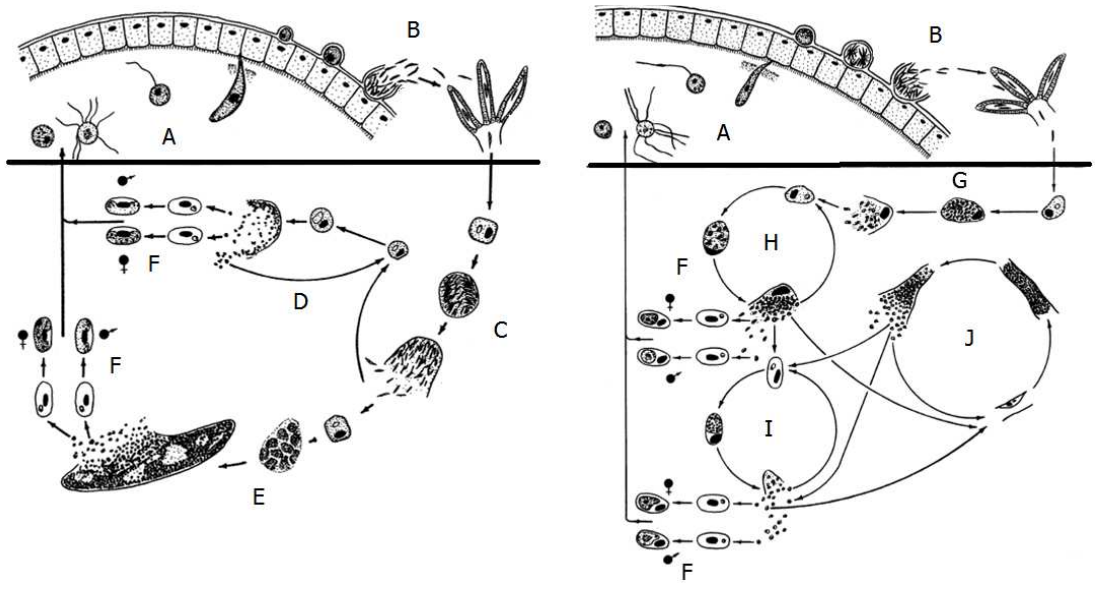

Figure 1. Life cycle of *Haemoproteus* (left) and *Plasmodium* (right), the horizontal line separates the stages occurring in vector (above) and bird (bottom) A: gametogenesis, fertilization, development of both ookinete and oocyst. B: development, releasing and migration to salivary glands by sporozoites. C: merogony and releasing of merozoites into endothelial cells. D: merogony in spleen cells. E: merogony in skeletal muscle. F: gametocytes developmetno in erythrocytes. G: merogony in several organs such as liver, spleen and lungs, H: merogony in macrophages. J: merogony in endothelial cells. I: merogony in erythrocytes. Graphics modified from Valkiūnas (2005).

Taxonomy and phylogenetic relationships

Valkiūnas (2005) included 135 *Haemoproteus* species and 39 *Plasmodium* species, in a comprehensive guide for the identification of avian Haemosporidia. However, since this book was published, the number of species has increased with the description of: *Haemoproteus parabelopolskyi* (Valkiūnas et al. 2007b), *H. vacuolatus* (Valkiūnas et al. 2008), *H. cyanomitrae* (Iezhova et al. 2010), *H. pallidulus* (Krizanauskiene et al. 2010), *H. multipigmentatus* (Valkiūnas et al. 2010), *H. catharti* (Greiner et al. 2011), *H. micronuclearis*, *H. paranucleophilus*, *H. nucleofascialis*, *H. homobeiopoisifyi* (Iezhova et al. 2011), *H. jenniae* (Levin et al. 2012), *H. nucleocondensus* (Krizanauskiene et al. 2012), *H. valkiūnasi* (Merino et al. 2012), *H. multivolutinus*, *H. paramultipigmentatus* (Valkiūnas et al. 2013), *Plasmodium ashfordi* (Valkiūnas et al. 2007a), *P. megaglobularis*, *P. globularis* (Valkiūnas et al. 2008), *P. lucens*, *P. multivacuolaris*, *P. parahexamerium* (Valkiūnas et al. 2009), *P. polymorphum* (Zehtindjiev et al. 2012a) and *P. homonucleophilum* (Ilgūnas et al. 2013).

The morphological features for the identification of *Haemoproteus* species include the different developmental stages found in blood as early, young and growing gametocytes, as well as the identity of the infected bird identified to the level of Order. Regarding early and young gametocytes, the features used include shape, edge envolepe type and position within the host cell. On growing and developed gametocytes the features examined are

shape, edge envolope type, arrangement respect to host cell nucleus, number and shape of the pigment granules, extent of displacement from the host cell nucleus and presence of unfilled zones between parasite and host cell envelope.

In the case of *Plasmodium* the features for morphological identification include trophozoites, gametocytes and meronts stages; about trophozoites their shape and position are important. The meronts features include size, number, cytoplasm amount, size of merozoites and extent of nucleus of the host cell displacement. For gametocytes, the shape, disposition relative to host cell and position of the parasite nucleus are taken into account.

Besides the microscopic diagnosis of blood smears, these parasites are detected by means of PCR using the Cyt *b* gene: Specialized Malavi database reports over 1000 unique lineages (Bensch et al. 2009)

The *Haemoproteus* genus currently is divided into two subgenera *H*. (*Haemoproteus*) and *H*. (*Parahaemoproteus*), this division is supported by the type of vector: species of the Hippoboscidae or Ceratopoginidae families respectively (Martinsen et al.2008). Traditionally, besides vector information *H*. (*Haemoproteus*) was thought to only infect Columbiformes (Valkiūnas 2005); however with molecular studies it was found that the species of this subgenus *H*. *iwa* and *H*. *jenniae* may infect Charadriiformes and Pelecaniformes orders (Levin et al. 2011, Levin et al. 2012). Also it was considered that the *H*. (*Parahaemoproteus*) subgenus only infects non-Columbiformes birds, but molecular data showed that *H*. *sacharovi* and *H*. *turtur*, widely distributed species which only infect Columbiformes belongs to the subgenus *Parahaemoproteus* (Krizanauskiene et al. 2013). The monophyly of the *Haemoproteus* genus is controversial since Krizanauskiene et al. (2013) has show a monophyletic status but several studies have shown a paraphyletic status (Martinsen et al. 2008, Perkins 2008, Santiago-Alarcon et al. 2010).

The genus *Plasmodium* contains five subgenera whose species infect birds: *P*. (*Haemamoeba*), *P*. (*Giovannolaia*), *P*. (*Novyella*), *P*. (*Bennettinia*) and *P*. (*Huffia*). They are diagnosed based on the shape of the gametocytes, relative size of merontes and type of blood cells infected (Valkiūnas 2005). The monophyly of the subgenera *Giovannolaia* and *Novyella* has been rejected using Cyt *b* or the combination of two mitochondrial genes (Martinsen et al. 2007, Valkiūnas et al. 2009). Moreover, *Bennettinia* and *Huffia* have been considered as monophyletic groups although with special considerations in each case: the sequences of the unique species belonging to *Bennettinia* are identical and clustered in a single clade. Regarding *Huffia* there are sequences available only for *P*. *elongatum*, so further information on other species could change the status of monophly. Finally *Haemamoeba* species often appear forming politomies with species from other subgenera (Valkiūnas et al. 2008, Valkiūnas et al. 2009, Zehtindjiev et al. 2012b) so their phylogenetic relationships with the data available today, remains unclear.

Topics on avian haemosporidians

Pathogenicity

Several researches have suggested that infection by haemosporidians has low pathogenicity in wildlife; however, this conclusion is biased because the capture method most frequently used is mist nets. Applying this methodology, birds captured are mainly those in chronic phase of the disease, probably only those who were able to survive acute period of the disease. Thus, it is not surprising that the majority of natural infections recorded have low number of parasites circulating.

Experimental studies with several *Plasmodium* species have shown that, in the acute phase of infection, there is a peak of blood parasitic forms associated with anemia and excess of biliverdin in feces. Additionally, there is an increase in activity of macrophages which phagocyte parasitized blood cells causing liver and spleen hypertrophy. When meronts are formed in endothelial cells, the capillaries could be blocked causing anoxia and necrosis of several tissues (Valkiūnas 2005, Atkinson 2008a, Palinauskas et al. 2008).

Pathologic effects reported for the infection with *Haemoproteus* spp. include inflammation, necrosis, hemorrhage and myopathy. Lesions are mainly associated with development of meronts in organs and its rupture releasing merozoites; tissues often affected are lungs, liver, spleen, heart and skeletal muscle, although negative effets have been reported on kidneys and gizzard (Atkinson 2008b). Traditionally, *Haemoproteus* infection is considered less pathogenic than *Plasmodium* infection; however there are reports of sudden death by haemorrhage in coelom and liver, and vascular lesions generated during pre-erythrocytic phase of infection (Donovan et al. 2008, Olias et al. 2011, Pacheco et al. 2011).

Chronic infection can also cause negative effects on bird reproductive success. Additionally, it has been shown that treatment with antimalarial drugs could counteract the negative effect, improving hatching rate and mass of nestling (Knowles et al. 2010). The avian malaria infection implies an energetic cost invested in immune defense and repair of tissues damaged (Wobeser 2008); also it cause anorexia that can lead to decrease in mobility, difficulty to obtain food and increase of vulnerability to predators as demonstrated by MØller and Nielsen (2007).

Ecology

Several studies have explored the relationship between blood parasites, including *Plasmodium* and *Haemoproteus*, and a range of abiotic, ecological and life history features to try to explain differences in parasite prevalence found among families and host species. Results have shown an inverse relationship between frequency of haemoparasites and elevation/latitude (Bears 2004, Merino 2008, Latta and Ricklefs 2010, Zamora-Vilchis et al. 2012, Van Rooyen et al. 2013), likely associated with changes in vector fauna or negative effect of low temperature in the parasite development.

Features as habitat, migration, nest type and gregariousness have showed results for and against for an association to high frequency of infection with *Haemoproteus* or *Plasmodium* (Scheuerlein and Ricklefs 2004, Ribeiro et al. 2005, MØller et al. 2009, Fecchio et al. 2013). Nest height above the ground has shown a positive relationship with prevalence of haemoparasites (Garvin and Remsen 1997). Also, it has been reported that time of egg incubation is inversely related to haemoparasite prevalence likely longer incubation time allows a better immune system development (Ricklefs 1992, Tella et al. 1999).

Habitat degradation could affect the parasite prevalence, since it modifies the vector-host-parasite interaction. For *Haemoproteus* there are contrasting results: the tendency of lower prevalence in urbanized and degraded habitats relative to conserved ones has been observed (Geue and Partecke 2008, Chasar et al. 2009, Loiseau et al. 2010), Fokidis et al. (2008) reported not significant differences for parasite prevalence between urban and rural areas, and .

In the case of *Plasmodium* the results are variable: Loiseau et al. (2010) and Bonneaud et al. (2009) reported lower prevalence in modified habitats, whereas Geue and Partecke (2008) did not find differences among types of habitat studied. However, this could occur when parasites are considered at the genera level. Chasar et al. (2009) did not find differences among *Plasmodium* prevalence in two habitats studied; but when the pattern was examined for each *Plasmodium* species separately, they found prevalent species in degraded areas whereas another group of species were more prevalent in undisturbed habitats.

Sexual selection

It has been hypothesized that the development of secondary sexual features such as plumage brightness and singing in birds may be associated with an increased responsiveness to parasites, since generation of such features represent a physiological investment for the increase of hormones which are immunosuppressive (Martínez 2010). The role of parasites in sexual selection of birds has been studied for avian malaria and related parasites, with contradictory results (Horak et al. 2001, Doucet and Montgomerie 2003, Durrant et al. 2007, Madsen et al. 2007, Garamszegi and Moller 2012, Jacquin et al. 2011), indicating that the relationship may be different for each avian species studied.

Conservation

The Hawaiian Islands have provided an opportunity to study the effect of foreign pathogens on endemic wildlife. The avifauna in the islands had not been exposed to avian malaria, so the introduction of *Culex quinquefasciatus* as a vector and exotic birds infected with *Plasmodium relictum,* produced an outbreak of avian malaria causing high mortality in resident birds and even extinction of several endemic species (van Riper III et al. 1986). Studies have shown that *Plasmodium relictum* lineage GRW4 is the cause of mortality in Hawaiian birds, although this and other strains belonging to the same parasite species are widely distributed but range with respect to virulence when they infect bird species in other geographical areas (Beadell et al. 2006); this result would be associated with the time of host-parasite coevolution.

The topography of the islands has allowed analyzing the effect of elevation on parasite prevalence, showing that although host-parasite-vector may coincide geographically, it is the temperature what can decrease the parasite development within the vector, thus interrupting the transmission cycle (LaPointe et al. 2012). Taking into account that previous evidence, the effect of climate change on *P*. *relictum* transmission at Hawaiian Islands has been studied; results suggest that in the last 10 years the parasite has extended its altitudinal range and as a consequence an increase in the optimum isotherm for the parasite development (Freed et al. 2005). Simulations to year 2100 have shown that a 2°C increase in temperature could lead to a 13°C isotherm reaching the highest elevation limit at the Hakalau Forest National Wildlife Refuge, an area for many endangered species that potentially could go extinct by infection with avian malaria (LaPointe et al. 2012).

Due to the evidence of death in native birds because of foreign parasites, research on endemic species in island systems has increased. Howe et al. (2012) assessed the presence of avian malaria in native and introduced birds in New Zealand: two important cases of infection were found: an endemic kiwi species and an introduced blackbird died both by infection with *Plasmodium* sp. lineage LINN1; another endemic and endangered bird species was found dead and infected by *P*. *relictum* lineage GRW4. However, it is speculated that native birds have already been in contact with several lineages of parasites since other endemic birds have been found infected with *Plasmodium* (*Novyella*) sp. and *Plasmodium* (*Huffia*) sp although with low parasitemias. In this territory, there are also potential vectors.

Another example of studies in island systems include Valkiūnas et al. (2010) and Santiago-Alarcon et al. (2010) who found high frequency of *Haemoproteus multipigmentatus* in the endemic Galapagos dove. Similar lineages were found in other areas in the New World Columbiformes, so it is theorized that this parasite is widely distributed and was brought to the Galapagos Islands by another species of pigeon from the continent land. Similar Levin et al. (2013) reported *Plasmodium* lineajes restricted to resident birds of Galapagos Islands but also parasite lineajes shared between Neartic migrants birds and endemic species.

Infection with avian malaria and related parasites is also important in zoological gardens, where exotic bird species can potentially enter in contact with new virulent parasites and carry fatal outcomes as reported by Donovan et al. (2008). However, native species getting infected in zoological gardens have been reported by Pacheco et al. (2011). In their study, they reported death cases in an endangered partridge subspecies; several individuals raised at zoological garden died from injuries caused during the pre-erythrocytic stage infection of *Plasmodium* and *Haemoproteus*; this study concluded that two other native species present at the zoological garden and showed the same parasite genotypes, possibly were the source of infection.

Host-parasite relationship

Two types of host-parasite association have been found: (1) generalist parasites that can infect several host species, both birds and vectors, (2) parasites infecting few host species or closely related host species (Hellgren et al. 2009, Loiseau et al. 2012). The degree of specificity of a parasite with its host is closely related to a process of reciprocal adaptation as well as their geographical distribution. In the first case, the parasite virulence depends

on how long is the association with the host; where long associations result in low host mortality since this is beneficial to the parasite. In recent associations the virulence is higher as it occurs with the *Plasmodium relictum* infection on Hawaiian birds (Beadell et al. 2006). The range of bird species that a parasite is able to infect depends at first on the composition of hosts in a particular environment, blood meal preferences of vectors and host-parasite compatibility (Hellgren et al. 2008, Medeiros et al. 2013).

Phylogeography

Phylogeographic studies are based on phylogenetic analyses of closely related species and the congruence with their geographical distribution. The reconstruction of the biogeographic history for species allows identifying the historical reasons for separation of populations and the effect of vicariance or dispersion. In a global context parasite phylogeography has been used to study coevolution processes involved in parasite virulence, colonization by exotic parasites of new hosts/areas and selection effects on alleles for drug resistance (Criscione et al. 2005).

For avian *Plasmodium* and *Haemoproteus*, the composition of parasite lineages present in one or few bird species through a geographical area have been explored (Durrant et al. 2007, Pagenkopp et al. 2008, Beadell et al. 2006, Kimura et al. 2006, Ishtiaq et al. 2010, Silva-Iturriza et al.2012). The findings of these researchers are variable and applicable only to the region studied; nevertheless they coincide in that the existence of spatial structure is not always determined by bird population structure or by geography, but rather by complex patterns of existence, abundance, compatibility and exposure to vectors.

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1. Chapter I. Avian haemosporidians from Neotropical highlands: evidence from morphological and molecular data

Hemosporidios aviares de tierras altas neotropicales: evidencia morfológica y molecular

Los hemosporidios aviares han sido escasamente estudiados en tierras altas Neotropicales a pesar de su alta diverisidad de aves y ecosistemas unicos. Los objetivos de este estudio fueron examinar la diversidad de *Haemoproteus* y *Plasmodium* con base en datos morfológicos y moleculares, asi como, explorar la concordancia entre esos dos enfoques. Se muestrearon 1487 aves pertenecientes a 166 especies, en siete localidades de la región Andina de Colombia con alturas entre los 2100 y 3950 m sobre el nivel del mar. Se tomaron tres extendidos sanguíneos por cada ave y se almacenó sangre en buffer SET para análisis moleculares.Se reportan 14 morfoespecies de las cuales seis no han sido descritas. Se encontraron 25 linajes de citocromo *b* de parásitos de los cuales 18 se reportan por primera vez. Se dan información morfológica e ilustraciones, as si como linajes de citocromo *b* para siete morfoespecies de hemosporidios aviares: *Haemoproteus columbae, Haemoproteus witti, Haemoproteus coatneyi, Haemoproteus vireonis, Plasmodium lutzi, Plasmodium unalis,* and *Plasmodium hermani*. Hasta donde es del conocimiento de los autores este es el primer reporte donde se vincula la identificación morfológica y molecular para *Haemoproteus witti* y *P*. *hermani*. En cuento a la concordancia ente identificación morfológica y molecular, citocromo *b* mostró ser útil para la determinación. Las diferencias en la composición de parásitos entre tierras bajas (estudios previos) y tierras altas en la región neotropical, halladas en este estudio, sugieren un remplazo de la fauna de *Plasmodium*. Se encontraron linajes de parásitos restringidos a especies de aves migratorias boreales y otros restringidos a aves residentes; el único linaje compartido no fue encontrado en aves no migratorias de Norte América. Como han exhortado recientemente diferentea autores, aquí se genera información valiosa mediante el uso de datos moleculares y morfológicos que representan relaciones hospedero parasito reales y se incrementa el muestreo de taxones de hemosporidios aviares.

Palabras clave: hemoparásitos aviares, Colombia, *Plasmodium*, *Haemoproteus*

Este artículo abarca ampliamente los tres objetivos del proyecto de maestría, *al dar la determinación morfológica y molecular de las especies de Plasmodium y Haemoproteus encontrada en aves andinas residentes en Colombia*. *Así como el análisis de la concordancia entre los dos enfoques*. *Mi contribución a este artículo radicó en la lectura de extendidos sanguíneos*, *toma de fotografías y medidas así como en la identificación morfológica de las especies encontradas*, *síes de ellas nuevas para el mundo y actualmente se encuentran en proceso de descripción*. *También realicé las búsquedas en bases de datos* (*GenBank y MalAvi*) *y los análisis filogenéticos*. *Participé en escritura de todo el artículo*.

Avian haemosporidians from Neotropical highlands: evidence from morphological and molecular data

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1.1 Abstract

Avian haemosporidian parasites have been scarcely studied in the Neotropical highlands despite their high avian diversity reported and the uniqueness of these ecosystems. The aims of this study were to examine the *Haemoproteus* and *Plasmodium* diversity based on morphological and molecular data, as well as, explore the concordance between these two approaches. We sampled 1487 birds belonging to166 species, in seven localities of the Andean region of Colombia at elevations ranging from 2100 and 3950 m above sea level. Three blood smears were obtained from each bird and blood was also stored in SET buffer for molecular analysis. Here, we report fourteen morphological parasite species, of which six are undescribed. Twenty-five parasite cytochrome b lineages are reported, 18 of them for the first time. We provide morphological information and illustrations, as well as, cytochrome b lineages for seven avian haemosporidian morphospecies: *Haemoproteus columbae, Haemoproteus witti, Haemoproteus coatneyi, Haemoproteus vireonis, Plasmodium lutzi, Plasmodium unalis,* and *Plasmodium hermani*. To the authors's knowledge this is the first report allowing a linkage between morphology and a molecular lineage for *Haemoproteus witti* and *P. hermani*. Regarding the concordance between morphological and molecular identification, cytochrome b gene showed to be useful for species determination as a barcoding. Differences on the parasite composition between lowlands (previously reported) and highlands in the study sites suggest a replacement of avian *Plasmodium* fauna. Parasite lineages restricted to either Colombian resident or Neartic migrantory birds were found; but a single lineage common in both has not been recorded in Neartic non-migratory birds. As it has been lately encouraged by different authors, we generate valuable information by using both morphological and molecular data representing real host-parasite relationships and increasing the taxon sampling of avian haemosporidian.

Keywords: avian haemoparasites, avian malaria, Colombia, *Plasmodium*, Haemoproteus.

1.2 Introduction

Haemosporida order includes four genera *Fallisia*, *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* which infect bird species; these parasites have a broad range of vertebrate host and vectors worldwide (Valkiūnas 2005). These parasites have acquired significance because they have been implicated in population decrease of endemic bird species in Hawaii and mortality in captive birds (van Riper et al. 1986, Donovan et al. 2008, Pacheco et al. 2011).

The avian haemosporidian taxonomy is traditionally based on morphological and morphometric features, type of host cell infected and host taxonomy. With the PCR diagnosis, the number of studies on that subject have increased, but generally excluding morphological identification (Valkiūnas et al. 2008). Recently, evidence of abortive infections has been published, which demonstrates that true host-parasite relationships should be addressed by both approximations (Valkiūnas et al. 2014).

