Acta Tropica 159 (2016) 83–94

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Haemoproteus erythrogravidus n. sp. (Haemosporida, Haemoproteidae): Description and molecular characterization of a widespread blood parasite of birds in South America

Juan S. Mantilla[a], Angie D. González[a], Ingrid A. Lotta[a], Michaël Moens[b], M. Andreína Pacheco[c], Ananias A. Escalante[c], Gediminas Valkiūnas[d], Ligia I. Moncada[e], Javier Pérez-Tris[b], Nubia E. Matta[a,*]

[a] *Universidad Nacional de Colombia, Sede Bogotá-Facultad de Ciencias, Departamento de Biología, Grupo de Investigación Caracterización genética e inmunología, Carrera 30 No. 45-03, Bogotá 111321, Colombia*
[b] *Departamento de Zoología y Antropología Física, Facultad de Biología, Universidad Complutense de Madrid, Calle José Antonio Novais 12, 28040 Madrid, Spain*
[c] *Institute for Genomics and Evolutionary Medicine (igem), Temple University, Philadelphia, Pennsylvania, USA*
[d] *Nature Research Centre, Akademijos 2, LT-08412 Vilnius, Lithuania*
[e] *Universidad Nacional de Colombia, Sede Bogotá-Facultad de Medicina, Departamento de Salud Pública-Grupo de Investigación Infecciones y Salud en el Trópico, Carrera 30 No. 45-03, Bogotá 111321, Colombia*

ARTICLE INFO

Article history:
Received 6 September 2015
Received in revised form 15 February 2016
Accepted 29 February 2016
Available online 16 March 2016

Keywords:
Haemoproteus
Blood parasite
New species
Avian malaria
Haemosporida
Neotropics
Zonotrichia capensis

ABSTRACT

The great diversity of birds and ecosystems in the Andean mountains has been understudied in terms of their parasite species. We describe a new *Haemoproteus* parasite, *H.* (*Parahaemoproteus*) *erythrogravidus* infecting *Zonotrichia capensis* (Rufous-Collared Sparrow) in South America. The description of this blood parasite species is supported by morphological and molecular data based on a fragment of *cytochrome b* gene (*cyt b*) and complete mitochondrial genome sequences. The new species is closely related to *H.* (*Parahaemoproteus*) *coatneyi*, and it can be readily distinguished from the latter parasite due to morphology of its blood stages, particularly 1) the formation of a marked protrusion on envelope of infected erythrocytes by the majority of developing gametocytes, a feature which is unique for this *Haemoproteus* species and 2) the extremely attenuated width of the growing dumbbell-shaped macro- and microgametocytes. Additionally, *Haemoproteus erythrogravidus* is shown to be a monophyletic taxon that diverges from *Haemoproteus coatneyi* at the molecular level. We provide the complete mitochondrial DNA genome for both *H. coatneyi* and *H. erythrogravidus*. Molecular and morphological evidences indicate that *H. erythrogravidus* is present in Ecuador and Colombia, and genetic lineages with 100% of identity for the *cyt b* gene were reported in Chile, Perú, and Venezuela. Our study also indicates that *H. erythrogravidus* and *H. coatneyi* are sympatric sister taxa sharing *Z. capensis* as a host species across its distribution, which could be the result of sympatric speciation or complex biogeographic processes. Further studies on the distribution and evolutionary history of *Z. capensis* and its parasites *H. erythrogravidus* and *H. coatneyi* insight for our better understanding of the factors and dynamics driving parasite speciation.

1. Introduction

Avian haemosporidian parasites (Haemosporida) belong to four genera: *Leucocytozoon*, *Haemoproteus*, *Plasmodium*, and *Fallisia*. These blood parasites are characterized by heteroxenous life cycles involving sexual stages and sporogony in blood-sucking dipterans and asexual stages and development of gametocytes in vertebrate hosts. Gametocytes of these parasites develop in blood cells and possess clear sexually dimorphic characters, which help to distinguish these haematozoa from other intracellular blood parasites (Valkiūnas, 2005). Species of *Haemoproteus* are found worldwide, and they belong to two subgenera: *H.* (*Haemoproteus*) and *H.* (*Parahaemoproteus*), which are transmitted by hippoboscid flies (Hippoboscidae) and *Culicoides* biting midges (Ceratopogonidae), respectively (Garnham, 1966; Valkiūnas, 2005; Atkinson, 2008).

* Corresponding author at: Carrera 30 No. 45-03 ciudad universitaria edificio 421 oficina 133, Bogotá 111321, Colombia.
E-mail address: nemattac@unal.edu.co (N.E. Matta).

http://dx.doi.org/10.1016/j.actatropica.2016.02.025
0001-706X/

This subgeneric classification has been supported by phylogenetic studies (Santiago-Alarcon et al., 2010; Dimitrov et al., 2014; Matta et al., 2014; Bukauskaitė et al., 2015).

Early studies considered *Haemoproteus* infections as relatively benign to their avian hosts (Bennett et al., 1993). However, recent observations provided evidence that some species can affect their host's fitness and even cause death (Marzal et al., 2005; Olias et al., 2011; Pacheco et al., 2011b; Shurulinkov et al., 2012). Moreover, intense *Haemoproteus* infections can be virulent and even lethal for their natural vectors and other blood-sucking insects (Valkiūnas and Iezhova, 2004; Valkiūnas et al., 2013). Over 140 morphological species of *Haemoproteus* have been described (Iezhova et al., 2011), and molecular studies suggest an even greater species diversity (Ricklefs et al., 2004; Bensch et al., 2004; Perkins, 2014). Additional morphological, molecular and ecological characterization of parasite species is important in order to understand better their life cycles, epidemiology, pathology, and their potential impacts on wildlife.