Despite a great number of studies on avian haemoparasites, only a few surveys have been made for avifauna up 2000 m above sea level around the world. Also in the Neotropical region, few studies have been developed on highlands (Valkiūnas et al. 2003, Munro et al. 2009, Rodríguez et al. 2009, Lotta et al. 2013, Mantilla et al. 2013a, 2013b, Jones et al. 2013). On the other hand, the richest avian fauna around the world is in the Neotropical region (Jetz et al. 2012) including the Andes and Amazon Basin regions, which are species-richness hotspots (Orme et al. 2005). Such biodiversity is associated to complexity in landscape, heterogeneous topography and climatic conditions (Rahbek and Graves 2001) factors that produce a high variety of habitats and endemism. High biodiversity plus scarcity of studies at highlands of the Neotropical region provides an opportunity to find new host-parasite-relationships, and analyze their specific features.

The aims of this study were (1) to provide a morphological survey of *Haemoproteus* and *Plasmodium* species infecting the avifauna from the highlands of the Andean region of Colombia, (2) to identify molecular lineages of parasites using a fragment of cytochrome *b* gene, (3) to explore the concordance of parasite identification between morphological and molecular approaches. Here we report for the first time the parasite cytochrome b lineages for *Haemoproteus* (*Parahaemoproteus*) *witti* and *Plasmodium* (*Huffia*) *hermani*. We also discuss the new findings with respect to previous reports for the Neotropical region, and hypothesize a differential distribution of parasites associated with altitude, suggesting an adaptation of these parasites to the biotic and abiotic conditions of highlands.

1.3 Materials and methods

1.3.1 Ethics and sampling permits

In order to obtain parasite DNA, we first collected a small amount of blood from sampled birds. This Bird handling followed hygiene requirements and short capture times to minimize individual animal stress. All captured individuals were released. The bird sampling

methodology for this study was approved by the Comité de Bioética of Departamento de Ciencias para la Salud Animal of Facultad de Medicina Veterinaria y de Zootecnia (act 005 of 2010). Sample collection was performed under permits supplied by Unidad Administrativa Especial del Sistema de Parques Nacionales Naturales de Colombia UAESPNN Subdirección técnica (agreement 09 of 2009, SUT 010701 of 2010) and Autoridad Nacional de Licencias Ambientales ANLA (file 4120E1104774 of 2011, file 4120E183893 of 2011, and resolution 0787 of 2013).

1.3.2 Study area, samples and blood film examination

This study included localities from two mountain ranges in Colombia, sampled from September 2009 to February 2014. Ecosystems and abiotic features are shown in Table 1, including vegetation into an urban area, grasslands, high Andean forest and Paramo. Birds were captured using mist nets, and identified by using first the field guide from Hilty and Brown (1986) and then reported according to the South American Classification Committee (Remsen et al. 2012).

Blood samples were collected by puncturing the brachial vein or by toenail clipping. Three blood smears were prepared from each bird, fixed with methanol for 5 min in field and then they were Giemsa's stained for 45 min in the laboratory. Blood was stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA pH 8.0) kept at room temperature in the field and later at –20 °C in the laboratory.

Table I 1. Localities sampled in this study. Features related to elevation, vegetation and number of host sampled are provided.

Elevation (m above sea level)	Geographic Coordinates	Ecosystems	Sample size	Avian Families/ Genera/ Species
2100	04°42' N 75°32' W	High Andean forest	71	13 /36 /37
2400	04°42' N 75°29' W	High Andean forest	157	15 /44 /51
2560	04°38' N 74°05' W	Urban area with vegetation patches	259	15 /33 /39
2900	04°41' N 73°50' W	High Andean forest	287	17 /37 /54
3100	04°37' N 73°43' W	Paramo	126	11 /24/29
3300	04°43'N 75°27'W	Grasslands and high Andean forest	265	13 /34 /45
4000	04°46'N 75°24'W	Paramo	323	13 /20 /20

Blood smears were examined using a Leica DM750 microscope (Leica Microsystems, Heerbrugg, Switzerland), first at low magnification (100x) for 10 min, and then at magnification under oil-immersion (1000x) for at least 20 min. The taxonomic determination of parasites was made following (1) Valkiūnas (2005) keys, (2) recent descriptions of new species (Valkiūnas et al. 2010, 2013, 2007, 2009a, Iezhova et al. 2010, 2011, Križanauskienė et al. 2010, 2012, Greiner et al. 2011, Merino et al. 2012, Zehtindjiev et al. 2012, Levin et al. 2012, Mantilla et al. 2013a); (3) original description for the species found

(Telford and Forrester 1975, Gabaldon and Ulloa 1976, White et al. 1979, Bennett et al. 1987, Burry-Caines and Bennett 1992), and (4) comparing our parasites with parahapantotype material of *Haemoproteus coatneyi* (Accession no. IRCAH 30671), *Haemoproteus vireonis* (Accession no. IRCAH 82659), *Plasmodium vaughani* (Accession no. IRCAH 33021), and *Plasmodium nucleophilum* (Accession no. IRCAH 73800) from the International Reference Collection for Avian Haematozoa IRCAH at the Queensland Museum and Science centre, Australia.

Digital images were prepared using a Leica EC3 digital camera, and processed with LAS EZ software (Leica Microsystems Switzerland Limited, 2012). All slides from infected birds were deposited in the collection Grupo de Estudio Relación Parásito Hospedero (GERPH), Department of Biology, Universidad Nacional de Colombia, Bogotá, Colombia. Digital images of blood stages are available on request from GERPH.

1.3.3 DNA extraction, PCR amplification, and sequencing

Total genomic DNA from blood was extracted by the phenol chloroform method (Sambrook et al. 1989), only from infected blood samples diagnosed by microscopy. Despite having identified 154 infections, we were only able to obtain 57 parasite sequences due to low parasitemia and coinfections. A fragment of 488 bp of the cytochrome *b* gene from the parasite was amplified using a nested PCR modified from Hellgren et al. (2004). First reaction with HaemNFI/HaemNR3 primers was made in a total volume of 12.5 µl and included 5.624 µl of GoTaq® Green Master Mix (Promega, USA), 50 ng DNA template and 0.6 µM of each primer. Second PCR reaction was made with primers HAEMF/HAEMR2 in a 25 µl total volume and included 11.249 µl of GoTaq® Green Master Mix (Promega, USA), 2 µl DNA template from the previous PCR and 0.6 µM of each primer. The thermal profile of the first reaction consisted of an initial step of 94 °C for 3 min, followed by 5 cycles with denaturation at 94 °C for 1 min, annealing at 45 °C for 1.5 min and extension at 72 °C for 1.5 min. Then 25 cycles with denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 45 sec, followed by a final extension at 72 °C for 10 min. The second thermal profile was conducted as described by Hellgren et al. (2004) except that an annealing temperature of 52 °C was used instead of 50 °C.

Amplified products were precipitated with ammonium acetate and 95% ethanol (Bensch et al. 2000) and sequenced in both directions in a 3730xlDNA analyzer (Applied Biosystems, Foster City, California) by Macrogen Inc.

1.3.4 Phylogenetic analysis

Sequences were edited using Sequencher 4.1.4 (Gene Codes, USA), and then aligned with Clustal W (Thompson et al. 1994) implemented in BioEdit version 7.0.5.3 (Hall 1999). Each sequence with at least one different nucleotide was consider a cytochrome b lineage, in order to assign a lineage name, sequences were compared with the specialized MalAvi

database (Bensch et al. 2009) using its local Blast tool (available on: http://mbio-serv2.mbioekol.lu.se/Malavi/blast.html); only sequences with 100% of coverage were used for comparison. A lineage not found either in the MalAvi or Genbank databases was considered a new one and named using the scientific host name and a consecutive number. Alignments were performed separately for *Haemoproteus* and *Plasmodium* lineages; each alignment included unique lineages from our sequences and one lineage representing each morphological species reported in the MalAvi database (alignments available in Online Appendix 1, 2). Our lineages were compared with Genbank database using Blast search in order to record the geographic distribution. The genetic distances were calculated using the Kimura two parameters model of substitution, implemented in the software MEGA version 5.05 (Tamura et al. 2011).

The phylogenetic reconstruction was performed by Bayesian inference. The nucleotide substitution models were selected by jModeltest 2.1.3 using the corrected Akaike Information Criterion (Posada 2008), and corresponds to General Time Reversible including invariable sites and variation among sites (GTR+I+G) for both alignments. The Bayesian analysis was run in MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) through the CIPRES Science Gateway version 3.3 (Miller et al. 2010). Two independent runs of $1.2x10^7$ generations were conducted with four chains, sampling every 500 generation. Convergence was assessed using average standard deviation of split frequencies between the two runs below 0.01 and graphically using Tracer (Rambaut and Drummond 2006). The runs were combined and a 25% of the trees were discarded as burn-in period; in all, 36000 trees were used to construct a majority rule consensus tree. The phylogeny was visualized using FigTree version 1.3.1 (Rambaut 2006).

1.4 Results

1.4.1 Morphological analysis

A total of 1487 birds belonging to 13 orders, 31 families and 166 species were analysed. The overall prevalence was 10%, 56 birds were infected with *Plasmodium* (4%) and 103 with *Haemoproteus* (7%). Five birds showed co-infeccion with both *Plasmodium* and *Haemoproteus* and, 24 birds (1.6%) showed multiple infections with at least two morphospecies belonging to the same genus. All birds captured and their infection statuses are shown in Online Appendix 3.

Eight morphospecies were determined: *Haemoproteus* (*Haemoproteus*) *columbae, Haemoproteus* (*Parahaemoproteus*) *witti, Haemoproteus* (*P.*) *coatneyi, Haemoproteus* (*P.*) *vireonis, Plasmodium* (*Haemamoeba*) *lutzi, Plasmodium* (*Novyella*) *unalis, Plasmodium* (*Huffia*) *hermani*, and *Plasmodium* (*N.*) *rouxi* (Fig. I 1, 2). Moreover, other six undescribed morphospecies were found: *Haemoproteus* (*P.*) sp. 1-3, *Plasmodium* sp. 1-3. Summary of infected bird species and abundance of each parasite morphospecies are provided in Table I 2 and Fig. I 3 respectively.

Haemoproteus (*P*.) *witti* was found in three hummingbird species: *Eriocnemis cupreoventris* (Coppery-bellied puffleg), *Eriocnemis derbyi* (Black-thighed puffleg) and *Eriocnemis vestita* (Glowing puffleg). *Haemoproteus* (*P*.) *witti* had large number of pigment granules (25±3.3 n=44) and its gametocytes did not encircle the nucleus of erythrocytes, instead the nucleus was displaced markedly (Fig. I 1 A-D); that allowed to differentiate this species from *Haemoproteus* (*P*.) *trochili* and *H*. (*P*.) *archilochus* which infect also birds belonging to Trochilidae family.

Haemoproteus (*P*.) *coatneyi* was a relatively common species, it was found in 13 Passeriformes species, with a prevalence of 2%. This species showed gametocytes which are oppressed by both envelope and nucleus of erythrocyte, and did not displace it; the central part of growing gametocytes frequently do not fill up the envelope causing a dip; the pigment granules are medium in size and roundish or oval in form (Fig. I 1 E-H). *Haemoproteus* (*P*.) *vireonis* was only found in *Vireo olivaceus* (Red-eyed vireo), this species was morphologically close to *H*. (*P*.) *coatneyi* except by the presence of medium and small pigment granules in similar proportions (Fig. I 1 I-L).

Haemoproteus (*H*.) *columbae* was found in one *Columba livia* (Rock Pigeon) and was distinguished from other *Haemoproteus* which infect Columbiformes, because this species had gametocytes with pigment granules clumped into compact masses (Fig. I 1 M-P); this feature was more evident in microgametocytes.

Plasmodium (*Huffia*) *hermani* was found in a *Trogon personatus* (Masked trogon); this parasite showed meronts infecting erythroblast. Young meronts had pigment granules golden colored (Fig. I 2 A-D), the fully grown meronts presented a mean number of merozoites of 7±0.7 (n=27), 52% of meronts were arranged as rosettes (Fig. I 2 C-D). The remaining was arranged in row or fan shape; gametocytes were not observed in the slides. This bird also showed co-infection with *P*. (*Novyella*) *rouxi* (Fig. I 2 I-L), which had commonly binuclear meronts and a maximum number of four merozoites; it did not show evident refractive globule but it did resemble to *P*. *rouxi*.

Plasmodium (*Haemamoeba*) *lutzi* was found in five avian species and the main distinctive features were: meronts and gametocytes exceed the length of erythrocyte nucleus (Fig. I 2 F-H), the gametocytes were roundish and their pigment granules clearly tends to clump into one spot, this feature was also observed in trophozoites (Fig. I 2 E-H).

Plasmodium (*Novyella*) *unalis* was found in *Turdus fuscater* (Great thrush) and was determined based on the following features: gametocytes were elongated (Fig. I 2 P); both trophozoites and meronts had refractive globule, vacuole and a single pigment granule (Fig. I 2 M-O).

In reference to the distribution of the parasite species identified, three species of *Haemoproteus* (*H*. *coatneyi*, *H*. sp.1, and *H*. sp. 2) and three of *Plasmodium* (*P*. sp 1, *P*. *unalis*, *P*. *lutzi*) were the most abundant (Fig. I 3, A), 31% of the infections were diagnosed only to the genus level. The frequency of each of the morphospecies was different among elevations, *H*. *coatneyi, H*. sp. 3, *P*. sp 1, and *P*. *lutzi* were present in a wide range of localities (Fig. I 3 B,C), whereas other morphospecies were confined to one or two localities.

Figure I 1. *Haemoprotesus* morphospecies found in this study. A-D: *Haemoproteus* (*Parahaemoproteus*) *witti* from *Eriocnemis derbyi* (Black-thighed puffleg) lineage TROAED20. E-H: *Haemoproteus* (*P.*) *coatneyi* from *Arremon brunneinucha* (Chestnut-capped Brush-finch) lineage ARBRU01. I-L: *Haemoproteus* (*P.*) *vireonis* from *Vireo olivaceus* (Red-eyed Vireo) lineage VIOLI04. M-P: *Haemoproteus* (*Haemoproteus*) *columbae* from *Columba livia* (Rock Pigeon) lineage HAECOL1. A, B, E, F, I, J, M: immature gametocytes. C, G, K, O, P: macrogametocytes. D, H, L, N: microgametocytes. Scale bar = 10 µm.

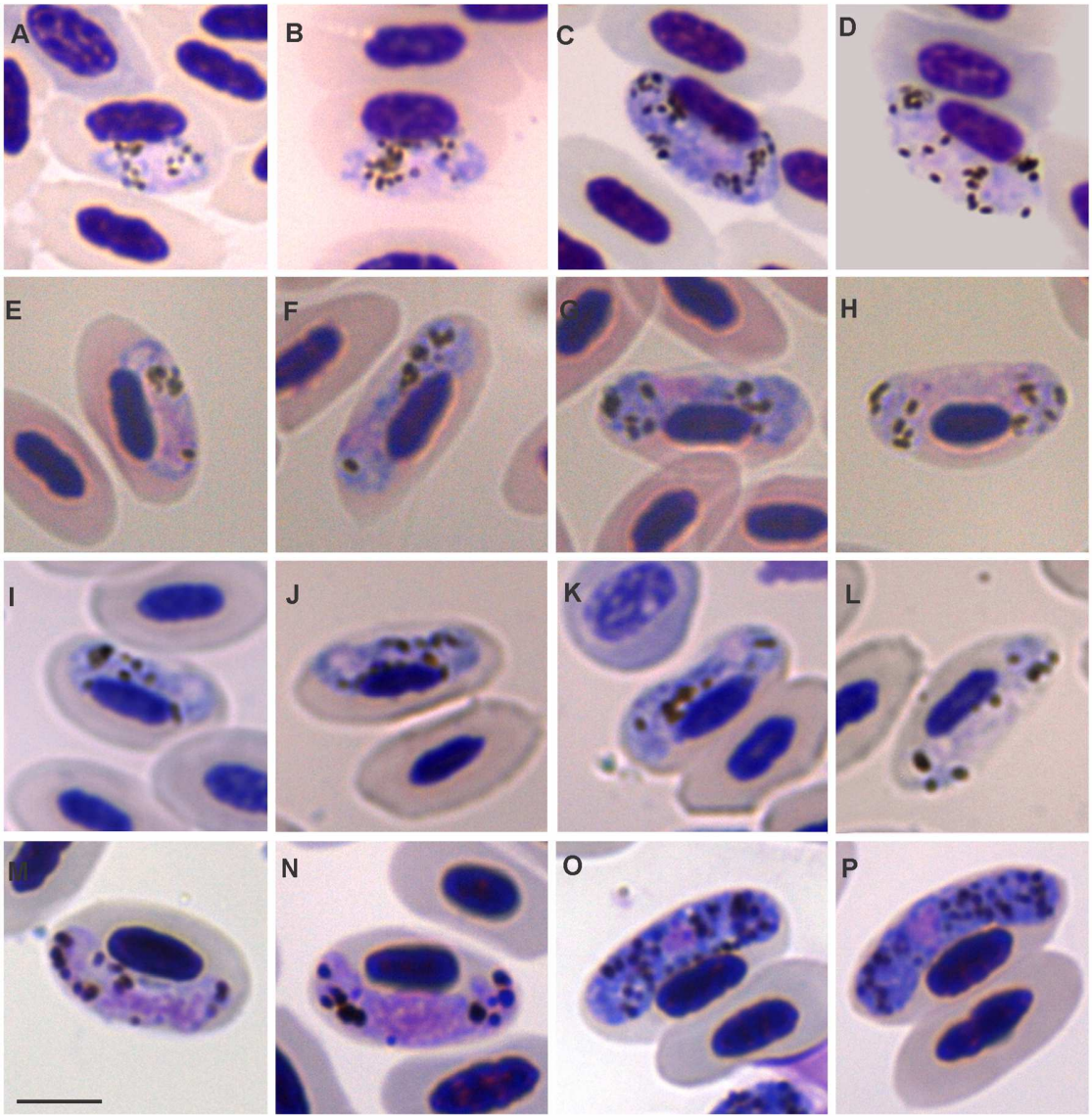


Figure I 2. *Plasmodium* morphospecies found in this study. A-D: *Plasmodium* (*Huffia*) *hermani* from *Trogon personatus* (Masked trogon) lineage TRPER01. E-H: *Plasmodium* (*Haemamoeba*) *lutzi* from *Diglossa cyanea* (Masked Flowerpiercer) lineage DIALB01. I-L: *Plasmodium* (*Novyella*) *rouxi* from *Trogon personatus* (Masked trogon). M-P: *Plasmodium* (*Novyella*) *unalis* from *Turdus fuscater* (Great thrush) lineage TFUS06. E, M: trophozoites. A, B, I: young meronts. C, D, F, J, K, N, O: mature meronts. G, L, P: macrogametocytes. H: microgametocytes. Scale bar = 10 µm.

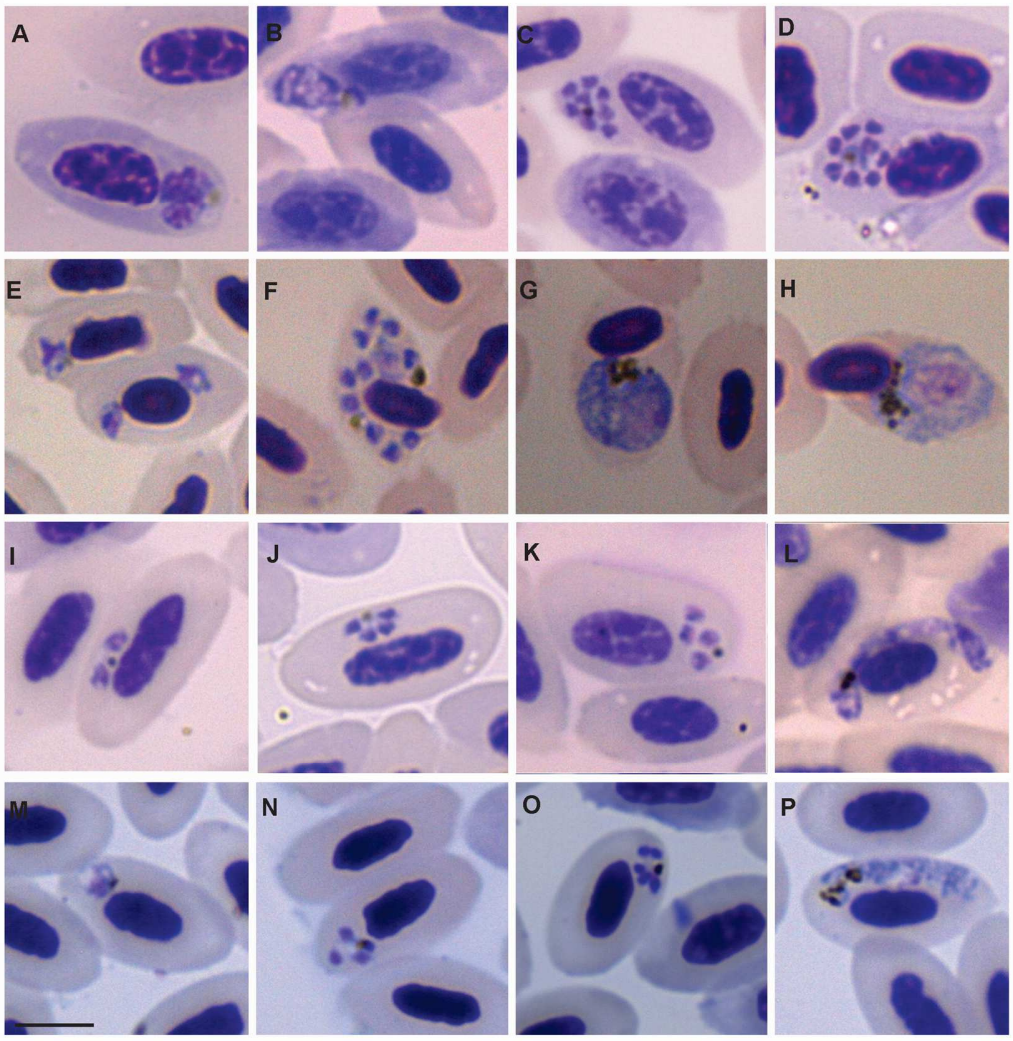
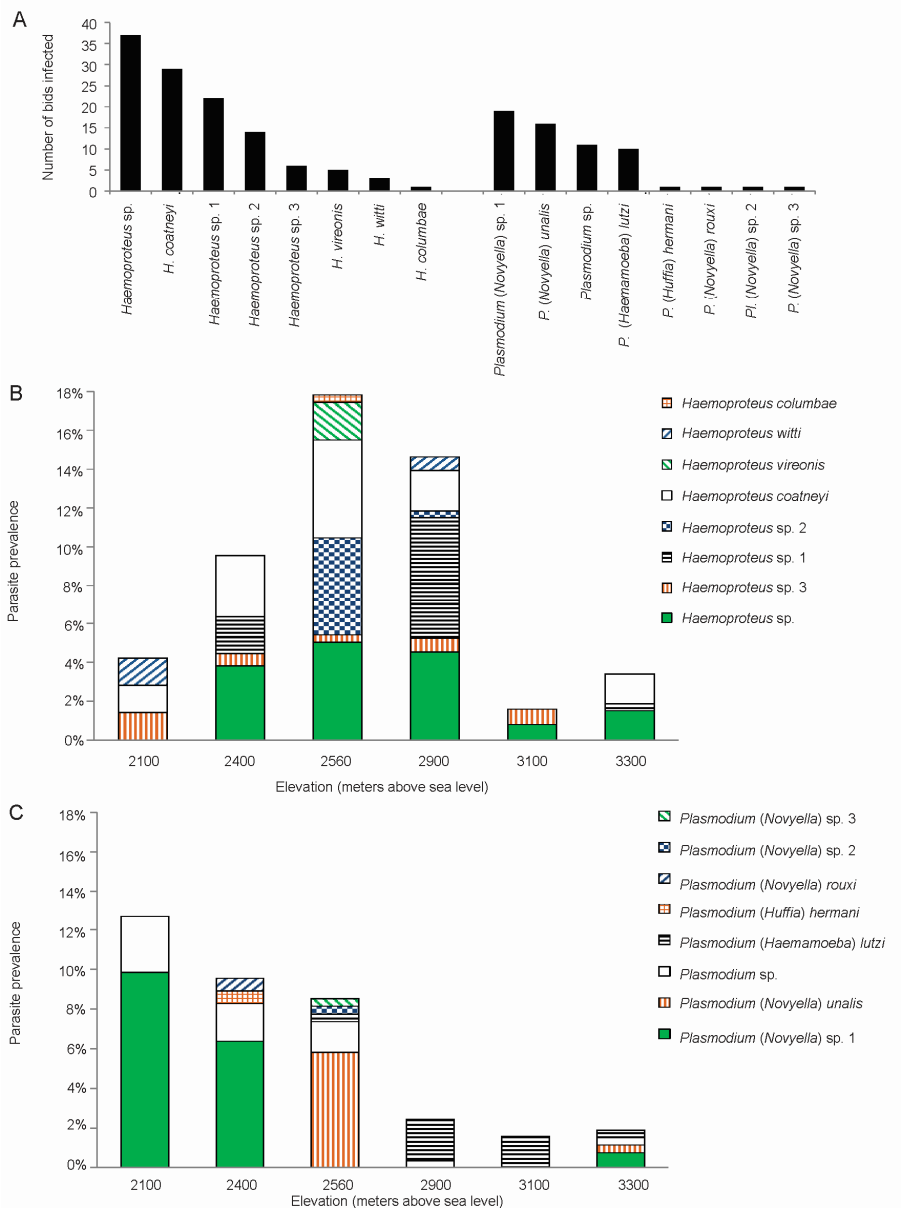


Table I 2. Summary of host-parasite relationships found in this study. In front of parasite lineage is given the host species found in this study and geographic areas where it has been reported in Genbank, if any. [a]: migratory species; [b]: lineage previously reported. n.a.: lineage no identified; *H.* (*P.*): *Haemoproteus* (*Parahaemoproteus*); *P.* (*Hu.*): *Plasmodium* (*Huffia*); *P.* (*N.*): *Plasmodium* (*Novyella*.); *P.* (*Ha.*): *Plasmodium* (*Haemamoeba*).

Parasite morphospecies (parasite lineage)	Host species and geographic areas where has been reported in Genbank
Haemoproteus sp.	*Anisognathus igniventris, Anisognathus lacrymosus, Arremon brunneinucha, Atlapetes pallidinucha, Atlapetes schistaceus, Basileuterus nigrocristatus, Buhtraupis montana, Tangara vassorii, Dendroica fusca* [a]*, Diglossa caerulescens, Diglossa lafresnayii, Hemispingus atropileus, Hemispingus verticalis, Vermivora peregrina* [a]*, Vireo olivaceus* [a]*, Zonotrichia capensis*
H. (*P.*) *witti* (TROAED20 [b])	*Eriocnemis cupreoventris, Eriocnemis derbyi, Eriocnemis vestitu*; Geographic areas reported in Genbank: Peru
H. (*H.*) *columbae* (HAECOL1 [b])	*Columba livia*; Geographic areas reported in Genbank: Botswana, Singapore, USA, India
H. (*P.*) *vireonis* (n.a.)	*Piranga rubra* [a]
H. (*P.*) *vireonis* (VIOLI04)	*Vireo olivaceus* [a]
H. (*P.*) *vireonis* (VIOLI05)	*Vireo olivaceus* [a]
H. (*P.*) *vireonis* (VIOLI06)	*Vireo olivaceus* [a]; Geographic areas reported in Genbank: Peru
H. (*P.*) *coatneyi* (n.a.)	*Anisognathus somptuosus, Arremon brunneinucha, Atlapetes albinucha, Atlapetes pallidinucha, Dendroica fusca* [a]*, Hemispingus superciliaris, Tangara vassorii, Zonotrichia capensis*
H. (*P.*) *coatneyi* (ARBRU01)	*Arremon brunneinucha*
H. (*P.*) *coatneyi* (ATPLA02)	*Atlapetes pallidinucha*
H. (*P.*) *coatneyi* (HEATR02)	*Tangara vassorii, Hemispingus atropileus, Hemispingus atropileus*
H. (*P.*) *coatneyi* (PACPEC02 [b])	*Piranga rubra* [a]; Geographic areas reported in Genbank: Papua New Guinea, Brazil
H. (*P.*) *coatneyi* (PIOLI03)	*Piranga olivacea* [a]
H. (*P.*) *coatneyi* (TANIG01)	*Tangara nigroviridis, Hemispingus superciliaris, Tangara vassorii, Vireo olivaceus* [a] Geographic areas reported in Genbank: Peru
H. (*P.*) *coatneyi* (ZOCAP08)	*Zonotrichia capensis*
H. (*P.*) sp. 1(n.a.)	*Anisognathus igniventris, Anisognathus somptuosus, Buhtraupis Montana, Hemispingus superciliaris, Hemispingus verticalis, Tangara vassorii*
H. (*P.*) sp. 1 (ANIGN01)	*Anisognathus igniventris*
H. (*P.*) sp. 2 (n.a.)	*Zonotrichia capensis*
H. (*P.*) sp. 2 (ZOCAP09)	*Zonotrichia capensis*; Geographic areas reported in Genbank: Peru, Chile
H. (*P.*) sp. 2 (ZOCAP10)	*Zonotrichia capensis*; Geographic areas reported in Genbank: Peru, Venezuela
H. (*P.*) sp. 3 (n.a.)	*Diglossa lafresnayii, Hemispingus atropileus, Hemispingus superciliaris, Tangara nigroviridis, Zonotrichia capensis*
Plasmodium sp. (n.a.)	*Anisognathus igniventris, Arremon brunneinucha, Vermivora peregrina* [a]*, Piranga rubra* [a]*, Vireo olivaceus* [a]*, Zonotrichia capensis*
Plasmodium sp. (VEPER01)	*Vermivora peregrina* [a]
P. (*Hu.*) *hermani* (TRPER01)	*Trogon personatus*
P. (*N.*) *rouxi* (n.a.)	*Trogon personatus*
P. (*Ha.*) *lutzi* (n.a.)	*Diglossa cyanea, Turdus fuscater*
P. (*Ha.*) *lutzi* (DIALB01)	*Diglossa cyanea, Diglossa lafresnayii, Diglossa cyanea, Diglossa cyanea, Anisognathus lacrymosus, Diglossa albilatera*; Geographic areas reported in Genbank: Peru
P. (*Ha.*) *lutzi* (DICYA02)	*Diglossa cyanea*
P. (*Ha.*) *lutzi* (DILAF01)	*Diglossa lafresnayi*
P. (*Ha.*) *lutzi* (TFUS05 [b])	*Turdus fuscater*
P. (*N.*) *unalis* (n.a.)	*Turdus fuscater*
P. (*N.*) *unalis* (TFUS06 [b])	*Turdus fuscater*
P. (*N.*) sp. 1 (n.a.)	*Chlorospingus flavigularis, Myioborus ornatus, Zonotrichia capensis*
P.(*N.*) sp. 1 (BAEBIC02 [b])	*Anisognathus somptuosus, Atlapetes albinucha, Basileuterus coronatus, Zonotrichia capensis;* Geographic areas reported in Genbank: USA, Costarica, Peru
P. (*N.*) sp. 2 (CATUST07)	*Catharus ustulatus* [a]

P. (*N.*) sp. 3 (VIOLI07) *Vireo olivaceus* [a]

Figure I 3. Number of birds infected and prevalence for each morphospecies found in this study. In total 56 birds were infected with *Plasmodium*, 103 with *Haemoproteus* and 1487 were examined. The summary of infection in the graphics may not coincide with total birds infected due to co-infection. *Haemoproteus* sp. and *Plasmodium* sp. indicate cases where identification was carried out to genus level. A: number of birds infected with each morphospecies. B: prevalence of each *Haemoproteus* morphospecies in function of elevation. C: prevalence of each *Plasmodium* morphospecies in function of elevation.



1.4.1 Phylogenetic analysis of cytochrome b lineages

Parasite DNA was successfully amplified from 57 blood samples (GenBank nos. KF537276-KF537331, KF780795, Online Appendix 3), from which 25 lineages were identified, 17 of them were present in resident birds and nine in migratory birds (Table I 2). A single lineage (TANIG01) was shared between a resident and a Neartic migratory bird. Eighteen lineages are reported for the first time in this study (Table I 2).

Both *Plasmodium* and *Haemoproteus* phylogenetic reconstructions showed low phylogenetic resolution, making impossible to infer deep relationship among species. However, cytochrome b lineages showed a good correspondence in six of eight morphospecies for which more than one individual was analyzed. For species: *H*. *witti*, *H*. sp. 1, *H*. sp. 2, *P*. sp. 1, *P*. *unalis,* and *P*. *lutzi* were found to exhibit intraspecific genetic variation of 0.0-0.4%. For *H*. *coatneyi* and *H*. *vireonis* we found mean intraspecific genetic variation of 1.9% and 2.1% respectively.

The phylogenetic reconstruction of *Haemoproteus* spp. showed two well-supported clusters corresponding to each subgenus (Fig. I 4). The cluster A shows the subgenus *Haemoproteus* (*Haemoproteus*), in which *Haemoproteus columbae* lineage HAECOL1 cluster, and appeared closely related with *H*. *multipigmentatus* (posterior probability = 1). Cluster B included the subgenus *Haemoproteus* (*Parahaemoproteus*). *H*. *coatneyi* from which we found more than one lineage do not appear as monophyletic group. *H*. *vireonis* appeared collapsed in the three.

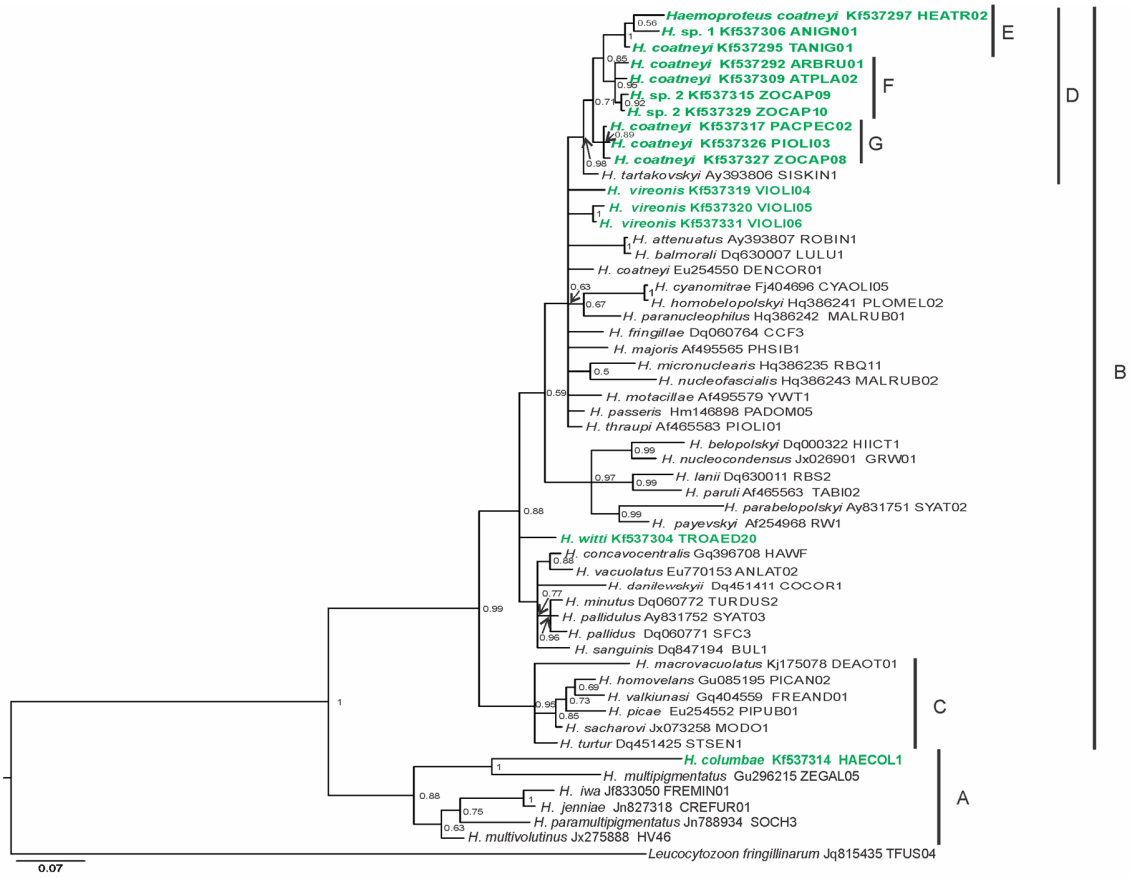
Haemoproteus (*P*.) *witti* was found in three hummingbird species: *E*. *cupreoventris, E*. *derbyi* and E. vestita; all sequences were identical and corresponded to lineage TROAED20. This is the first lineage reported that allow a linkage with the *H*. *witti* morphoespecies, however a blast search found identical sequences reported in Peru from seven Trochilidae species (JQ988105, JQ988133, JQ988445, JQ988268, JQ988331, JQ988294, JQ988312), as well as two Passeriformes species (JQ988559, JQ988210). *Haemoproteus* (*P*.) *witti* lineage TROAED20 infects species of order Apodiformes, but it did not cluster within the group C that was formed by parasite species that infect non-Passeriformes hosts (Fig. I 4). *Haemoproteus witti* did not show to be closely related with any morphospecies.

Lineages VIOLI05 and VIOLI06 from *H*. *vireonis* found in this study clustered into a well-supported cluster. However, neither these nor VIOLI04 lineages showed be closely related with any other mophospecies (Fig. I 4).

Ten out fourteen *Haemoproteus* (*Parahaemoproteus*) lineages recorded in this study cluster on the well-supported cluster D (posterior probability =0.98), which includes four species: *H*. *tartakovsky* lineage SISKIN1, *H*. *coatneyi, Haemoproteus* sp. 2, and *Haemoproteus* sp. 1 (Fig. I 4). The cluster E included *Haemoproteus* sp. 1 lineage ANIGN01 and two lineages of *H*. *coatneyi*, these three lineages appeared infecting five Thraupidae species and one Vireonide species. The cluster F included two lineages of *Haemoproteus* sp. 2 (genetic distance=0.2%) and two lineages of *H*. *coatneyi*, these four

lineages were infecting host species from the Emberizidae family. The cluster G was composed by three lineages of *H. coatneyi*. ZOCAP08 lineage was recorded in one resident bird belonging to host family Emberizidae, and PACPE02 and PIOLI03 were found in migratory birds of Cardinalidae family.

Figure I 4. Bayesian phylogeny of *Haemoproteus* spp. based on 54 cytochrome b lineages (488 bp). *Leucocytozoon fringillinarun* TFUS04 was used as outgroup. Names of the morphological species followed by GenBank accession number and MalAvi lineage. Lineages reported in this study are given in green bold. Nodal support indicates posterior probability. Scale bar indicates amount of change.



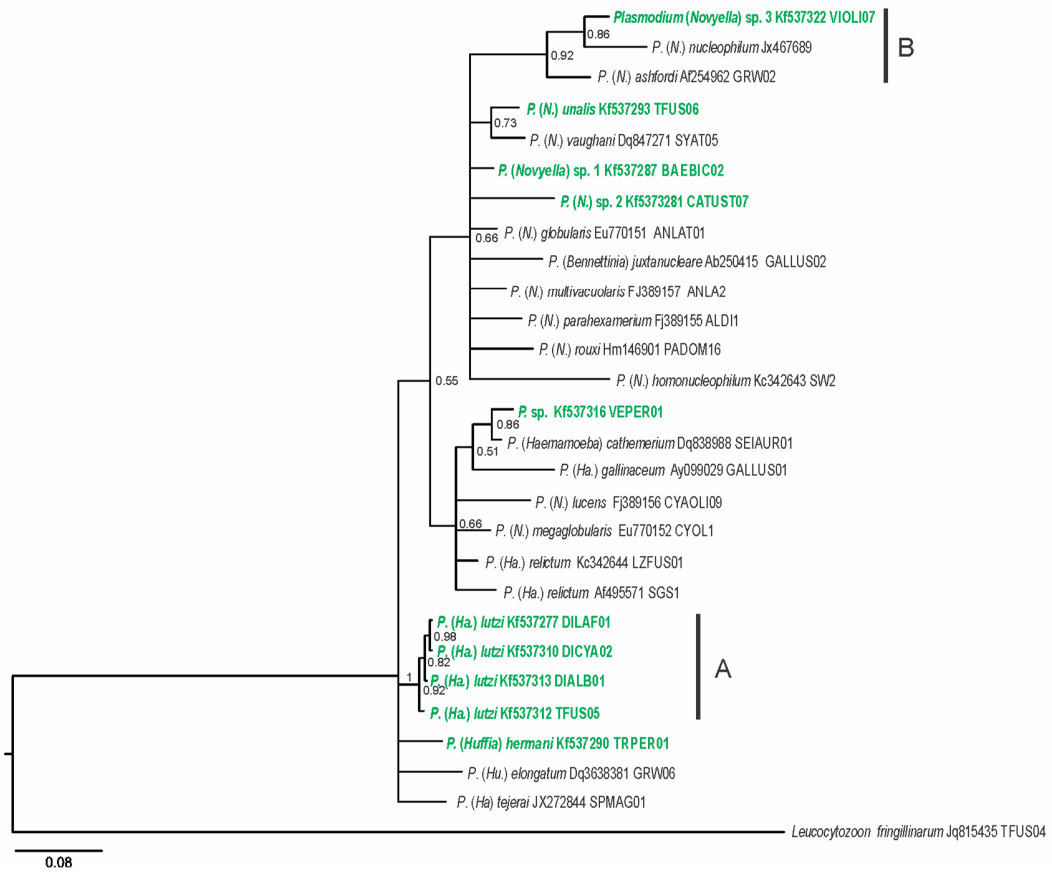
Regarding *Plasmodium* phylogeny (Fig. I 5),), it attract attention that none of the subgenera from which we included more than one morphospecies (*Novyella* 13 sps., *Huffia* 2 sps., *Haemamoeba* 5 sps.) turned out monophyletic. *P*. (*Haemamoeba*) *lutzi* clustered in the well-supported clade A (Fig. I 5) which were found infecting both Thraupidae and Turdidae species. *P*. *lutzi* exhibited an intraspecific genetic distance of 0.2%-0.8% (mean=0.4%).

Plasmodium (*N*.) sp. 3 VIOLI07 lineage from *Vireo olivaceus* grouped in the well-supported cluster B (Fig. 5, posterior probability = 0.92) along with *P*. (*N*.) *ashfordi* and *P*. (*N*.) *nucleophilum*, this later species showed genetic distance of 3.8% with VIOLI07.

Plasmodium (*N*.) *unalis* lineage TFUS06 included identical sequences from six individuals and seems to have preference to infect only Turdidae taxa, particularly *Turdus fuscater*. *P*. (*N*.) *vaughani* lineage SYAT05 was the most similar lineage to *Plasmodium* (*N*.) *unalis* with genetic distance of 3.0%. On the other hand, *Plasmodium* (*Novyella*) sp. 1 BAEBIC02 lineage included identical sequences from 10 individuals from three families of Passeriformes. This parasite lineage has been recorded previously in *Baelophus bicolor* (AF465555)*, Geothlypis trichas* (EU328171), and *Tangara icterocephala* (JN819334).

Plasmodium lineage VEPER01 from *Vermivora peregrina* appeared closely related with *P*. (*Ha*.) *cathemerium* SEIAUR01. However, neither *P*. (*Huffia*) *hermani*, *Plasmodium* (*Novyella*) sp. 2, nor *P*. (*N*.) sp. 1 cluster with other morphospecies.

Figure I 5. Bayesian phylogeny of *Plasmodium* spp. based on 28 cytochrome b lineages (488 bp). *Leucocytozoon fringillinarun* TFUS04 was used as outgroup. Names of the morphological species followed by GenBank accession number and MalAvi lineage. Lineages reported in this study are given in green bold. Nodal support indicates posterior probability. Scale bar indicates amount of change.



Blast searches of *Plasmodium* and *Haemoproteus* (*Parahaemoproteus*) lineages found in this study showed identical or more often similar (99% identity) sequences from both migratory and resident bird species, distributed in the New world. However, some lineages were only detected in migratory birds: *H*. *vireonis* lineages VIOLI04-05 were only found in *Vireo* spp., *Plasmodium* (*N*.) sp. 2 lineage CATUST07 in *Catharus* spp. and *Myadestes* spp, and *Plasmodium* (*N*.) sp. 3 lineage VIOLI07 in *Vireo olivaceus*. In the other hand *Haemoproteus* (*P*.) sp. 1 lineage ANIGN01 was only found infecting Thraupidae resident species.

1.5 Discussion

The Neotropical region offers a great opportunity for research on avian haemoparasites, this study found new host–parasite relationships, probably due to high avian diversity associated with the uplift of the Andes, and a scarce number of studies on this topic made in the region (Valkiūnas et al. 2003, Munro et al. 2009, Rodríguez et al. 2009, Lotta et al. 2013, Mantilla et al. 2013a, 2013b, Jones et al. 2013). An increasing number of lineages from avian haemosporida have been recorded in the last decade. However, only few studies have also determined morphologically the parasites associated with those lineages (Valkiūnas et al. 2008). Additionally, recent information demonstrates a high frequency of abortive infections (Olias et al. 2011, Levin et al. 2013) that incorrectly, could be taken as true when only molecular analysis are made (Valkiūnas et al. 2014).

This study has shown that the association between morphospecies and molecular lineages is variable since most of species identified in this study showed a strong correspondence, with molecular lineage with genetic distances close to 0%, such as the cases of *H*. (*P*.) *witti*, *P*. (*Ha*.) *lutzi, P*. (*N*.) *unalis*, *Plasmodium* (*N*.) sp. 1, *Haemoproteus* (*P*.) sp. 1, and *Haemoproteus* (*P*.) sp. 2; whereas, only two morphospecies exhibited different molecular lineages and corresponded to the same morphology such as *H*. (*P*.) *coatneyi* and *H*. (*P*.) *vireonis*, the latter may be examples of cryptic species in haemosporida as has been reported by (Sehgal et al. 2006); however, studies of species delimitation must be addressed by using both mitochondrial and nuclear evidence.

Valkiūnas et al. (2009a), Valkiūnas et al. (2010) and Hellgren et al. (2007), have remarked that morphospecies with genetic distances larger than 5% for cytochrome b can be morphologically distinguishable; however different morphospecies may have genetic distances smaller than 5% as the cases of *H*. *minutus-H*. *pallidus* (Hellgren et al. 2007). This cut point of genetic and morphological similarity is different for *Plasmodium* spp. and *Haemoproteus* spp., most of *Haemoproteus* species described have intraspecific variation less than 5%; whilst for *Plasmodium* the 99.6% of pair-wise comparisons for the *Plasmodium* morphospecies included in this study showed values larger than 3% (data no shown), as was reported in *Plasmodium* species infecting lizards (Perkins 2000). Such differences between *Plasmodium* and *Haemoproteus* species could be due to the fact that for *Plasmodium* taxonomy there are more morphological features available to distinguish between species, for example features related with trophozoites and meronts.

Regarding phylogenetic relationships, cytochrome b marker exhibited low phylogenetic resolution, for both genera. Additional molecular markers with different substitution rates to

assess the evolutionary history of haemosporidian parasites are desirable, as well as, an increase of taxa sampling specially in parasite species from America and non-Passeriformes birds.

The highlands of the Neotropical region have been scarcely sampled, and a potential large number of new host–parasite relationships remain unknown. Thus, almost half of the parasite morphospecies recorded here still wait to be described. The lineages associated with these parasites are likely to have a distribution throughout the Andean ecosystems, since they were found infecting several host taxa with Andean distribution. Previously 34 *Haemoproteus* and 21 *Plasmodium* species have been recorded in Neotropical birds (Apendix I 1). From which we found five species restricted to America (*H*. (*P*.) *witti; H*. (*P*.) *vireonis, P*. (*Ha*.) *lutzi, P*. (*N*.) *unalis* and *P*. (*Hu*.) *hermani*) and three widely distributed (*H*. (*H*.) *columbae, H*. (*P*.) *coatneyi* and *Plasmodium* (*N*.) *rouxi*).

So far *Haemoproteus* (*P*.) *witti* has been reported only for the type locality, Jamaica (White et al. 1979, Valkiūnas 2005); the present results expand the distribution of this parasite to Colombia and Peru, since the same lineage was recorded there. Moreover, this is also the first report that allows a linkage between morphology of *H*. (*P*.) *witti* with the cytochrome b lineage TROAED20.

The same lineage of *Haemoproteus* (*P*.) *witti* found in Colombia was reported in two Passeriformes species (JQ988559, JQ988210) in Peru. This result widens the family host specificity reported to *H*. (*P*.) *witti*, although this could be an artifact, as a result of using only molecular tools. Since the PCR technique is able to amplify sporozoites DNA from a recent vector bite (Valkiūnas et al. 2009b), an abortive infection cannot be discarded. A true new host-parasite relationship should be confirmed by the presence of gametocytes in blood.

We found co-infection of *Plasmodium* (*Hu*.) *hermani* and *Plasmodium* (*N*.) *rouxi* in *Trogon personatus*, this finding is interesting since infected Trogoniformes are rarely reported. Currently, there are only two reports of *Plasmodium* infection in Trogoniformes (White et al. 1978, Bennett et al. 1993). Additionally, we report the first cytochrome b lineage associated with *Plasmodium* (*Hu*.) *hermani* TRPER01. In spite of co-infection, it is possible to compare TRPER01 lineage with *P*. (*N*.) *rouxi* PADOM16 lineage and since the difference between lineages is 9%, it was possible to associate TRPER01 to *Plasmodium* (*Hu*.) *hermani*.

The most common *Plasmodium* subgenera found were *P*. (*Novyella*) and *P*. (*Haemamoeba*), however the latter was only represented by *P*. (*H*.) *lutzi*. Rodriguez et al. (Rodríguez et al. 2009) reported birds infected with *P*. (*Novyella*) sp. and *P*. (*Haemamoeba*) sp. at 3100 m above sea level in Colombia. We double checked the material deposited in the GERPH collection and we were able to determine that these species corresponded to *P*. (*N*.) *unalis* and *P*. (*Ha*.) *lutzi*. Interestingly, we did not find infection by *P*. (*N*.) *vaughani* or *P*. (*Ha*.) *relictum*, which are very frequent and cosmopolitan parasites (Valkiūnas 2005); and they have been reported in lowland localities of Neotropical countries (Bennett et al. 1980, Sousa and Herman 1982, Woodworth-Lynas et al. 1989, Young et al. 1993). The apparent absence of these common parasites in the localities sampled may be due to a

differential distribution of haemoparasites through altitudinal ranges, associated with changes in temperature, hosts and vectors. LaPointe et al. (2010) reported the inability of *P*. (*Ha*.) *relictum* to develop sporozoites below 13°C, this temperature coincides with a 1800 m above sea level belt; up of this elevation the prevalence of *P*. (*H*.) *relictum* is low. Bearing in mind that all data gathered in this study come from elevations up to 2100 m above sea level, our results suggest a replacement of avian haemoparasite fauna, where the same subgenera in highlands, but represented by other species able to develop their climatic conditions.

Regarding the altitudinal distribution, the parasites taxa and their prevalences were not homogeneous among localities. At each elevation, was found *Haemoproteus coatneyi* accompanied with *Plasmodium* and *Haemoproteus* species prevalent only in some localities (*Haemoproteus* sp. 1, *Haemoproteus* sp. 2, *Plasmodium* sp. 1, *Plasmodium unalis*, *Plasmodium lutzi*). Specific bird species and vector composition at each site may be affecting transmission cycles and thus parasites found. One example may be the urban area of Bogotá (2560 m above sea level) which presents a strong association between the two most abundant bird species (*Turdus fuscater* and *Zonotrichia capensis*) with two high prevalent parasites (*Plasmodium unalis* and *Haemoproteus* sp. 2) which have restricted host range.

The parasites lineages of *H*. (*P*.) *vireonis* (VIOLI04, VIOLI05, VIOLI06)*, Plasmodium* (*N*.) sp. 2 (CATUST07) and *Plasmodium* (*N*.) sp. 3 (VIOLI07) were found only in migratory species after a blast search indicating that the infection could have been acquired in the breeding area. Differences in vector or avian fauna, as well as parasite-host compatibility or the low intensity of infection (Valkiūnas et al. 2003, Medeiros et al. 2013) could hamper the establishment of these lineages in Colombia. Levin et al. (2013), reported a remarkable case in the Galapagos Islands, where three lineages shared between resident and migratory birds were detected using a molecular survey; however, infective stages for vector (gametocytes) were not found in any of 3726 resident birds sampled, suggesting that transmission cycle is not established in the Galapagos Islands.

In contrast, in the present study only one *H*. (*P*.) *coatneyi* lineage (TANIG01) was shared between three resident and one Neartic migratory bird species (*Vireo olivaceus*); we did not find this lineage in North American non-migratory birds after a blast search. In order to explain this result we have three hypothesis: (1) *Vireo olivaceus* is being infected with TANIG01 in the non-breeding area, but it could clear the infection before arriving to the breeding area, (2) parasites are cleared from the blood stream when birds are in the breeding area and they reappear after migration in the non-breeding area to be suitable for a competent vector, and (3) the parasite is highly virulent and kill the host. The Neotropical ecosystems represent an important opportunity to develop research on patterns of transmission, infection and pathogenicity of avian haemoparasites between resident and migratory bird species.

As concluding remarks, we present evidence regarding the importance to generate basic information supported by both morphological and molecular approaches; which would avoid considering as true some host-parasite relationships that in fact may be abortive infections. Cytochrome *b* proved to be useful for species determination despite its limited phylogenetic resolution, supporting its use as barcode sequence. Other molecular markers are

necessary to improve resolution at deeper nodes and explore to which extent cytochrome b lineages are representing reproductively isolated lineages. Our findings show great potential to discover new host-parasite relationships in the Andean landscapes and point out to the importance of implementing different capture methods in addition to mist nets to focus research on non-Passeriforme birds.

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1.8 Appendix

Appendix I 1. Distribution of *Haemoproteus* and *Plasmodium* species reported for Neotropical region (Woodworth-Lynas et al. 1989; Valkiūnas 2005; Valkiūnas et al. 2010; Levin et al. 2012; Mantilla et al. 2013a). [a]: Haemoproteus species reported in bird families distributed only in America.

Species	Geographic distribution
Haemoproteus (*Haemoproteus*) *janniae*	Only Neotropic
Haemoproteus (*H.*) *multipigmentatus*	Only Neotropic
Haemoproteus (*Parahaemoproteus*) *apodus*	Only Neotropic
Haemoproteus (*P.*) *bucconis*	Only Neotropic
Haemoproteus (*P.*) *circumnuclearis*	Only Neotropic
Haemoproteus (*P.*) *cracidarum*	Only Neotropic
Haemoproteus (*P.*) *formicarius*	Only Neotropic
Haemoproteus (*P.*) *furnarius*	Only Neotropic
Haemoproteus (*P.*) *ortalidum*	Only Neotropic
Haemoproteus (*P.*) *souzalopesi*	Only Neotropic
Haemoproteus (*P.*) *tinnunculi*	Only Neotropic
Haemoproteus (*P.*) *trochili*	Only Neotropic
Haemoproteus (*P.*) *witti*	Only Neotropic
Plasmodium (*Giovannolaia*) *gabaldoni*	Only Neotropic
Plasmodium (*G.*) *pinottii*	Only Neotropic
Plasmodium (*Haemamoeba*) *lutzi*	Only Neotropic
Plasmodium (*Ha.*) *tejerai*	Only Neotropic
Plasmodium (*Huffia*) *huffi*	Only Neotropic
Plasmodium (*Novyella*) *bertii*	Only Neotropic
Plasmodium (*N.*) *columbae*	Only Neotropic
Plasmodium (*N.*) *forresteri*	Only Neotropic
Plasmodium (*N.*) *paranucleophilum*	Only Neotropic
Haemoproteus (*P.*) *archilochus* [a]	Only Neotropic

Haemoproteus (*P.*) *beckeri* [a]	Neotropic and Neartic
Haemoproteus (*P.*) *quiscalus* [a]	Neotropic and Neartic
Haemoproteus (*P.*) *tyranny* [a]	Neotropic and Neartic
Haemoproteus (*P.*) *vireonis* [a]	Neotropic and Neartic
Plasmodium (*G.*) *pedioecetae*	Neotropic and Neartic
Plasmodium (*N.*) *hexamerium*	Neotropic and Neartic
Haemoproteus (*H.*) *iwa*	Neotropic and Neartic
Haemoproteus (*P.*) *borgesi*	America and other continets
Haemoproteus (*P.*) *coatneyi*	America and other continets
Haemoproteus (*P.*) *fallisi*	America and other continets
Haemoproteus (*P.*) *handai*	America and other continets
Haemoproteus (*P.*) *orizivorae*	America and other continets
Haemoproteus (*P.*) *trogonis*	America and other continets
Plasmodium (*Bennettinia*) *juxtanucleare*	America and other continets
Plasmodium (*N.*) *rouxi*	America and other continets
Haemoproteus (*H.*) *columbae*	America and other continets
Haemoproteus (*H.*) *sacharovi*	Cosmopolitan
Haemoproteus (*P.*) *fringillae*	Cosmopolitan
Haemoproteus (*P.*) *nettionis*	Cosmopolitan
Haemoproteus (*P.*) *noctuae*	Cosmopolitan
Haemoproteus (*P.*) *passeris*	Cosmopolitan
Haemoproteus (*P.*) *plataleae*	Cosmopolitan
Haemoproteus (*P.*) *syrnii*	Cosmopolitan
Haemoproteus (*P.*) *wenyoni*	Cosmopolitan
Plasmodium (*G.*) *circumflexum*	Cosmopolitan
Plasmodium (*G.*) *polare*	Cosmopolitan
Plasmodium (*Ha.*) *cathemerium*	Cosmopolitan
Plasmodium (*Ha.*) *relictum*	Cosmopolitan
Plasmodium (*Hu.*) *elongatum*	Cosmopolitan
Plasmodium (*N.*) *nucleophilum*	Cosmopolitan
Plasmodium (*N.*) *vaughani*	Cosmopolitan

Online Appendix captions

Online Appendix 1. Alignment of 54 cytochrome *b* lineages (488 bp) of *Haemoproteus* in fasta format.

Online Appendix 2. Alignment of 28 cytochrome *b* lineages (488 bp) of *Plasmodium* in fasta format.

Online Appendix 3. Avian species analyzed in this study, individuals captured and infected are shown. The excel spreadsheet shows for each bird captured the following data: elevation (m above sea level), host bird taxonomy, infection status of each genera and parasite species, co-infection status, Genbank number of sequence isolated and parasite lineage.

2. Chapter II. Description and molecular characterization of *Plasmodium* (*Novyella*) *unalis* sp. nov. from the Great Thrush (*Turdus fuscater*) in highland of Colombia

Descripción y caracterización molecular de *Plasmodium* (*Novyella*) *unalis* sp. nov. en la mirla común (*Turdus fuscater*) en tierras altas de Colombia

Se encontró *Plasmodium* (*Novyella*) *unalis* sp. nov. en la mirla común, *Turdus fuscater* (Passeriformes, Turdidae) en Bogotá, Colombia a 2560 m sobre el nivel del mar donde la transmisión activa ocurre. Este parásito es descrito con base en la morfología de sus estados sanguíneos y un fragmento de gen mitocondrial citocromo b (linaje UN227). Se presentan ilustraciones de los estadios sanguíneos de la nueva especie y el análisis filogenético identifica las especies y linajes de parásitos cercanamente relacionados. La nueva especie es similar a *P*. (*N*.) *vaughani* (linaje SYAT05), un parasito de malaria aviar de distribución cosmopolita; esos parásitos son también genéticamente cercanos con una diferencia genética de 3.2% entre ellos. *Plasmodium unalis* puede ser fácilmente diferenciado morfológicamente de *Plasmodium* (*Novyella*) *vaughani* primariamente debido a (1) la presencia de un único y grande granulo de pigmento de forma circular, tanto en merontes como en trofozoitos eritrociticos, (2) la presencia de prominentes vacuolas en trofozoitos y merontes en crecimiento, (3) la presencia de merontes eritrociticos predominantemente en forma de abanico. Linajes de citocromo b con alta similitud al de la nueva especie han sido reportados en Costa Rica, Brasil, Chile and EU. Es probable que la nueva especie de malaria aviar sea ampliamente distribuido en el Nuevo Mundo. Este parasito ha sido reportado únicamente es *Turdus fuscater* en el sitio de estudio y podría tener y restringido rango de hospedero aviares. El registro de *P*. *unalis* es de particular interés teórico debido a si activa transmisión en tierras altas de los Andes. Se discute la posible influencia de la urbanización en la transmisión de este parasito de malaria aviar en Bogotá.

Palabras clave: malaria aviar, Colombia, hemosporidios, tierras altas, nueva especie, urbanización.

Este artículo abarca parcialmente los tres objetivos del proyecto, *al dar la descripción morfológica y molecular de una especie nueva de parásito encontrada en Colombia en un ave residente de distribución Andina*. *Mi contribución a este artículo radicó en la colecta de muestras en campo*, *la lectura de extendidos sanguíneos y el desarrollo de los análisis filogenéticos para la caracterización molecular de la nueva especie*. *También participé en la escritura del artículo en lo que corresponde a metodología*, *resultados y discusión de la caracterización molecular*.

Description and molecular characterization of *Plasmodium* (*Novyella*) *unalis* sp. nov. from the Great Thrush (*Turdus fuscater*) in the highlands of Colombia

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2.1 Abstract

Plasmodium (*Novyella*) *unalis* sp. nov. was found in the Great Thrush, *Turdus fuscater* (Passeriformes, Turdidae) in Bogotá, Colombia at 2560 m above sea level where the active transmission occurs. This parasite is described based on the morphology of its blood stages and a fragment of the mitochondrial cytochrome *b* gene (lineage UN227). Illustrations of blood stages of the new species are given and the phylogenetic analysis identifies closely related species and lineages of avian malaria parasites. The new species is most similar to *Plasmodium* (*Novyella*) *vaughani* (lineage SYAT05), a cosmopolitan avian malaria parasite; these parasites are also closely related genetically, with a genetic difference of 3.2 % between them. *Plasmodium unalis* can be readily distinguished from the latter species morphologically, primarily due to (1) presence of a single large, circular shaped pigment granule in the erythrocytic trophozoites and meronts, (2) presence of prominent vacuoles in throphozoites and growing meronts, 3) presence of predominantly fan-like shaped erythrocytic meronts. Cytochrome *b* lineages with high similarity to the new species have been reported in Costa Rica, Brazil, Chile and USA. It is probable that the new species of malaria parasite is widely distributed in the New World. This parasite has been reported only in the Great Thrush at the study site and might have narrow range of avian hosts. Records of *P. unalis* are of particular theoretical interest due to its active transmission at highlands in Andes. Possible influence of urbanization on transmission of this malaria parasite in Bogotá is discussed.

2.2 Introduction

The evolution of vector-borne parasites involves interactions with host and vector implying complex relationships, during which parasites need to survive under the pressure of the immunological and physiological barriers both in vector and vertebrate host. On the other hand, behaviour and ecology of vectors and birds, as well as environmental factors, may

restrict the development of the parasite, especially in vectors (Shahabuddin and Costero 2001, Higgs and Beaty 2004, Santiago-Alarcon et al. 2012). The restrictions are important for spread of vector-borne parasitic infections, including avian malaria.

The Great Thrush *Turdus fuscater* is not a migratory species and has a broad distribution along the Andes from Venezuela to Bolivia (Ridgely et al. 2012). In Colombia, it has been reported between 1400 and 4100 m above sea level. This is an opportunistic bird species that is well adapted to urban ecosystems. Habitats of this bird are associated with open and grass areas, characterized by hedges, short grass pastures and isolated patches of shrubs. The Great Thrush has been reported less frequently in wooded areas, on the roofs of buildings, or near water bodies.

With recent advances in molecular biology, the large hidden diversity of avian haemoparasites has attracted the attention of researcher. However few studies address linkages between morphospecies and DNA lineages of parasites; this is particularly true for avian malaria parasites in the Neotropics. Molecular characterization is important for the diagnosis of parasitic infections, but this is a difficult task because of the predominantly low intensity of infection and the widespread haemosporidian co-infections; it is not always possible to determine readily which parasite DNA sequence was amplified from a sample, when several parasites are visible under the microscope (Zehtindjiev et al. 2012a). That calls for a careful and rigorous analysis in studies addressing the event of develop molecular markers for certain parasite morphospecies.

The aims of this study were (1) to provide the morphological descriptions of one new *Plasmodium* (*Novyella*) species, (2) to determine mitochondrial cytochrome *b* gene (Cyt *b*) sequences for molecular identification and diagnosis of this parasite, (3) to discuss the classification of the new species and its relationship with other species of the subgenus, based on the molecular phylogenies and morphological data, (4) to discuss possible explanations for the active transmission of this malaria parasite at high elevation.

2.3 Materials and methods

2.3.1 Study area

The study site was the main campus of Universidad Nacional de Colombia-Bogotá (UNAL) located at 2560 m above sea level (04°38' N, 74°5' W). This site has patches of vegetation within an anthropogenic matrix, which, together with the parks and wetlands of the Bogotá savannah, harbours a great diversity of birds; nearly 200 bird species have been reported at the study site (Molina et al. 1997, Asociacion Bogotana de Ornitologia 2000). The climate is characterized by a bimodal distribution of precipitation, which is influenced by shifts in the intertropical convergence zone. The average annual rainfall is 1788 mm, and the average temperature is 15 °C. However, there is a variation in mean daily temperature between 10 °C and 20 °C throughout the year (Department of Geosciences Universidad Nacional de Colombia 2011).

2.3.2 Samples and blood films examination

A total of, 268 individual birds belonging to 39 species were captured using mist nests; the classification of birds is given according to the South American Classification Committee (SACC) (Remsen et al. 2012). Forty-four Great Thrushes were sampled at the study site; all these birds were resident (non-migrating). Among them, 38 were adult birds and 6 juveniles; the latter were born at the study site. Three thin smears were prepared from each bird using blood obtained from the brachial vein. The smears were air dried, then fixed in methanol for 5 min and stained with Giemsa (pH 7.2) for 45 min. About 50 µl of whole blood was taken in microcapillaries and stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA pH 8.0) for molecular analysis (these samples were kept at room temperature in the field and later at –20 oC in the laboratory). Blood smears were examined using a Leica DM750 microscope (Leica Microsystems, Heerbrugg, Switzerland), first at low magnification (100×) for 10 min, and then at high magnification (1000×) for 20 min. Digital images were prepared using a Leica EC3 digital camera, and processed with LAS EZ software (Leica Microsystems Switzerland Limited, 2012). Measurements were taken from digital images using ImageJ (Schneider et al. 2012). Entire positive blood smears with the new species were examined microscopically; over 200 images of parasites (trophozoites, gametocytes and erythrocytic meronts) of the new species were seen and analysed. Intensity of infection was estimated by counting 100 fields at a magnification of 1000× by moving the slide in areas where the blood cells formed a monolayer. A single monolayer field contained approximately 100 cells under 1000× magnification.

Morphology of *Plasmodium* (*Novyella*) *unalis* sp. nov. from the Great Thrush was compared with type and voucher material of the *Plasmodium* (*N.*) *vaughani* from its type vertebrate host the American robin *Turdus migratorius*, and the additional vertebrate host the Blackbird *Turdus merula* (accession nos. 635, 639, 654, 655) in the Garnham Collection at the Natural History Museum, London. The methodology for this study has been approved by bioethics committee of Veterinary Medicine Faculty, of Universidad Nacional de Colombia, acta 005 of 2010).

2.3.3 DNA extraction, PCR amplification, and sequencing

DNA was extracted with phenol chloroform (Sambrook et al. 1989); it was used for a nested PCR amplifying a segment of 489 bp (Hellgren et al. 2004) or 1034 bp (Pacheco et al. 2011); both are segments of the parasite cytochrome *b*. The first protocol was ran exactly as described by Hellgren et al. (2004). The Pacheco et al. (2011) protocol was modified as follows: the initial PCR reaction included 50 ng of total genomic DNA, 0.2 mM of each dNTP, 0.6 mM of each primer, 0.3 units Taq DNA polymerase (Thermo Scientific Fermentas, USA). The thermal profile was conducted with the following modifications 94 °C for 4 min, followed by 36 cycles of touch-up PCR with denaturation at 94 °C for 1 min, the initial annealing temperature of 47.9 oC (with an increase of 0.3 oC per cycle) 1 min, and extension at 72 oC for 2 min, followed by a final extension at 72 oC for 10 min. Nested PCR reaction used 1 µl of template, 2 mM MgCl2 and 53.7 °C for annealing; other conditions

were identical to the first reaction (without touch-up). The amplification was evaluated by running 2 µl of the final PCR on a 2 % agarose gel.
Every reaction was accompanied by negative and positive controls; no false positives or negatives were recorded. Amplified products were precipitated with ammonium acetate and 95 % ethanol (Bensch et al. 2000) and sequenced on a 3730xlDNA analyzer (Applied Biosystems, Foster City, California). Sequencing was done in both directions using forward and reverse primers.

2.3.4 Phylogenetic analysis

Sequences were edited using BioEdit version 7.0.5.3 (Hall 1999), and then aligned with Clustal W (Larkin et al. 2007) implemented in MEGA v5.05 (Tamura et al. 2011); the final alignment included 25 sequences of 489 nucleotides. We used only Cyt *b* sequences, which were precisely linked with their morphospecies in GenBank and MalAvi database (Bensch et al. 2009).

The phylogenetic reconstruction was done using Bayesian Inference in MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) through the CIPRES Science Gateway V. 3.3 (Miller et al. 2010). We used the General Time Reversible model including invariable sites and variation among sites (GTR+I+G), selected for the corrected Akaike Information Criterion implemented in jModeltest 2.1.1 (Darriba et al. 2012). Two independent runs of $1.5x10^7$generations were conducted with four chains, sampling every 500 generation. Convergence was assessed using average standard deviation of split frequencies between the two runs below 0.01 and graphically using Tracer (Rambaut and Drummond 2007, available at: http://tree.bio.ed.ac.uk/software/tracer/). In all, 25 % of the trees were discarded as burn-in period; 22500 trees were used to construct a majority rule consensus tree. The phylogeny was visualized using FigTree v1.3.1 (Rambaut 2006, available at: http://tree.bio.ed.ac.uk/software/figtree/). The sequence divergence between the different lineages was calculated using a Kimura-2 parameter model of substitution, implemented in the program MEGA v5.05 (Tamura et al. 2011).

2.4 Results

Microscopic examination of blood smears revealed the presence of *Plasmodium* (*Novyella*) *unalis* in 13/44 (29.5%) of Great Thrushes. Of these birds, eleven showed single infections with *P*. *unalis*, and only three samples were amplified by PCR; two birds were co-infected with different haemosporidian parasites, as determined by microscopic examination of blood smears. One bird was co-infected with *P*. *unalis* (intensity of infection 1%) and *P*. (*Haemamoeba*) *lutzi* (0.01%); this individual was an adult female, sometimes with multiple infections of *P*. *unalis* in the same erythrocytes. It is worth mentioning that this bird had deteriorated plumage and unusually low weight (110 g) compared to the other captured birds from the same species (mean: 149 g, n: 43). Another individual bird was co-infected with *Leucocytozoon majoris* (intensity of infection 0.01%) and *P*. *unalis* (0.5%). The new parasite was observed in both adult (12 of 38 (31.6%) and juveniles birds (1 of 6, 16.6%) demonstrating that active transmission occurs at the study site.

2.4.1 Description

Plasmodium* (*Novyella*) *unalis sp. nov. (Figs. 1, 2 Table II 1)

All blood stages develop mainly in mature erythrocytes, but trophozoites and mature meronts were occasionally seen in inmature erythrocytes.

Trophozoites (Fig. II 1a-c): Roundish or oval, variable in margins, but do not possess clearly defined long outgrowths. The 'ring' stage was not seen; refractive globules present (Fig. II 1a,b). One or several small (≤1 μm in diameter) vacuoles (Fig. II 1a) present in the cytoplasm. Advanced trophozoites possess a prominent brown pigment granule of circular shape, (Table II 1, Fig. II 1a-c) characteristic of this species development. The nucleus is prominent with pink pigmentation (Fig. II 1a-b). Trophozoites usually do not touch nuclei of infected erythrocytes; they assume polar or sub-polar position in the host cells (Fig. II 1a,c). The influence of parasites on infected erythrocytes is not pronounced.

Erythrocytic meronts (Fig. II 1d-t)*:* Cytoplasm is readily visible in young meronts (Fig. II 1f) and is scanty and even invisible in mature meronts (Fig. II 1i-j,n-t). A prominent dark-brown, of circular shape pigmented granule, which area ranging between 0.2 and 0.8 μm^2 (on average 0.5±0.15 μm^2) is present in growing and mature meronts, a characteristic feature of the development of this species (Fig. II 1d-t). The size of fully-grown meronts does not exceed the size of the nuclei of infected erythrocytes. Parasite nuclei are large in binuclear meronts (Fig. II 1g); the size of nuclei markedly decreases as the parasite matures (compare Fig. II 1g with Fig. II 1q-t). The growing meront possesses one vacuole of irregular shape (Fig. II 1d,e); remnants of the vacuole sometimes are visible in maturing meronts (Fig. II 1r). Refractive globules are present in some meronts (Fig. II 1f,m). Meronts produce between 3 and 8 (more often 5) merozoites. Nuclei predominantly are arranged as fans (Fig. II 1h,m-o,q-s), sometimes as rosettes (Fig. II 1i,j) and occasionally, irregularly located in mature parasites (Fig. II 1k,l,p). Merozoites persist in the erythrocytes after segmentation of mature meronts (Fig. II 1t). In some cases meronts can touch the nuclei of infected erythrocytes, but it is uncommon. The influence of parasites on infected erythrocytes is not pronounced except in cases where the same cell has been infected multiple times with several parasites (Fig. II 1e).

Macrogametocytes (Fig. II 2f-l)*:* Elongated slender bodies, extending laterally along nuclei of erythrocytes. The maximum width of fully grown macrogametocytes was 2.6 μm (Table II 1). The parasite nucleus is compact, small, of variable shape, and frequently seen in a median position. Numerous vacuoles present in the cytoplasm (Fig. II 2l); they are randomly scattered throughout the cytoplasm. Fully grown gametocytes usually touch the envelope of infected erythrocytes and sometimes touch the nucleus of the erythrocytes; they do not fill the infected erythrocytes to their poles. Pigment granules are roundish or oval, of small (<0.5 μm), medium (0.8-1 μm) or large (>1 μm) size, not numerous (Table II 1), usually clumped into one or several groups (Fig. II 2f-l). The influence of gametocytes on infected erythrocytes is not pronounced or only slightly pronounced, and the nuclei of infected erythrocytes can be slightly displaced laterally (Fig. II 2l).

Figure II 1. Trophozoites (a-c) and erythrocytic meronts (d-t) of *Plasmodium* (*Novyella*) *unalis* sp. nov. (lineage UN227) from the blood of the Great Thrush. Long arrows: nuclei of parasites. Short arrows: pigment granules. Triangle: vacuoles. Simple arrowheads: refractive globules. Giemsa-stained thin blood smears. Bar = 10 µm.

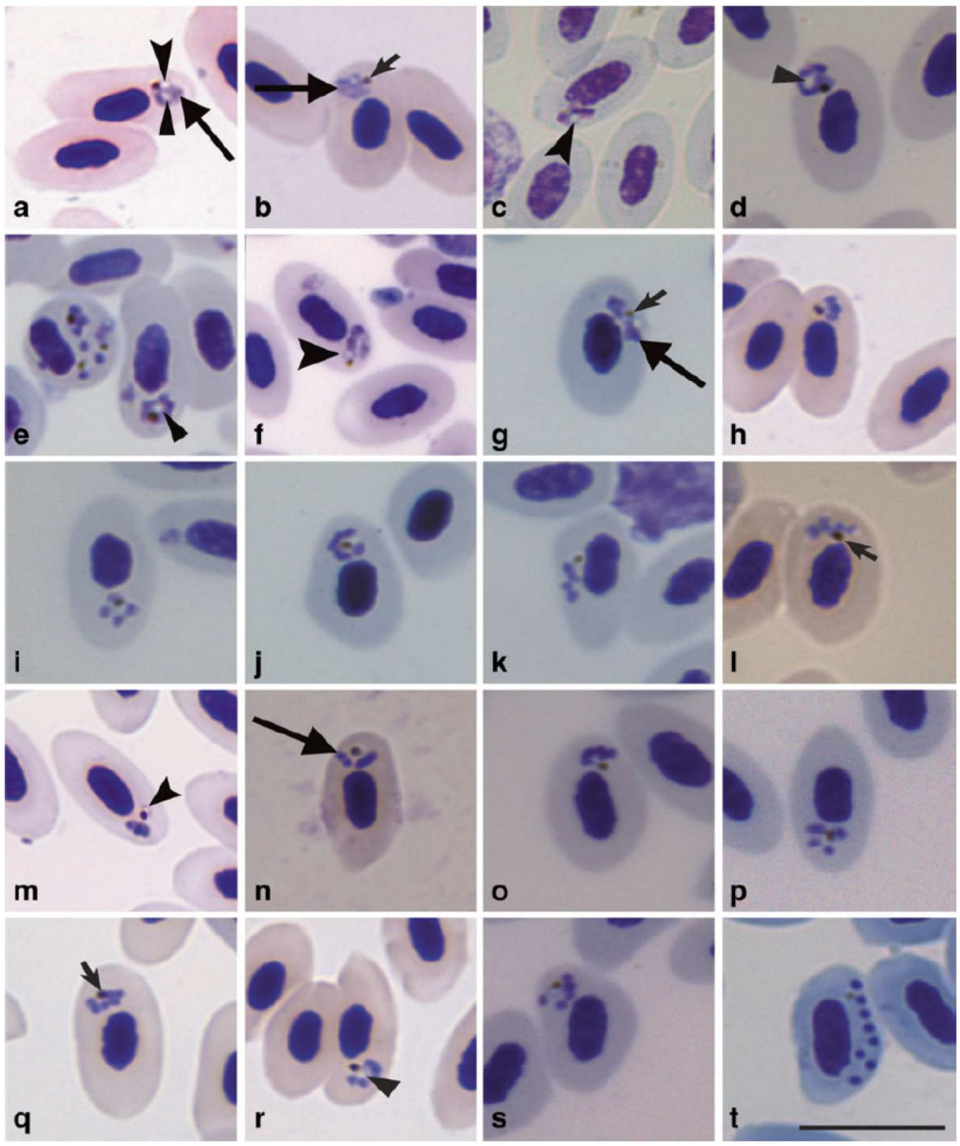
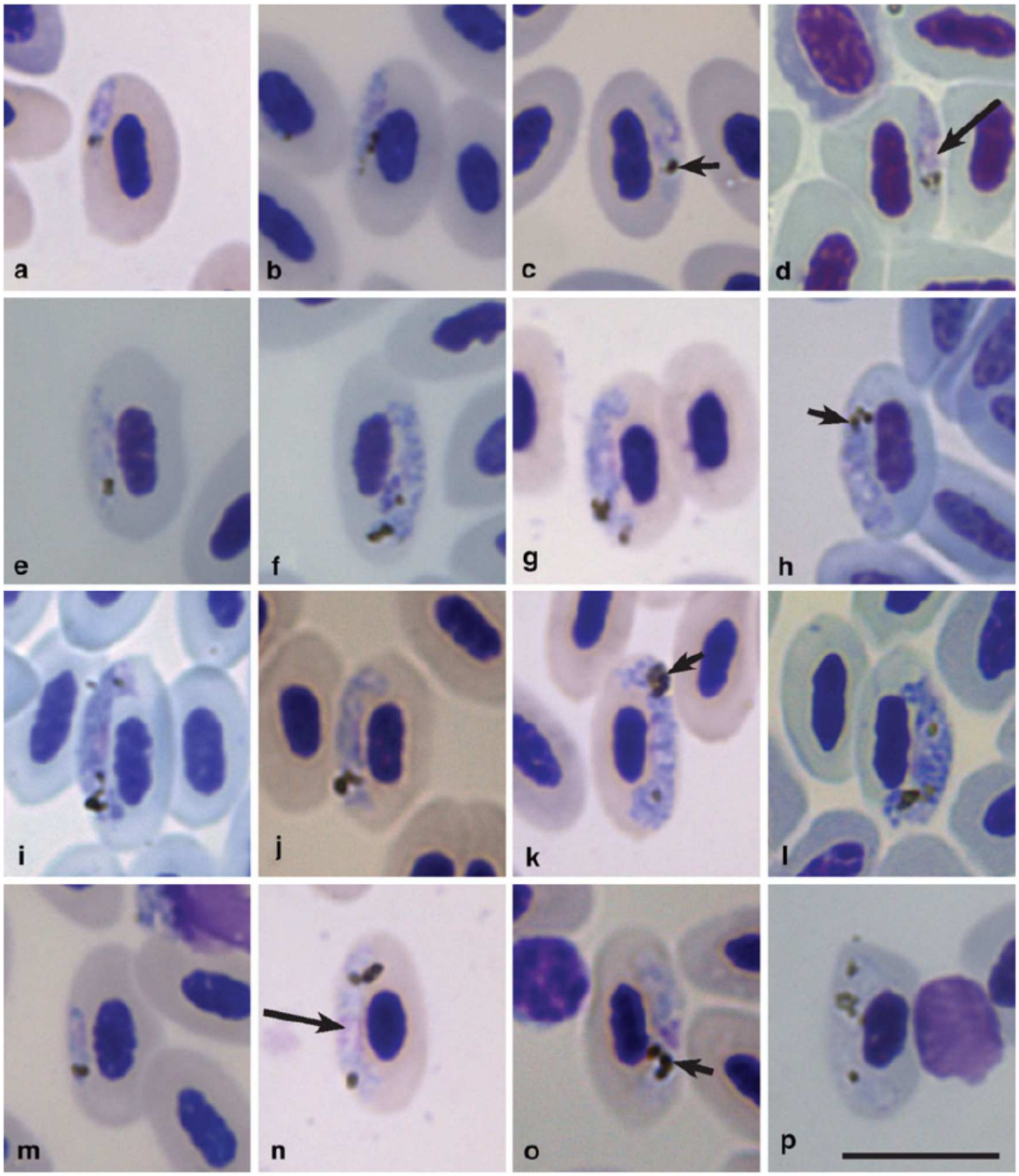


Figure II 2. Gametocytes of *Plasmodium* (*Novyella*) *unalis* sp. nov. (lineage UN227) from the blood of the Great Thrush. a-e, m young gametocytes; f-l macrogametocytes; n-p microgametocytes. Long arrows: nuclei of parasites. Short arrows: pigment granules. Giemsa-stained thin blood films. Bar = 10 µm.



Young gametocytes (Fig. II 2a-e,m): Elongated shape from the earliest stages of their development. Occasionally possess short finger-like outgrowths (Fig. II 2b,e). Multiple infection of the same host cell is uncommon. Nucleus of parasite is of central position and irregular in shape. The cytoplasm is plentiful, with granular appearance and few vacuoles present. Large and dark-brown pigment granules usually roundish or with slightly oval shape, often clumped (Fig. II 2a,c,e); their number varies between 1 and 5. Young gametocytes do not displace the nuclei of erythrocytes and usually do not touch the nuclei.

Table II 1. **Morphometric parameters of mature blood stages of *Plasmodium* (*Novyella*) *unalis* sp. nov. (lineage UN227). [a]:All measurements are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.**

Features	Measurements [a]
Meronts	n= 40
Length	2.2-9.1 (3.2±1.1)
Width	1.6-2.8 (2.2±0.3)
Area	3.8-13.3 (5.1±1.7)
Area of globule	0.1-0.4 (0.2±0.1)
No. merozoites	3-8 (5.0±0.6)
No. of pigment granules	1
Area of pigment granules	0.2-0.7(0.4±0.1)
Macrogametocytes	n=20
Length	11.3-12.6 (11.3 ±0.8)
Width	1.4-2.6 (2.2±0.3)
Area	19.4-29.1 (24.1±2.9)
Gametocyte nucleus	
Length	1.6-2.5 (1.9±0.3)
Width	1.0-1.8 (1.4±0.3)
Area	1.4-3.8 (2.1±0.6)
No. of pigment granules	4-11 (6.3±1.9)
Microgametocytes	n=20
Length	10.2-12.9 (11.6±0.9)
Width	1.8-2.8 (2.1±0.3)
Area	17.6-26.6 (23.3±3.3)
Gametocyte nucleus	
Length	2.5- 4.8 (3.5±0.6)
Width	1.3-1.9 (1.6±0.3)
Area	2.5-4.8 (4.5±1.0)
No. of pigment granules	5-9 (6.2±1.5)

Microgametocytes (Fig. II 2n-p): Maximum width of fully grown microgametocytes was 2.8 µm (Table II 1), the general configuration is similar to macrogametocytes with the usual haemosporidian sexual dimorphic characters. Parasite nucleus is granular in appearance. Cytoplasm possesses small evident vacuoles. The proportion of micro and macrogametocytes is 1:4 in the type material.

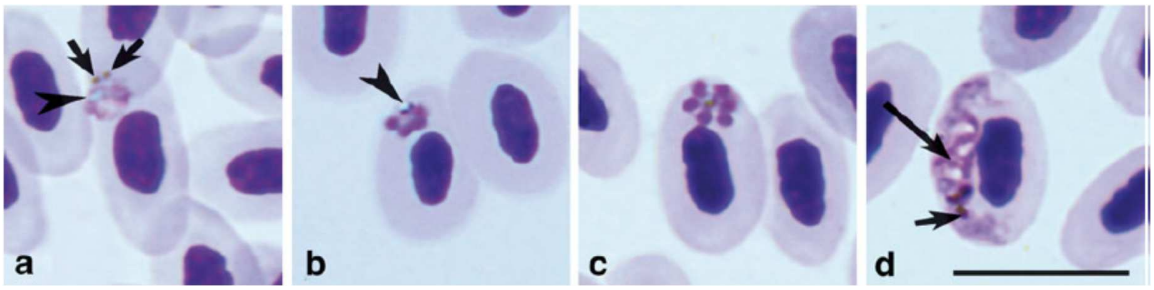
2.4.2 Remarks

Plasmodium unalis is particularly similar to *P*. *vaughani*; both these parasites belong to the subgenus *Novyella*. *P*. *unalis* can be readily distinguished from the latter species, primarily due to (1) presence of a single large circular shaped pigment granule in the erythrocytic trophozoites and meronts, (2) presence of readily distinguishable vacuoles in trophozoites and growing meronts, (3) predominant fan-like shape of erythrocytic meronts. These features are not characteristic of *P*. *vaughani* (Valkiūnas 2005, Zehtindjiev et al. 2012b). To facilitate comparison of these two similar parasites, microphotographs of the latter species are given from their type vertebrate host species, the American robin (Fig. II 3): note the presence of two small distinct pigment granules both in trophozoites (Fig. II 3a) and meronts (Fig. II 3b), and absence of vacuoles in trophozoites and meronts (Fig. II 3a-c). These features are not characteristic of *P*. *unalis* sp. nov (Fig. II 1). Gametocytes of these species are morphologically similar (Fig. II 2, Fig. II 3d).

Other species of *Novyella* are distinguishable from the new species (1) morphologically, primarily due to morphology of pigment granules in trophozoites and erythrocytic meronts (Valkiūnas 2005, Valkiūnas et al. 2009) and (2) genetically, based on a fragment of 489 bp of Cyt *b* (Fig. II 3).

Plasmodium unalis was found in 13 Great Thrushes, however, the amplification was done only in three samples. The three sequences: UN227 (GenBank KC771248), UN182 (GenBank KF182186) and UN06 (GenBank KC771247) were identical between them. The genetic analysis showed that *P. vaughani* (lineage SYAT05, GenBank DQ847271), was the closest *Plasmodium* species related to *P. unalis* with a genetic distance in Cyt *b* gene of 3.2 % between them. Both parasites locate in a clade containing mostly *P.* (*Novyella*) species. Genetic distance among lineages of this clade is between 3.2 and 8.9 % (Table II 2). Four monophyletic groups (Fig. II 4A-D) contain lineages with genetic distances of ≤ 4 % among them (Fig. II 4, Table II 2); these clades contain readily distinguishable morphological species.

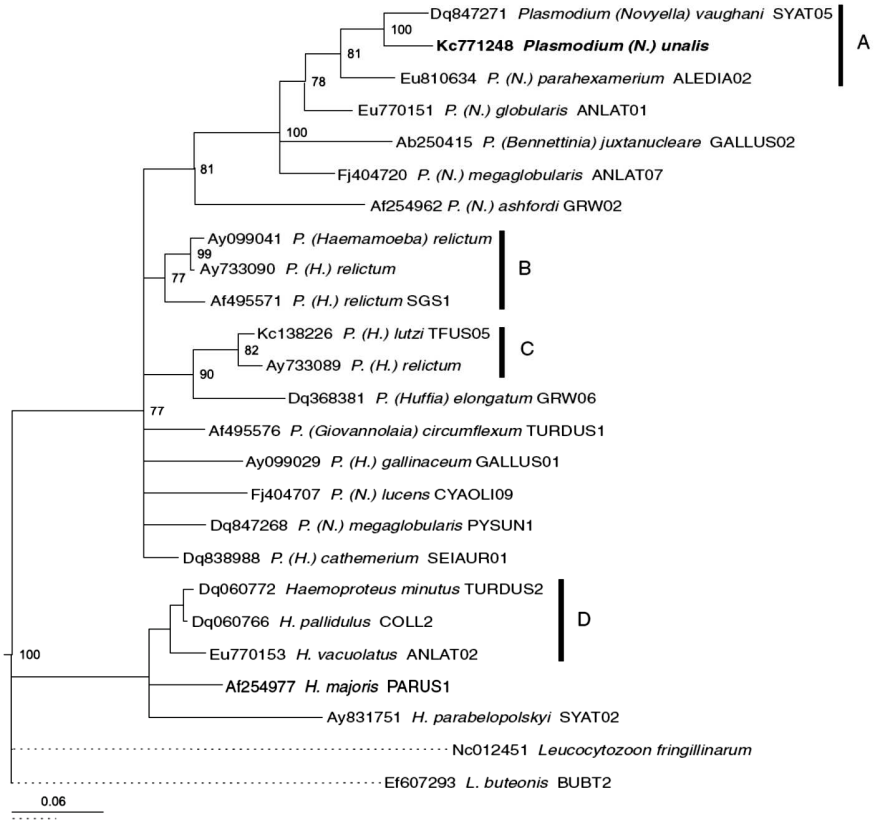
Figure II 3. Blood stages of *Plasmodium* (*Novyella*) *vaughani* from the blood of its type vertebrate host the American robin *Turdus migratorius*. a trophozoite; b-c erythrocytic meronts; d microgametocytes. Note the presence of 2 small pigment granules in trophozoites (a) and meronts (b), and of one refractive globule in trophozoites and meronts (a-c). Long arrow: nucleus of parasite. Short arrows: pigment granules. Simple arrowheads: refractive globules. Giemsa-stained thin blood films. Bar = 10 µm.



2.4.3 Phylogenetic relationships of parasites

Molecular evidence suggests that the new species is likely distributed in the New World because Cyt *b* lineages of marked similarity (genetic distance ≤ 0.5 %) were reported in *Catharus ustulatus* (Swainson's Thrush, JN792135, Alaska, USA; Dodge et al. 2013), *Tangara icterocephala* (Silver-throated Tanager, JN819328, JN819340, Costa Rica,), *T. migratorius* (American Robin, AF465548, Ricklefs and Fallon 2002; HM222477, Ricklefs and Outlaw 2010), *T. amaurochalinus* (Creamy-bellied Thrush, JX021462, Brazil, Lacorte et al. 2013), *T. falcklandii* (Austral Thrush, EF153639, Chile, Merino et al. 2008), *T. assimilis* (White-necked Thrush, JN819347, JN819343, Costa Rica), *Hylocichla mustelina* (Wood Thrush, HM222478, HM222479, Ricklefs and Outlaw 2010), and *Culex pipiens* (GQ471955, USA, Kimura et al. 2010). Morphological evidence is needed to determine if these lineages belong to *P. unalis*.

Figure II 4. Bayesian phylogeny based on 489 bp of cytochrome *b* sequences of morphologically identified *Plasmodium* spp. (18 lineages) and *Haemoproteus* spp. (5 lineages). Two *Leucocytozoon* spp. lineages were used as outgroup. Vertical bars indicate groups of species with a genetic distance ≤ 4 % between them. The new species is indicated in bold. GenBank accession numbers are provided before the parasite species names. Values of posterior probabilities over 70 are shown. The branch lengths are drawn proportionally to the amount of change, the scale bars show substitutions per site.



2.4.4 Taxonomic summary

Type host: Great Thrush *Turdus fuscater* (Turdidae, Passeriformes).

Type locality: Bogotá, D.C., Campus of Universidad Nacional de Colombia (04°38'N, 74°05'W), Colombia.

Type specimens: Hapantotype (accession no. GERPH-06004 in the collection Grupo de Estudio Relación Parásito Hospedero (GERPH), Department of Biology, Universidad Nacional de Colombia, Bogotá, Colombia; intensity of infection is 1 %; collected by Juan S. Mantilla, 5 August 2012; lineage UN227, GenBank KC771248). Parahapantotypes

(accession nos. GERPH-060030, GERPH-02807, GERPH-02808, GERPH-05854, GERPH- 05855) were deposited in the biological collection GERPH at Universidad Nacional de Colombia, Bogotá, Colombia. Digital images of blood stages of the new parasite from the type preparations are available on request from GERPH.

DNA sequences: Mitochondrial Cyt *b* lineages UN227 (1034 bp, GenBank no. KC771248, from the hapantotype sample), UN06 (495 bp GenBank, accession no. KC771247, from the parahapantotype sample) and UN182 (495 bp GenBank, accession no. KF182186)

Site of infection: Mainly in mature erythrocytes, but trophozoites and mature meronts were occasionally seen in inmature erythrocytes.

Prevalence: In the type locality, 13 out of 268 investigated individual birds of all species were infected (the overall prevalence is 4.8 %), but the prevalence was 13 of 44 (29.5 %) in the type host.

Distribution: This species has been found only in the type locality.

Additional hosts: Additional hosts are unknown, but see the section 'Phylogenetic relationships of parasites'.
Etymology: The species name is derived from the abbreviation of the Universidad Nacional de Colombia (UNAL); the name indicated the type locality where infected birds were sampled.

2.5 Discussion

Species of Turdidae have been frequently reported to be parasitized by numerous haemosporidian parasites around the world (Valkiūnas 2005). Although we sampled 39 different bird species, *Plasmodium unalis* was found only in *Turdus fuscater* (Turdidae) at the study site. However, genetically related lineages (similarity of 0.5% or less in Cyt *b* gene) have been reported in six different species of the Turdidae as well as one lineage that was detected in a bird belonging to the Thraupidae (Ricklefs and Fallon 2002, Merino et al. 2008, Ricklefs and Outlaw 2010, Dodge et al. 2013, Lacorte et al. 2013). For this reason, this parasite seems to be not strictly specificic for *T*. *fuscater*. The absence of this parasite in many wild-caught birds at the study site might indicate (1) resistance, (2) high mortality or (3) vector preference to bite particularly *T*. *fuscater*. Further studies are needed to clarify this issue.

The Great Thrush has been parasitized by *Leucocytozoon dubreuili*, *L*. *fringillinarum*, *Haemoproteus fallisi*, *Plasmodium* (*Haemamoeba*) sp., *P*. (*Novyella*) sp. and *P*. (*Haemamoeba*) *lutzi* in Colombia (Rodríguez et al. 2009, Lotta et al. 2013, Mantilla et al. 2013). This bird species has been successfully established in rural areas with presence of vegetation, where it finds food sources and nesting sites (Asociacion Bogotana de Ornitologia 2000). This bird species does not migrate so the one infected juvenile bird certainly was infected at the study site. Based on phylogenetic relationships (Fig. II 4), *P*. *vaughani* is the sister taxon to *P*. *unalis*, however both avian host and geographical

distribution of these parasites are different. *P*. *vaughani* (lineage pSYAT05) is of worldwide distribution; it has been recorded in North America, Europe, Japan, New Zealand and Turkey (Martinsen et al. 2008, Kim and Tsuda 2010, Glaizot et al. 2012, Howe et al. 2012, Inci et al. 2012, Zehtindjiev et al. 2012b) infects many bird species belonging to several orders around the world, but *P*. *unalis* was found only in *Turdus fuscater* in Colombia. Based on available sequence information in GenBank, *P*. *unalis* seems to be restricted to the New World, however, this assumption is based only on limited molecular evidence and additional morphological information is needed to prove that.

Plasmodium parasites are rare at high elevations, probably due to negative effect of low temperature on sporogony in mosquito vectors. For instance, the Hawaiian strain of *P*. *relictum* does not complete sporogony below 12 oC and is not prevalent at 1800 m above sea level (LaPointe et al. 2010, Santiago-Alarcon et al. 2012). Several recent studies speculate about possible expansion of *Plasmodium* parasites at highest latitudes because of climate warming (Freed et al. 2005, Loiseau et al. 2012, Zamora-Vilchis et al. 2012). In Colombia, unidentified avian *Plasmodium* species (belonging to the subgenus *Novyella* and *Haemamoeba*) with overall prevalence of 8.1 % was reported at 3100 m above sea level, in páramo ecosystem where temperatures currently range between 0 to 14 °C thorough the year (Rodríguez et al. 2009). It is possible that some avian malaria parasites have adapted to successfully develop in the vector under these temperature conditions. *Plasmodium unalis* has a theoretical interest as an example of malaria parasite capable of active transmission in mountains of at least 2560 m above sea level.

The urban ecosystems (such as Bogotá) could affect the ecology of vector-borne parasites in different ways: first, the emission of greenhouse gases are implicated in climate change; this, coupled with increase of building areas, create urban heat islands due to the increase of surface for absorbing solar energy (Grimm et al. 2008). This phenomenon was reported in Bogotá by Angel et al. (2010); who stated the increase of temperature of about 3 °C in comparison to the periphery of the city where temperature is 12 °C on average throughout a year. Our study area was located in the middle of this heat island. That, might be favorable for the development of the parasites in the vectors.

Table II 2. Genetic distance (in percentage) among cytochrome *b* sequences of morphologically identified *Plasmodium* species. The genetic distance was calculated using Kimura 2-parameter model of substitutions. GenBank accession numbers are shown only for *Plasmodium* (*Haemamoeba*) *relictum* lineages because several lineages of this parasite were used; for the other species, the accession numbers are given in Fig. II 1. Name of the new species is given in bold font.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. ***P.* (*Novyella*) *unalis***	0																
2. *P.* (*N.*) *vaughani*	3.2	0															
3. *P.* (*N.*) *parahexamerium*	4.0	4.0	0														
4. *P.* (*N.*) *globularis*	5.1	4.9	4.2	0													
5. *P.* (*N.*) *megaglobularis*	7.9	8.9	8.6	8.6	0												
6. *P.* (*N.*) *multivacuolaris*	5.8	6.4	5.1	4.9	7.9	0											
7. *P.* (*N.*) *ashfordi*	7.7	8.4	8.6	7.9	7.9	8.9	0										
8. *P.* (*N.*) *lucens*	8.0	7.7	7.6	8.1	5.9	8.5	8.6	0									
9. *P.* (*Haemamoeba*) *lutzi*	7.3	7.9	7.3	8.9	6.1	6.7	8.4	6.2	0								
10. *P.* (*Ha.*) *cathemerium*	6.8	7.0	7.5	7.2	3.4	7.1	7.2	5.1	4.4	0							
11. *P.* (*Ha.*) *relictum* (AF495571)	7.7	8.2	7.9	7.9	3.7	8.4	8.2	5.9	5.9	3.4	0						
12. *P.* (*Ha.*) *relictum* (AY733089)	7.5	8.2	6.6	8.8	7.0	7.1	7.9	7.1	1.7	4.9	6.6	0					
13. *P.* (*Ha.*) *relictum* (AY099041)	7.8	8.4	8.0	7.9	4.3	8.0	7.5	5.3	5.5	3.1	2.8	5.7	0				
14. *P.* (*Ha.*) *relictum* (AY733090)	7.3	7.9	7.5	7.4	3.9	7.6	7.0	4.9	5.1	2.7	2.3	5.3	0.4	0			
15. *P.* (*Ha.*) *gallinaceum*	8.9	8.6	9.1	10.6	5.9	8.7	9.1	6.7	6.0	4.4	6.3	5.5	5.5	5.1	0		
16. *P.* (*Giovannolaia*) *circumflexum*	6.9	6.6	6.9	7.4	4.4	7.4	6.9	6.0	6.2	3.3	4.1	6.0	4.1	3.7	5.0	0	
17. *P.* (*Bennettinia*) *juxtanucleare*	6.0	7.0	5.7	5.6	8.4	5.8	9.6	7.4	6.9	6.8	8.2	8.0	7.3	6.9	8.5	7.6	0
18. *P.* (*Huffia*) *elongatum*	8.4	8.2	8.4	8.9	7.0	8.4	7.3	7.2	4.7	5.9	7.0	4.5	5.9	5.4	6.5	6.0	8.2

Second, immature stages of *Culex quinquefasciatus* mosquitos are often present in different kinds of naturals and artificial containers of polluted water. Such containers are numerous in Bogotá; that provides opportunities for this mosquito to develope in the city´s environment (Salazar and Moncada 2004). Moreover, Kimura et al. (2010) reported a *Plasmodium* lineage (GQ47195) in *Culex pipiens* (species complex with *Cx*. *quinquefasciatus*) and this lineage had a genetic similarity of 99% with the lineage UN227 of *P*. *unalis*, supporting an idea that this mosquito species can be involved in transmission of this parasite at the study site.

Thirdly, only the species adapted to the anthropogenic stress can be dominant in this ecosystem (Grimm et al. 2008, Møller et al. 2010). In this case, the population of the Great Thrush increase is associated with the anthropic intervention and is perhaps, the most dominant species in the study area (Asociacion Bogotana de Ornitologia 2000). This bird species has been frequently infected with several haemoparasites (Rodríguez et al. 2009, Lotta et al. 2013, Mantilla et al. 2013), so we hypothesized that the urban environment changes the so-called “Dilution effect” reducing the number of parasite-refractive hosts (Schmidt and Ostfeld 2001) but further investigations are needed to support this hypothesis.

The classification of avian malaria parasites using morphology has gaps. Discussion has been recently opened about shortcomings of this parasite classification, particularly at the level of *Plasmodium* subgenera (Corradetti and Scanga 1965, Martinsen et al. 2006, Santiago-Alarcon et al. 2012). The subgenus *Novyella* was established by Corradetti et al. (1963) for malaria parasites producing small meronts with scanty cytoplasm, elongate gametocytes and exoerythrocytic merogony in the reticuloendothelial system. Landau et al. (2010) suggested that the presence of globule is a characteristic feature of the *P*. (*Novyella*) species. Globules can be distinguished from vacuoles because the latter appear as transparent roundish structures of variable size and shape, while the globules are relatively hard structures (circular and possessing more or less permanent shape), often bluish and often remaining intact even in the mature meronts (when the vacuole usually disappear) (Chavatte et al. 2010, Landau et al. 2010). It is worth mentioning that our phylogenetic analysis does not support the taxonomic value of the character, because the species possessing the globules (*P*. *vaughani*, *P*. *globularis*) and the species lacking the globules (*P*. *parahexamerium*, *P*. *juxtanucleare*, *P*. *ashfordi*) appeared in the same clade in the phylogenetic tree (Fig. II 4). In the case of *P*. *unalis* the globule is present, but not in all erythrocytic meronts (Fig. II 1).

The number of haemosporidian genetic lineages that can be considered as distinct evolutionary entities is significantly greater than the number of species described using traditional biological characters (Ricklefs and Fallon 2002, Bensch et al. 2004, Martinsen et al. 2008). There is not a clearcut point that indicates when a morphological difference or molecular difference in Cyt *b* sequence could indicate a new avian haemosporidian species. The modern taxonomy approach involves different tools helping decide when a taxon is a new pecies. Hellgren et al. (2007) and Valkiūnas et al. (2009) suggested that in most cases, haemosporidian species with a genetic distance of over 5% in Cyt *b* gene could be morphologically differentiated. However, this trend certainly works only one direction. For example, haemosporidians *Haemoproteus minutus* and *Haemoproteus pallidus*, which differ by less than 1% in Cyt *b* gene, are readily distinguishable morphologically (Fig. II 4D); these species also have been shown likely to not hybridize *in vitro* (Valkiūnas et al. 2008a).

Another example can be seen in clade A (Fig. II 4): parasites of this clade are readily distinguishable due to morphological features, but the genetic distance between their lineages is between 3.2 and 4%. Therefore, to clarify the taxon identity, it is essential to use different approaches that involve traditional microscopy based diagnoses, sequence information, and if possible, also experimental *in vivo* hybridization assays.

Numerous DNA sequences of haemosporidians have been deposited in the GenBank, however, the majority of them do not have morphological identification, or might be erroneously identified to the species level. For instance, Valkiūnas et al. (2008b) reported the wrong identification of *P*. (*Haemamoeba*) *relictum* (GenBank AY733088); this lineage belongs to *Plasmodium* (*Huffia*) *elongatum*. A similar case happened with the sequence AY733089 from the American Type Culture Collection (ATCC), which was attributed to *P*. *relictum* but is genetically and phylogenetically (Fig. II 4C) closer to *P*. *lutzi*. The quality of samples is a bottleneck in the attempt to determine the identity of haemosporidian species. To avoid this difficulty, extensive sampling is required to be able to identify single infections with sufficient variety of blood stages and to make linkages between morphology and DNA sequences. Additionally, further research and standardization of other molecular markers is needed in order to obtain a higher power of resolution for deep nodes in the phylogeny and then we will be able to explain evolutionary questions.

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3. Chapter III. Identification of *Plasmodium* (*Haemamoeba*) *lutzi* (Lucena, 1939) from *Turdus fuscater* (Great Thrush) in Colombia

Identificación of *Plasmodium* (*Haemamoeba*) *lutzi* (Lucena, 1939) en *Turdus fuscater* (mirla común) en Colombia

Este estudio reporta la ampliación de rango altitudinal y un nuevo hospedero de *Plasmodium* (*Haemamoeba*) *lutzi* en Colombia. El estudio fue realizado en la ciudad de Bogotá, localizada en la cordillera oriental de Colombia a 2560 m sobre el nivel del mar, con una temperatura anual promedio de 15°C. Ciento cincuenta y seis especímenes de aves pertenecientes a 25 especies y 14 familias fueron capturadas con redes de niebla. Se colectaron muestras de sangre por punción de la vena braquial y se analizaron por microscopia de luz. *Plasmodium* (*H*.) *lutzi* fue encontrado únicamente en dos individuos de *Turdus fuscater* (mirla común). Este parasito había sido reportado previamente en *Aramides cajaneus* (antes: *Aramides cajanea*), un ave encontrada en tierras bajas de Brasil, Venezuela y Colombia. Los resultados proveen evidencia de una ampliación de rango de hospedero para *P*. *lutzi* que incluye al menos dos órdenes diferentes: Gruiformes y Passeriformes, también la expansión altitudinal de su distribución los estados sanguíneos fueron comparados con la descripción original del parasito y la secuencia de genoma mitocondrial (mtDNA) confirma que *P*. *lutzi* es el taxón hermano de *P*. *relictum*; como se había propuesto previamente.

Este artículo abarca parcialmente el primer objetivo, *al dar la redescripción morfológica de una especie de Plasmodium circulante en un ave de la cordillera oriental*, *reportada por última vez en tierras bajas de Venezuela en 1976*. *Mi contribución a este artículo radicó en la colecta de muestras en campo*, *lectura inicial de los extendidos e identificación morfológica de estadios sanguíneos que llevaron al hallazgo de Plasmodium* (*Haemamoeba*) *lutzi*, *colaboré en parte con la toma de medidas y fotografías de los parásitos*. *También participé en la escritura del artículo en lo que corresponde a la distribución y ecología de Turdus fuscater y características del sitio de muestreo*.

Identification of *Plasmodium* (*Haemamoeba*) *lutzi* (Lucena, 1939) from *Turdus fuscater* (Great Thrush) in Colombia

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3.1 Abstract

This study reports both broadening of the altitudinal range and a new host for *Plasmodium* (*Haemamoeba*) *lutzi* in Colombia. The study was conducted in the city of Bogotá, located in the eastern cordillera of Colombia at 2560 m above sea level, with an average annual temperature of 15°C. One hundred fifty six specimens of birds belonging to 25 species and 14 families were captured using mist nets. The blood samples were collected through venipuncture and analysed by light microscopy. *Plasmodium* (*H*.) *lutzi* was only found in two individual of *Turdus fuscater* (Great Thrush). This parasite has previously been reported in *Aramides cajaneus* (before: *Aramides cajanea*) (Grey-necked Wood Rail), a bird found in the lowlands of Brazil, Venezuela and Colombia. This finding provides evidence for a broad host range for *P*. *lutzi* that includes two different orders Gruiformes and Passeriformes, and also altitudinal expansion of its distribution. The blood stages were compared with the parasite's original descriptions and the sequence of the parasite's mitochondrial genome (mtDNA) confirms that *P*. *lutzi* is a sister taxon of *P*. *relictum*; as previously proposed.

3.2 Introduction

Avian haemoparasites use insects of the order Diptera as vectors, they have a worldwide distribution and have already been identified in 4000 bird species (Valkiūnas 2005). The parasites of the genus *Plasmodium*, which cause malaria in birds, comprise approximately 50 species with established morphological characteristics (Valkiūnas 2005, Valkiūnas et al. 2008). Molecular data show a high genetic diversity of these parasites, indicating that the number of species and their taxonomical diversity may be underestimated by the current classifications (Bensch et al. 2009).

The genus *Plasmodium* in birds is divided into the subgenera *Haemamoeba*, *Novyella*, *Giovannolaia*, *Huffia* and *Bennettinia* (Valkiūnas 2005). The parasite-host relationship of

Haemamoeba, which includes *Plasmodium* (*Haemamoeba*) *relictum*, is well characterized. This parasite has been found in more than 300 bird species belonging to different families around worldwide (Valkiūnas 2005), and it has been associated with the loss of bird diversity in Hawaii (van Riper III et al. 1986, LaPointe et al. 2012).

Plasmodium (*H*.) *lutzi* was first observed in Brazil by Lutz and Meyer in 1908, where it was parasitizing *Aramides cajaneus* (Gruiformes). The species was subsequently described by Lucena (1939) upon finding the parasite in the same bird species. Renjifo et al. (1952) also reported parasites similar to *P*. (*H*.) *relictum* in the same bird species in Colombia. However, Gabaldón and Ulloa (1976a), who found the parasite in birds at 3 sites in Venezuela, reported that the parasites found in the eastern plains of Colombia were likely P. (H.) lutzi. This article reports the finding of *P*. (*H*.) *lutzi* parasitizing *Turdus fuscater* (Great Thrush, Passeriformes), a resident bird inhabiting at 2560 m asl (above sea level) in the city of Bogotá, Colombia.

3.3 Materials and methods

The study site was the campus of the Universidad Nacional de Colombia-Bogotá (UNAL), located at 2560 m asl (4° 38' N, 74° 5' W). This site has patches of vegetation within an anthropogenic matrix that, together with the parks and wetlands of the Bogotá savannah, harbors a great diversity of birds (Molina et al. 1997). The climate system is characterized by a bimodal distribution of precipitation, with 2 periods of relative maxima and 2 of relative minima that is influenced by Intertropical Convergence Zone. The average annual rainfall is 1,788 mm, and the average temperature is 15 C. However, there is significant variation in the daily temperature that ranges between 10 C and 20 C (Department of Geosciences, National University of Colombia 2011).

3.3.1 Samples

In total, 156 birds belonging to 25 species and 14 families were captured using mist nets at UNAL between September 2009 and July 2012, the classification of birds was performed according to the South American Classification Committee (SACC) (Remsen et al. 2012) (Table III 1). The blood samples were collected by puncturing the brachial vein. Three blood smears were fixed with methanol for 5 min and stained with Giemsa pH 7.2 for 45 min, as described by Rodríguez and Matta (2001). The blood was also stored in EDTA.

The smears were scanned in a double-blind at low magnification (X100) for 10 min and then for 20 min at higher magnification (X1000). Once the diagnosis was made, the slides were scanned again for photographic and morphometric records. The parasitemia was established by counting 100 fields at X1,000 magnification by focusing on the areas where the blood cells formed a monolayer. A single monolayer field under a X100 oil objective contained approximately 100 cells (1 infected cell/10,000 erythrocytes) (Muñoz et al. 1999).

3.3.2 Microscopic analysis

All of the blood samples were observed by microscopy. Two out of the 37 individuals of *Turdus fuscater* were positive by microscopy. One of the specimens was exclusively infected with *P*. (*H*.) *lutzi*, with a parasitemia of 0.03% as determined by microscopic quantification (Muñoz et al. 1999, Valkiūnas 2005). All the photographic records and blood stages description were obtained from this one sample (Figs. III 1-12). The second specimen was co-infected with another *Plasmodium* of the subgenus (*Novyella*) species with a parasitemia of 1 % for the *P*. (*Novyella*) sp. and 0.01% for *P*. *lutzi* species. The morphological and morphometric characteristics of gametocytes proposed by Gabaldón and Ulloa (1976a) and Valkiūnas (2005) were used for diagnosis and identification. The morphometric features (Table III 2) were those defined by Valkiūnas (2005). The digital images were captured by a Leica EC3 camera and processed with the LAS EZ software (Leica Microsystems [Switzerland] Limited, 2012) using a Leica DM750 microscope (Leica Microsystems, Heerbrugg, Switzerland).

3.3.3 DNA extraction and amplification of parasite mitochondrial genome (mtDNA)

DNA was extracted from blood spots of the one Great Thrush that was exclusively infected with *P*. (*H*.) *lutzi* by using QIAamp® DNA Micro Kit (Qiagen GmbH, Hilden, Germany). We amplified approximately 5,889 bp of the parasite mitochondrial genome (mtDNA) using oligos Forward 5' GA GGA TTC TCT CCA CAC TTC AAT TCG TAC TTC/Reverse 5' CAG GAA AAT WAT AGA CCG AAC CTT GGA CTC with TaKaRa LA TaqTM Polymerase (TaKaRa Mirus Bio Inc., Shiga, Japan) as described elsewhere (Pacheco et al. 2012). Briefly, PCR amplifications were carried out in a 50-µl volume using 20 ng of total genomic DNA. The PCR conditions were: a partial denaturation at 94 C for 1 min and 30 cycles of 30 sec at 94 C and 7 min at 68 C, followed by a final extension of 10 min at 72 C. At least 2 independent PCR products (bands of approximately 6 kb) were excised from the gel, purified using QIAquick® Gel extraction kit (Qiagen), and cloned in the pGEM®-T Easy Vector systems (Promega, Madison, Wisconsin) following manufacturers' directions. Each clone was sequenced using an Applied Biosystems 3730 capillary sequencer with pUC/M13 sequencing oligos (5´GTT TTC CCA GTC ACG AC3' and 5'CAG GAA ACA GCT ATG AC3´) and the following internal forwards primers: IF1: 5'GAC TTG TGT TGT AAC CTT AC3', IF2: 5'ACA GAT GCC AGG CCA ATA AC3', IF3: 5'ACT CAG AAT AGA ATA AGA ACT C3', IF4: 5'GCT TCT GAT ATA ATT ATT GAT AAC3', IF5: 5'TAC GCC CAA ACG TTA AGA TC3', IF6: 5'GAT CTC CAG AAT TAG CTT ATC3', IF7: 5'TAT GTA TAC AAC AGG TCT AG3', IF8: 5'TTA TGG ATT TCT TTT AGG AAT AG3', IF9: 5'TTA TGC AAT GTT AAA AAC CAT TC3'. The internal primers were designed to provide at least 300 bp overlap between internal sequences. Both strands were sequenced from at least 3 clones. The mtDNA sequence reported in this investigation was deposited in GenBank under the accession number KC138226.

3.3.4 Phylogenetic analysis

In order to establish the phylogenetic relationship between *P*. (*H*.) *lutzi* and other Haemosporidian parasites, the mitochondrial genome was chosen, given that there is extensive data set available in the GeneBank that includes many avian malarias (National

Center for Biotechnology Information, National Institutes of Health). *Plasmodium* (*H*.) *lutzi* mtDNA sequence was aligned with 28 previously reported mitochondrial genomes from other Haemosporidian with ClustalX v2.0.12 and Muscle using SeaView v4.3.5. We used 3 complete mtDNA genomes available for *Leucocytozoon* species as an out-group, as has been proposed before (Martinsen et al. 2008). Phylogenetic relationships were estimated by using Bayesian methods as implemented in MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). We used a general time reversible+gamma model (GTR+Γ), this model had the lower number of parameters that best fit the data as estimated by MEGA v5.0 (Tamura et al. 2011). The alignment was divided into 4 categories where each gene (COX3, COX1 and CYTB) was used as a separate partition plus the non-coding regions (Ronquist and Huelsenbeck 2003). Bayesian support for the nodes was inferred in MrBayes using 6 $x10^6$ Markov Chain Monte Carlo (MCMC) steps, and after convergence was reached, we discarded the 50% of the sample as a burn-in. Sampling was performed every 100 generations. Convergence is reached after the average standard deviation of the posterior probability is below 0.01 and the value of the potential scale reduction factor (PSRF) is between 1.00 and 1.02 (Ronquist and Huelsenbeck 2003). In order to compare the divergence among specific pair of species, we estimated their genetic divergences on the complete mtDNA genomes using the Kimura-2 parameters model as implemented in MEGA v5.0.

3.4 Results

3.4.1 Redescription

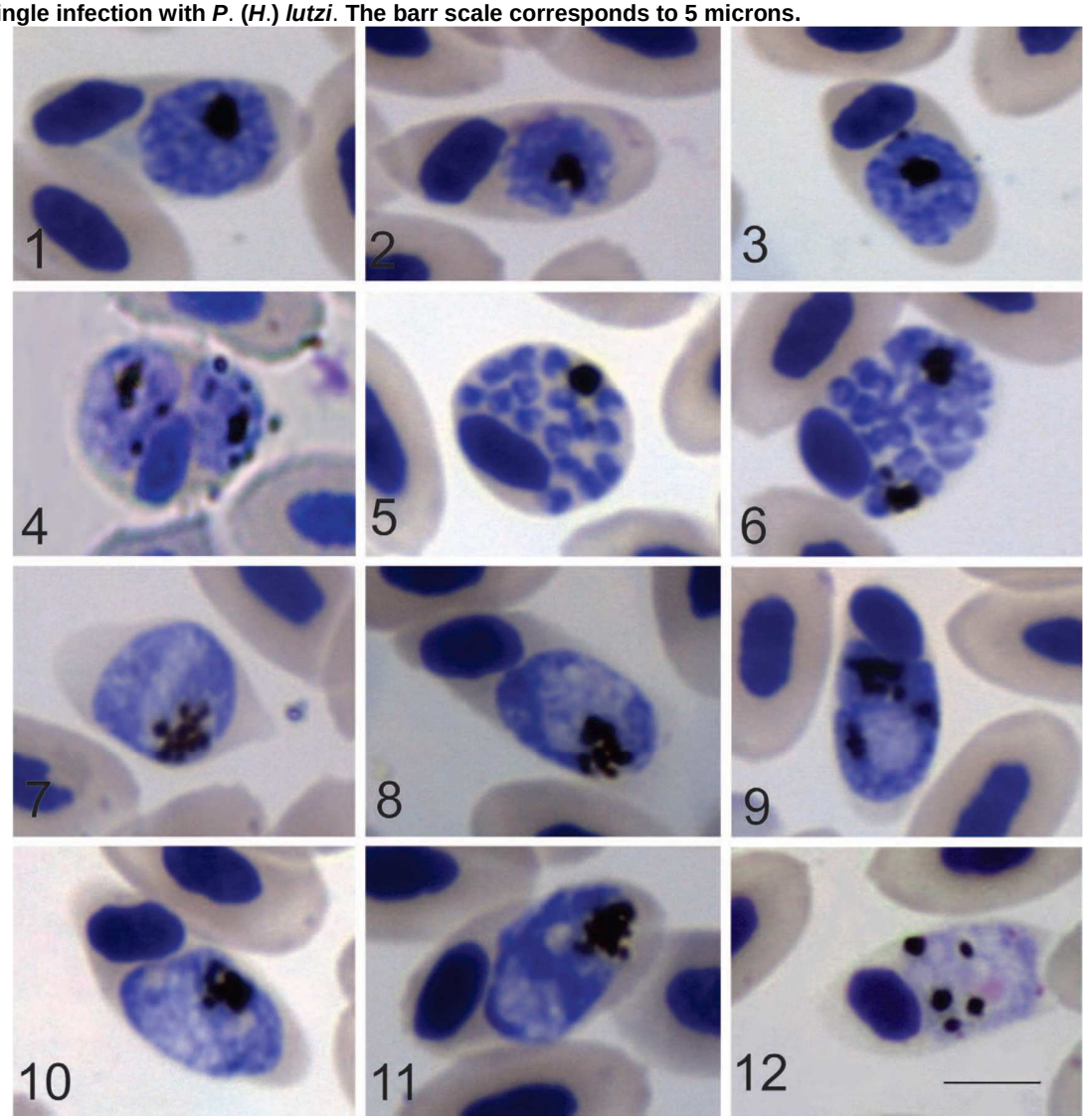
Plasmodium* (*H*.) *lutzi (Lucena 1939) (Figs. III 1-12; Table III 2)

Schizonts (Figs. III 1-6): Merozoites round and large with a prominent nucleus in the mature schizonts (Figs. III 5-6), without vacuoles. The presence of pre-segmented schizonts (Figs. III 1-4) is characteristic of *P. lutzi* as reported by Gabaldón and Ulloa (1976a).

Macrogametocytes (Figs. III 7-11): Macrogametocytes round or oval, and their nucleus was either large and round with irregular edges or elongated and band shaped. The intensely blue stained cytoplasm sometimes contained vacuoles (Figs. III 7-11). Its contents were heterogeneous and contained medium-sized (>0.5 µm), rounded, and dark-brown pigmented granules that were difficult to count due to their tendency to cluster (Figs. III 10,11). In contrast to the findings of Gabaldón and Ulloa (1976a), the gametocytes in our study often touched the sides of the red blood cells and the host cell nucleus (Fig. III 9).

Microgametocytes (Figs. III 12): Microgametocytes oval with a diffuse, large nucleus and dark-brown granules that bundled within the parasite cytoplasm, albeit in smaller numbers than those observed within the macrogametocytes. The microgametocytes: macrogametocytes proportion was 1:16; similar findings were reported by Zehtindjiev et al. (2012) for *Plasmodium polymorphum*.

Figures III 1-12. Blood forms of *Plasmodium* (*H*.) *lutzi* found in *Turdus fuscater*. 1-3: presegmented schizonts. 4: double infection in the same erythrocyte of indeterminate form. 5,6: segmented schizonts. 7,11: macrogametocytes. 12: microgametocyte. The pictures correspond with the bird that showed a single infection with *P*. (*H*.) *lutzi*. The barr scale corresponds to 5 microns.



3.4.2. Remarks

The sizes of the infected and uninfected cells of *T*. *fuscater* were smaller than those reported by Gabaldón and Ulloa (1976a) in *A*. *cajaneus* and by Hartman and Lessler (1963). There are several diagnostic differences between *P*. (*H*.) *lutzi* and *P*. *relictum*, which is the closest related species based on morphology: (1) *P*. (*H*.) *lutzi* schizonts produce fewer merozoites in mature schizonts (6-26), (2) its pigment granules tend to cluster at the poles,

(3) multiple parasites can be observed within a host cell, and (4) it cannot experimentally infect canaries (Lucena 1939, Gabaldón and Ulloa 1976a, Valkiūnas 2005). *Plasmodium* (*H*.) *lutzi* gametocytes in *A*. *cajaneus* can be found in polychromatic erytrocytes¸ however, we only found them in mature erythrocytes and cause the displacement and enlargement of the host cell nucleus, at times expelling it (Fig. III 7). Multiple infections were observed in a single host cell (Figs. III 4,6).

Table III 1. Species of caught birds and frequency of infection of *P*. *Lutzi*. Some of these birds were infected with other haemosporidia, but for the purpose of this paper, we only report those infected with *P*.*lutzi*

Birds species, family and order	Number examined	Number infected with *P. lutzi*
Apodiformes	18	0
Trochilidae	18	0
Adelomyia melanogenesis	1	0
Colibri coruscans	17	0
Columbiformes	13	0
Columbidae	13	0
Zenaida auriculata	13	0
Cuculiforme	2	0
Cuculidae	2	0
Coccyzus americanus	2	0
Passeriformes	122	0
Emberizidae	29	0
Zonotrichia capensis	29	0
Hirundinidae	14	0
Orochelidon murina	13	0
Hirundo rustica	1	0
Icteridae	13	0
Icterus nigragularis	1	0
Molothrus bonarensis	12	0
Parulidae	4	0
Leiothlypis peregrina	4	0
Thraupidae	7	0
Conirrostrum rufum	1	0
Diglossa sittoides	6	0
Troglodytiidae	1	0
Troglodites aedon	1	0
Turdidae	40	2
Catharus fuscescens	1	0
Catharus ustulatus	8	0
Turdus fuscater	31	2
Tyrannidae	5	0
Empidonax trailli	1	0
Pyrocephalus rubinus	3	0
Tyrannus melancholicus	1	0
Cardinalidae	4	0
Piranga rubra	4	0
Fringillidae	5	0
Astragalinus psaltria	5	0
Pelicaniformes	1	0
Ardeidae	1	0
Butorides striatus	1	0

Total	156	2

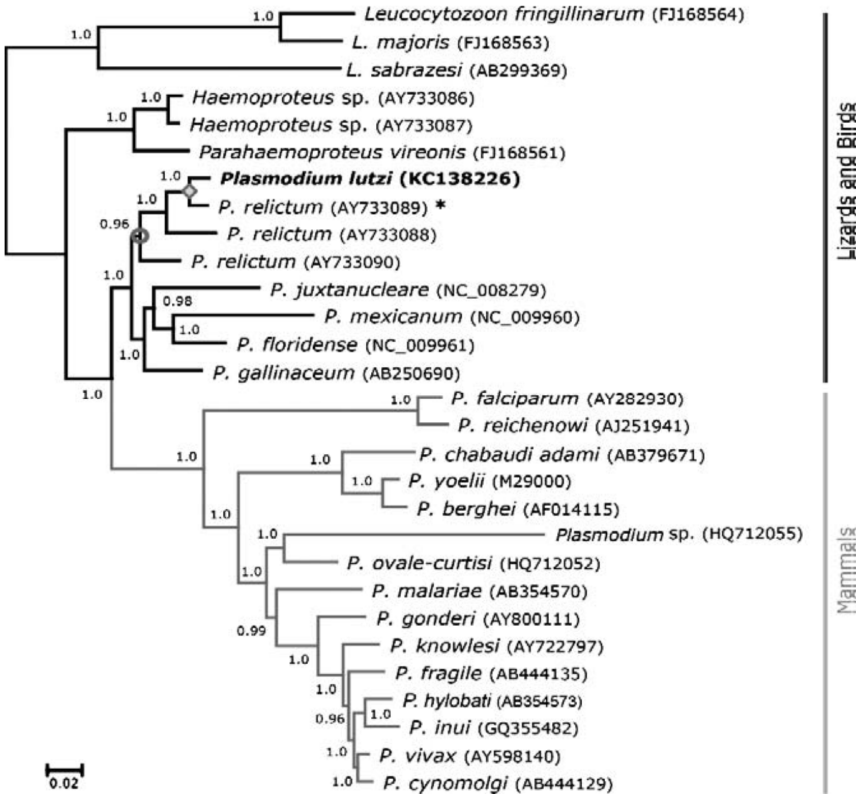
Table III 2. . Morphometric measures of the blood stages found in Turdus fuscater (present study) and *Aramides cajanea* (Gabaldón & Ulloa 1976). [a]: minimum and maximum values are provided followed in parentheses by the arithmetic mean and stantard desviation. [b]: taken from Hartman and Lessler (1963).

	Size (lm)*	
	Turdus fuscater [a]	*Aramides cajanea* [a b]
Uninfected erythrocytes	n=60	n=20
Length	11.8±0.67	12.9± 0.78
Width	6.3±0.62	7.2 ± 0.5
Nucleus length	5.2±0.42	5.5±0.4
Nucleus width	2.2±0.25	3.3±0.37
Schizonts	n =5	n =10
Length	8.22-9.17(8.77±0.38)	6.5-9.8(7.58±0.85)
Width	6.09-7.07(6.55±0.30)	5.3-7.1(6.40±0.62)
Length/width	1.1-1.4(1.3±0.8)	1.04-1.15(1.20±0.22)
Area	28.2-46.3(35.9±6.5)	N/A
Number of merozoites	16-23(18.5±3.1)	8-26(15.2±
Macrogametocytes	n =22	n =10
Length	6.04-7.92(6.7±0.57)	6.9-9.5(8.1±0.71)
Width	4.83-6.35(5.5±0.43)	5.5-7.7(6.6±0.69)
Length/width	30.26-33.22(31.46±1.14)	N/A
Area	1.02-1.65(1.27±0.2)	1.00-1.67(1.24±0.17)
Maximum number of pigment granules	16	16
Pigment granule size	0.57-1.18(0.8±0.2)	N/A
Microgametocytes	n =2	n =10
Length	6.64-9.45	6.3-8.6(7.5±0.64)
Width	4.91-5.63	5.3-7.4(6.3±0.68)
Length/width	33.1-36.61	N/A
Area	1.17-1.92	1.02-1.49(1.22±0.16)
Maximum number of pigment granules	15	19
Pigment granule size	0,76	N/A

3.4.3. Phylogenetic analysis and genetic distances

The phylogeny estimated with complete mitochondrial genomes is depicted in Fig. III 13. The phylogenetic analysis shows that *P*. (*H*.) *lutzi* shares a recent common ancestor with *P*. *relictum* (Fig. III 13). There are 3 complete mtDNA sequences identified by morphology as *P*. *relictum* with an average genetic divergence of 4.0% as was reported by Beadell and Fleischer (2005) (3.4-3.9%). The *P*. *relictum* isolate jb4. ATCC (strain ATCC 30141, American Type Culture Collection, sequence AY733089) is the closest related to *P*. *lutzi*; it was found in a mourning dove (*Zenaida macrura*) in 1937 in Nebraska, United States. By including *P*. *lutzi*, the 3 lineages identified as *P*. *relictum* form a paraphyletic group. The average genetic divergence, using the complete mitochondrial genome, between *P*. *lutzi* and any of the lineages identified as *P*. *relictum* was 3.3% (including the 3 lineages reported in Fig. III 13). The distance between *P*. *lutzi* and *P*. *relictum* AY733089 (the closest one to *P*. *lutzi*) was estimated to be 1.8%. As a comparison, the genetic distance estimated between sisters taxa of *Plasmodium* parasites from mammals was: 2.4% for *P*. *falciparum* - *P*. *reichenowi*; 1.8% for *P*. *yoelii* - *P*. *berghei*, and 1.2% for *P*. *vivax* and *P*. *cynomolgi* (see Fig. III 13).

Figure III 13. Phylogenetic tree of *Plasmodium* (*Haemamoeba*) *lutzi* based on complete mitochondrial genomes (approx. 5800 bp). The values above branches are posterior probabilities. * correspond to *P.relictum* from the ATCC isolate jb4.ATCC. Within parenthesis are the GenBank accession numbers for all the sequences analysed in this study.



3.4.4. Taxonomic summary

Type host*:* This parasite was described by Lucena (1939) in *Aramides cajaneus* in Brasil.

Additional hosts: *Turdus fuscater*

Material: The slides of the present study were stored in the Host-Parasite Relationship Research Group collection: avian haemoparasites model GERPH of the Universidad Nacional de Colombia (GERPH-0003182-0003194).

New locality*:* Bogotá, Colombia (4° 38' N, 74° 5' W 2560 m above sea level).

Distribution: This parasite has been recorded in Brazil, Venezuela, and now highlands in Colombia

DNA sequence*:* The mtDNA genome was deposited in GenBank under the accession number KC138226.

Site of infection*:* mature erythrocytes

Prevalence*:* 2 out 31 *Turdus fuscater* (6.45%) was infected at the locality of the study

3.5. Discussion

This investigation reports an enlarged of the geographic distribution range of *P*. (*H*.) *lutzi* and its host range. In epidemiologic surveys of avian malaria in Venezuela, Gabaldón and Ulloa (1976b) analysed haemoparasites in 21,201 samples belonging to 81 bird species, including *T*. *fuscater* (Passeriformes), but they only found *P*. (*H*.) *lutzi* in *A*. *cajaneus* (Gruiformes). Here, we report *T*. *fuscater* as a natural host for *P*. (*H*.) *lutzi*. This new record involves a host distantly related to those previously reported (Gruiformes vs. Passeriformes). Interestingly, the morphology of the parasites is maintained. It has been shown that the host can induce morphological changes in the parasite (Gabaldón et al. 1988). Furthermore, Hartman and Lessler (1963) reported that the differences in the sizes of infected red cells between 2 hosts can be explained by differences in the bird species. Unfortunately, there are no samples from this *P*. (*H*.) *lutzi* early description that allow us to compare them with our findings using molecular methods.

Although the mechanisms behind host switching in wildlife are not completely understood, they have been widely suggested in avian malarias (Garnham 1966, Valkiūnas 2005, Krizanauskiene et al. 2006). It may be possible that our finding is the result of a host switch or that *P*. (*H*.) *lutzi* actually exploits multiple hosts. Indeed, a broad host range is considered normal in avian malarias, as evidenced, for example, by the 310 hosts reported for *P*. *relictum* (Valkiūnas 2005). In some cases, host switching could increase virulence in 1 or multiple hosts (Van Riper et al. 1986, Toft and Karter 1990, Woolhose et al. 2001), but our data are not enough for supporting a host switch.

Turdus fuscater is distributed along the Andes from Venezuela to Bolivia (Ridgely et al. 2011). In Colombia, it is found between 1,400 m asl to 4,100 m asl. It is an opportunistic species that is well adapted to urban ecosystems. Its habitat includes open and cultivated areas, hedges, short grass pastures, weedy slopes and isolated shrub patches, and it has been less frequently observed in forested areas, on the roofs of buildings or near bodies of water. These characteristics make it one of the most abundant bird species in the study area.

Plasmodium (*H*.) *lutzi* has been reported in Brazil, Venezuela, and, possibly, in the Colombian plains (Lucena 1939, Renjifo et al. 1952, Gabaldón and Ulloa 1976b), all of which are lowland sites between 60 and 454 m asl in the eastern region of the continent. The type host, *A*. *cajaneus,* belonging to Rallidae family, is distributed from the south of México to the north of Argentina, where it can be found starting at sea levels up to 1,900 m

asl; in Colombia it has been found as high as 2,300 m asl. It is possible that *A*. *cajaneus* can exist sympatrically with *T*. *fuscater*, although each species would then be at their altitudinal distribution limit. In the lowlands, where *P*. (*H*.) *lutzi* has previously been reported, other species of *Turdus* can also be found. At higher altitudes, such as the altitude corresponding to Bogotá, there are other species of the Rallidae family, such as *Rallus semiplumbeus* (Hilty and Brown 1986). These observations, along with the feeding habits of the potential vectors, could explain the increase in the altitudinal range of *P*. (*H*.) *lutzi* that is reported in this work.

Our phylogenetic analyses confirmed that *P*. (*H*.) *lutzi* is indeed closely related to a lineage of *P*. *relictum*. Interestingly, there are 3 complete mitochondrial genomes identified as *P*. *relictum* by morphology. The relative position of *P*. *lutzi* makes *P*. *relictum* a paraphyletic group. The closest *P*. *relictum* lineage to *P*. *lutzi* was the isolate that is part of the ATCC (American Type Culture Collection). There are 2 alternative ways of interpreting these results; if we assume that the species were correctly identified, *P*. *lutzi* should not be considered a good morphological species. However, it seems obvious that such divergence among the 3 genomes cannot be considered as the polymorphism of 1 species (Beadell and Fleisher 2005, Beadell et al. 2006, Pacheco et al. 2012). Indeed, the divergence between *P*. *relictum* from the ATCC and *P*. *lutzi* is comparable with those found between pairs of sister species found in mammals, *P*. *vivax* - *P*. *cynomolgi* and *P*. *yoelii* - *P*. *berghei* (Fig. III 13). Thus, this option seems less likely.

Alternatively, our analysis indicates that there may be at least 2 species improperly identified as *P*. *relictum* or that morphology provides limited information for species identification (Beadell and Fleisher 2005, Beadell et al. 2006). We cannot discern between these 2 scenarios, since the morphological information was not described in detail (Beadell and Fleisher 2005, Beadell et al. 2006). Because we could morphologically identify this species as *P*. *lutzi* thanks to the excellent original description (Lucena 1939, Gabaldón and Ulloa 1976a), it seems important to highlight that it may be possible that many of the 310 reports of *P*. *relictum* (Valkiūnas 2005) could be indeed *P*. *lutzi* or other species. We can only speculate about the nature of *P*. *relictum* as an evolutionary lineage. Previous investigations indicate that it has extraordinary molecular diversity (Beadell et al. 2006) and we could indeed have many cryptic species. Overall, it is imperative to properly revise the limits of morphological information by integrating both morphology and molecular information.

Although knowledge of the ecology of the potential hematophagous vectors is crucial for the understanding of the mechanisms of avian haemoparasite transmission, to date the vector for *P*. *lutzi* in Colombia remains unknown. However, an abundance of *Culex quinquefasciatus* has been observed in the UNAL study site. Several groups have identified this Dipteran species as the vector of *P*. *relictum* (Rosen and Reeves 1954, LaPointe et al. 2005, Kimura et al. 2010) and for other *Plasmodium* species (Raffaele 1934, Huff 1950, Ejiri et al. 2008). In general, culicids have proven to be efficient vectors of various *Plasmodium* species (Klein et al. 1987, Santiago-Alarcon et al. 2012). Therefore, it is possible that *Cx*. q*uinquefasciatus* could be involved in *P*. (*H*.) *lutzi* transmission at the sampling location.

Neotropical countries are characterized by a low level of prevalence and parasitemia of avian haemoparasite infections when compared to other regions, such as the Nearctic (White et al. 1978). However, bird species belonging to the Turdidae family have been reported to be infected by parasites causing avian malaria worldwide (Beier et al. 1981, Young et al. 1993, Hatchwell et al. 2001, Rodríguez et al. 2009). This suggests that members of this family can mount an immunological response that can control the infection by keeping a low parasitemia, thus enabling the survival of both the parasite and the host. According to our study, this malaria parasite is being reported for the first time in Great Trush, whether it should be considered as a possible new pathogen requires additional investigation.

Our finding is of special relevance because the elevation in Bogotá exceeds that reported in previous studies for *Plasmodium* where the maximum boundary was considered to be 2,445 m asl (Loiseau et al. 2012). Meanwhile, *Plasmodium* has been identified from birds in Colombia at elevations up to 3.100 m asl (Rodríguez et al. 2009). Because UNAL is located in the central area of Bogotá, it is enclosed by a heat island produced by the albedo effect, and temperatures can reach up to 3 C higher than in areas located outside the city (Ángel et al. 2010). These anthropic changes, which are inherent to cities, could promote the spread of pathogens that otherwise would have narrower distributions. Because avian haemoparasites had not previously been studied in this area, it is impossible to know whether the parasite was previously established in the area or its presence is related to human activity (e.g., climate change). It is worth noting that Garamszegi (2011) reported that avian malaria has tripled in the last 70 yr in parallel with an increase in global temperature. Thus, anthropogenic process could change the geographic and altitude ranges of avian malarial parasites.

There are reports that demonstrate the development of *Plasmodium* in different vectors under variable temperature conditions (Santiago-Alarcon et al. 2012). The daytime temperature differences registered at the sampling location could be responsible for enabling the sexual of the parasite within the vector. The circulation of the parasite in this site demonstrates that the parasite-host-vector relationship is successful at both the altitude and temperature conditions of the area.

Although host switching has been a relevant evolutionary mechanism in avian malarial parasites, the process is still poorly understood. Whether or not a species have a naturally broad range of hosts and then occasionally switches to a new species is hard to demonstrate in nature. Thus, in order to ascertain the circumstances that facilitate the transmission of this parasite, we need to improve our understanding of the ecology of the invertebrate and vertebrate hosts and the ways in which it affect transmission in the study area. Finally, it is important to properly link morphological and molecular data whenever possible.

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Conclusions

The uplift of the Andes mountain range created new ecosystems, which were inhabited progressively from south to north following the progression of the mountains, as well as by organisms migrating from the lowlands to the new ecosystem at higher elevations. This process resulted in high species richness and endemisms in the Andes (Raven 2011). It is expected that an environment highly diverse favors complex interaction nets between organisms such as host-parasite relationships. Parasitism, in turn, is an important ecological interaction that modulates directly the population dynamics of their hosts and indirectly food webs in which they participate (Price et al. 1989). Within this frame, avian malaria and related parasites have been established as a model to study experimental infection and treatment, sexual selection, host and vectors specificity, diversity, ecology of parasite transmission in wildlife and the effect of foreign parasites in endemic and invasive bird populations (Marzal et al. 2011, Marzal 2012).

This study shows the Colombian Andes as a promising area for research on avian malaria and related parasites in the highlands. First new taxa have been discovered: seven new species, one recently described *Plasmodium* (*Novyella*) *unalis*. Second, new Host-parasite relationships were found for *Plasmodium rouxi* and *Plasmodium hermani* infecting a species of Trogoniformes. In addition, *Plasmodium lutzi* infecting a species of passerine is first reported here.

Concerning the distribution of the taxa found, this can be explored in several ways: macro-geographical, elevational and with respect to host range. *Plasmodium* and *Haemoproteus* species found in this study showed two geographical distribution patterns. Seven new species and other four previously described are distributed only in America. While, three species are distributed worldwide (*Haemoproteus columbae*, *Haemoproteus coatneyi* and *Plasmodium rouxi*). Regarding the elevational distribution, the *Plasmodium* species found were different to the reported in lowland previously.

With regard to host distribution, it is considered that species of *Plasmodium* infect a wide variety of birds even from different orders; whereas *Haemoproteus* show some degree of host specificity (Valkiūnas 2005). This study found three patterns of association with hosts for both genera (parasitic species infecting more than one individual): (1) species infecting a single bird species (*Plasmodium unalis*, *Haemoproteus* sp. 2), (2) species infecting birds from single family (*Haemoproteus witti*, *Haemoproteus* sp. 1) and (3) species widely distributed along bird taxa (*Haemoproteus coatneyi*, *Plasmodium* sp. 1, *Plasmodium lutzi*).

For protists taxonomy often few features are available to distinguish among species; therefore, it is necessary to use a molecular approach. This study confirm and for that reason also advice the use of both traditional and molecular techniques for research on haemosporidian diversity, as has been proposed by Valkiūnas et al. 2008 and Perkins et al. 2011. On one hand, the traditional approach allowed to describe the morphological features associated with species and provided evidence on true host-parasite associations (presence of gametocytes in blood of birds). On the other hand the molecular approach

supported new species descriptions, evaluate the presence of cryptic species and performed phylogenetic analyses.

In this study the use of cytochrome *b* sequences allowed to find new lineages, extend the knowledge on geographical and host distribution of these lineages and speculate about cross-transmission of parasites between resident and migratory birds.

This study reports cytochrome *b* lineages only from parasites confirmed by morphology since cytochrome b amplification of a parasites resulting from a recent bite by an infected vector or abortive infection could lead to erroneous conclusions about host distribution. However, in the future it would be appropriate to examine all samples available using molecular tools in order to have a broad view about what lineages are being potentially transmitted to a given bird and which eventually develop.

Cytochorome *b* gene shows limited resolution mainly at deep nodes, but it turns out useful at species level. Exploring larger sequences of the gene as well as whole mitochondrial evidence should provide better resolution to understand lineage diversification and parasite-host relationships. Other markers such as nuclear and apicoplast genes should also be explored.

Findigns allow concluding that:

(1) The relation between morphology and molecular lineage was close for six morphoespecies found: *H*. *witti*, *P*. *lutzi*, *P*. *unalis*, *Plasmodium* (*N*.) sp. 1 and *Haemoproteus* (*P*.) sp. 1, and *Haemoproteus* (*P*.) sp. 2.

(2) The Andes has great potential to discover new parasite taxa and host- parasite relationships as demonstrated by the finding of seven new species to the world and the description of ones: *Plasmodium* (*Novyella*) *unalis* sp. nov.

(3) There are at least 14 morphospecies of *Plasmodium* (7) and *Haemoproteus* (7) infecting birds from Colombian Andes.

(4) There are at least 25 cytochrome *b* lineages of *Plasmodium* and *Haemoproteus* circulating in the highlands of Colombia, 18 of them have been reported for the first time; of them 16 were found in resident birds.

(5) The Andes has great potential to discover new host- parasite relationships as is evidenced with: redescription of *P*. *lutzi* and expanding its elevational and host range, broadenring of geographic range for *Haemoproteus witti* a species restricted to hummingbirds, first determination of *Plasmodium* species infecting Trogoniformes, and differences in parasite composition between lowlands and highlands.

(6) *Plasmodium* (*Novyella*) *unalis* showed restricted host range (Turdidae family) in contrast to the broad geographic and host distribution of it the closest, morphologically and genetically species: *Plasmodium vaughani*.

(7) There were no parasite lineages from migratory birds infecting resident birds, suggesting that environmental and ecological conditions in this region have not permitted the establishment of their transmission cycles.

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Divulgation

Oral presentation

Matta NE, Gonzalez AD, Moncada LI (2012) Avian haemoparasites distribution in an altitudinal transect in Colombia. III International Congress of Neotropical Parasitology (Lima, Peru, 5-10 November). The Biologist (Lima) Abstract Book del III Congreso Internacional de Parasitología Neotropical 10: 82.

Matta NE, Mantilla JS, Gonzalez AD, Moncada LI (2013) Altitudinal range broadening and a new host for *Plasmodium* (*Haemamoeba*) *lutzi* in Colombia. III International Congress of Neotropical Parasitology (Lima, Peru, 5-10 November). The Biologist (Lima) Abstract Book del III Congreso Internacional de Parasitología Neotropical 10: 83.

Posters

Matta NE, Mantilla JS, Escalante AA, Pachecho AM, Gonzalez AD, Moncada LI (2013) *Plasmodium* (*Haemamoeba*) *lutzi* in Colombia International conference on Malaria and Related Haemosporidian Parasites of Wildlife (Vilnius, Lituania, 7-1 August). International conference on malaria and related Haemosporidian parasites of wildlife abstract book 2013, p.118.

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Scientific journals

Mantilla JS, González AD, Valkiūnas G, Moncada LI, Matta NE (2013) Description and molecular characterization of *Plasmodium* (*Novyella*) *unalis* sp. nov. from the Great Thrush (*Turdus fuscater*) in highland of Colombia. Parasitology research 112: 4193-4204.

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Plasmodium (Haemamoeba) lutzi in Colombia

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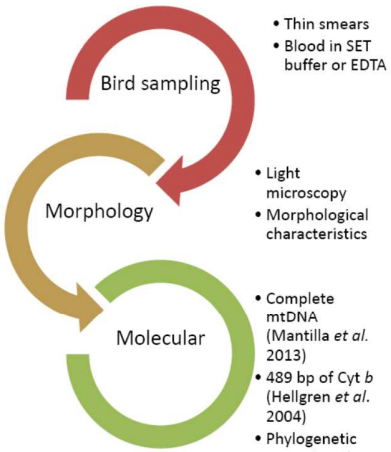
Plasmodium (Hemamoeba) lutzi was first observed in Brazil by Lutz and Meyer in 1908, where it was parasitizing *Aramides cajaneus* (Gruiformes).

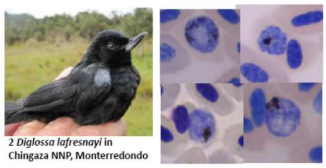
There were reports the finding of *P. (H.) lutzi* parasitizing three resident bird species of Passeriformes order, captured in highland of Colombia: the city of Bogotá and the Chingaza National Natural Park.

METHODOLOGY



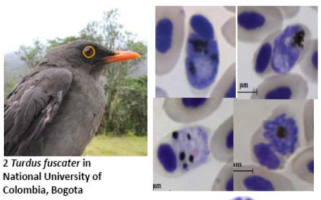
RESULTS

568 individuals, 79 species, 22 families. 243 Individuals from Bogotá and 325 from Chingaza NNP.

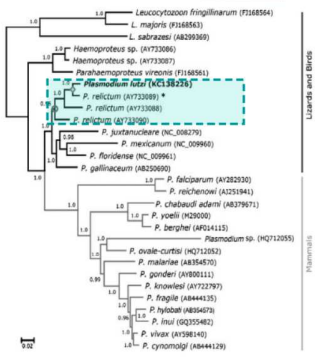


2 *Diglossa lafresnayi* in Chingaza NNP, Monterredondo

Diglossa albilatera in Chingaza NNP, Palacio



2 *Turdus fuscater* in National University of Colombia, Bogota



Phylogenetic tree of *P. (H.) lutzi* based on complete mtDNA (approx. 5,800 bp). The values above branches are posterior probabilities. Asterisk indicates *Plasmodium relictum* from the ATCC isolate jb4.ATCC. GenBank accession numbers for all the sequences analyzed in this study are given in parentheses.

Parasites in *Diglosa* spp. showed a Cyt *b* genetic distance (K2P) of 0.2% when compared with to parasite of *T. fuscater*.

When only Cyt *b* sequences are included, the most similar morphological species to *P. lutzi* are *P. (H.) tejerai*, another parasite from South America. We found similar Cyt *b* sequences to *P. lutzi* in America, Africa and Oceania (data no shown).

DISCUSSION

This finding provides evidence for a broad host range for *P. lutzi* that include two different orders, Gruiformes and Passeriformes. Similar parasites like *P. relictum* has been found in 11 avian orders, while *P. tejerai* has been reported in Galliformes and Sphenisciformes.

P. lutzi has been reported in Brazil, Venezuela, and, possibly, at the Colombian plains all of which are lowland sites between 60 and 454m. We reported an altitudinal expansion of the know altitudinal distribution for *P. lutzi* to high mountains located at 2650-3100 m asl.

Plasmodium has been identified in Colombia at altitudes up to 3100m (Rodriguez et al. 2009).

The circulation of the parasite in these sites demonstrates that the parasite-host-vector relationship is successful at both the altitude and temperature conditions of the area, because the infected bird species are not migratory, and have Andean distribution.

An abundance of *Culex quinquefasciatus* has been observed in the UNAL study site. Then, due that culicids have proven to be efficient vectors of various *Plasmodium* species, is possible that this mosquito could be involved in *P. (H.) lutzi* transmission at the sampling location.

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BIODIVERSITY OF AVIAN HAEMOPARASITES IN A HIGH ALTITUDE CITY OF COLOMBIA

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Universidad Nacional de Colombia-Sede Bogotá

Due to fast and uncontrolled expansion of cities, ecosystems are being affected by transformation, fragmentation and destruction of natural habitats, turning them into landscapes dominated by buildings. Urbanization alters the composition of wildlife communities, leading to decrease of biodiversity and changing the ecological interactions in these places. Although habitat loss and overexploitation are widely recognized as major causes of wildlife population declines, infectious diseases also produce significant impact on wildlife conservation.

The objective of this study was to analyze the diversity of avian blood parasites in an urban ecosystem in Colombia.

METODOLOGY:

Results: The general prevalence of parasites found by light microscopy were:

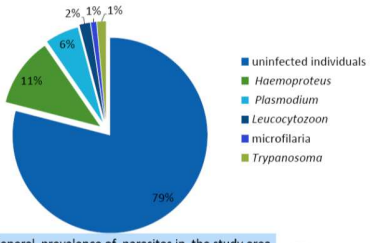
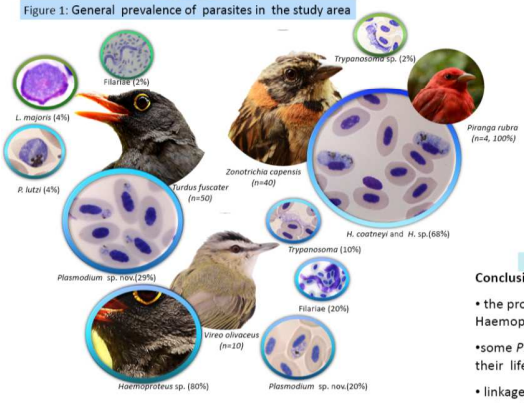


Figure 1: General prevalence of parasites in the study area



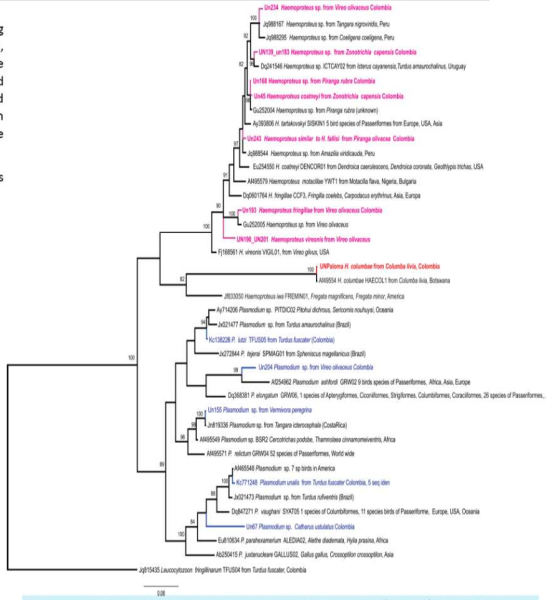


Fig. 2. Phylogenetic tree based on fragment of cytochrome *b* (490 bp). The phylogenetic reconstruction was done using Bayesian Inference in MrBayes version 3.1.2

Plasmodium sp. nov. is an issue of particular theoretical interest due to its ability to be transmitted actively at highlands in Andes, we found this parasite, in different localities between 2450m to 3100m. But the prevalence in Bogotá was more than twice that in other non-urbanized areas (Fig. 3), the urbanization process might be affecting transmission in different ways (Fig. 4), but more studies are necessary for a better understanding of this fact.

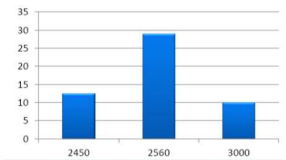


Fig 3. Prevalence of *Plasmodium* sp. nov. at different altitudes in Colombia, the places in 2450 m and 3100m are Naturals parks (protecting areas in Colombia, the place in middle altitude is the C-UN.

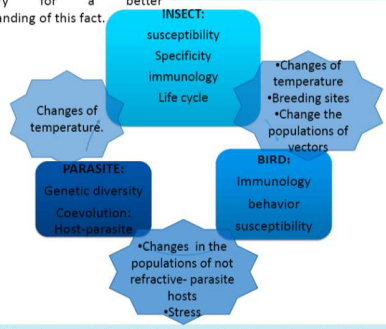


Fig. 4. Different ways in which urbanization may affect the ecology of vector-borne parasites

Conclusions:

- the process of urbanization in Bogotá probably is favoring the presence of some Haemoparasites in resident birds.
- some *Plasmodium* species have a successful development at high altitudes of the Andes, and their life cycles could be favored by human intervention.
- linkage between morphological and molecular are needed to obtain better phylogenetic and ecological conclusions in avian malaria.

References:
Mantilla JS, Matta NE, Pacheco MA, Escalante A, Gonzalez AD, Moncada LI (2013) Identification of *Plasmodium* (*Haemamoeba*) *lutzi* (Lucena, 1939) from *Turdus fuscater* (Great Thrush) in Colombia. J Parasitol. doi: 10.1645/12-138.1
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