Zonotrichia capensis (Rufous-collared Sparrow) is a passerine bird with a wide geographic range in the Americas, extending from Mexico to Argentina. It is commonly found on mountains between 1000 and 4700 m above sea level (m asl), but some populations are also present near sea level (BirdLife International, 2015). Its habitat includes open and semi-open green unforested areas, with different degrees of human intervention such as gardens, parks, croplands, grasslands, urban and suburban areas. Resident individuals are territorial, usually found in couples, with a reproductive season determined mainly by food availability. This species shows a high diversity of geographical song dialects between populations probably as the result of partial geographical isolation (Chapman, 1940; Lougheed and Handford, 1992; Fotheringham, 1995). Furthermore, different populations of *Z. capensis* show variable migratory behaviours, including sedentary and territorial tropical populations, altitudinal migrations among populations from Bolivia and Northern Argentina, and during the winter, there are latitudinal migrations from the Southern tip of Chile and Argentina to the Northern areas of these countries (Handford, 1985).

Z. capensis has been reported infected by a variety of haemosporidian parasites (Bennett and de Souza, 1980; Young et al., 1993; Valkiūnas, 2005; Durrant et al., 2006; Merino et al., 2008; Munro et al., 2009; Lacorte et al., 2013; Jones et al., 2013; González et al., 2014, 2015), however, the majority of recent reports are based on molecular diagnostics alone, lacking microscopy data that confirm parasite identity by morphology and report life cycle stages present in blood. It remains unclear if all reported haemosporidian lineages complete cycles and produce gametocytes in this bird species due to possible abortive development of infections (Valkiūnas, 2015). In fact, to date only three haemosporidian species have been conclusively demonstrated to infect *Z. capensis*: *Haemoproteus coatneyi*, *Plasmodium pinottii* and *Plasmodium homopolare* (Valkiūnas, 2005; Walther et al., 2014; González et al., 2015). However, there is genetic evidence to indicate that several yet unidentified lineages of Haemoproteus could infect this bird (Young et al., 1993; Durrant et al., 2006; Merino et al., 2008; Munro et al., 2009; Lacorte et al., 2013; Jones et al., 2013).

The aims of this study were (1) to describe a new haemosporidian parasite infecting *Z. capensis*, (2) to develop molecular characterization of this parasite in order to facilitate its identification and diagnosis and (3) to discuss the classification of the new species and its relationships with other haemosporidian parasites, based on molecular and morphological data.

2. Materials and methods

2.1. Ethical statement and sampling permits

Bird sampling methodology for this study in Colombia was approved by the Bioethics Committee of the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Nacional de Colombia Permit number: CBE-FMVZ-016 and the permit of Science Faculty act 05 of 2014. Sampling was authorized by "Unidad Administrativa Especial del Sistema de Parques Nacionales Naturales de Colombia UAESPNN—Subdirección técnica" (agreement 09 of 2009, SUT 010701 of 2010 and 01 of 2015), the Corporación autonoma regional de Risaralda CARDER and Autoridad Nacional de Licencias Ambientales, (ANLA) (file 4120E1104774 of 2011, file 4120E183893 of 2011, and resolution 0787 of 2013 and 001 of 2015). Genetic resources accession was authorized by contract No. 98 of March 26, 2014 of Ministerio de Ambiente y Desarrollo Sostenible de Colombia. The collection permits in Ecuador were provided by Ministerio del Ambiente, Loja (Research project 009-2012-IC-FAU-DPL-MA). Samples were transported under the exportation permits CITES 021/VS and 016-2012-IC-FLO-DPL-MAD.

2.2. Study area and sample collection

Samples for this study were collected at different sites in Colombia between January 2013 and July 2015 and in Ecuador between June and November 2012 (Table 1 and Appendix A in Supplementary material). We followed the taxonomical classification of birds of the South American Classification Committee (Remsen et al., 2014).

Birds were sampled by toenail clipping or by brachial vein puncture. Three thin blood smears were prepared and air-dried in the field. Blood smears were fixed in methanol for 5 min and stained with Giemsa 10% (pH 7.2) for 45 min in the laboratory. Additionally, approximately 50 μl of blood was taken using micro-haematocrit capillaries and stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA pH 8.0) or in absolute ethanol to preserve the DNA for molecular analysis. These samples were kept at room temperature in the field and later at −20 °C in the laboratory.

2.3. Blood film examination

Blood smears were examined using a Leica DM750 microscope (Leica Microsystems, Heerbrugg Switzerland) at high magnification (1000×). In order to detect malaria parasites, blood films were observed for 30 min. Then, positive slides were screened for two hours and at least 80 images representing different parasite blood stages of the new species were prepared using a Leica EC3 camera and processed with LAS EZ software (Leica Microcystems Ltd., Switzerland, 2012). Measurements of the parasites were obtained using ImageJ (Schneider et al., 2012). The morphology of the newly described parasite was compared to the type material of *Haemoproteus* (*Parahaemoproteus*) *coatneyi* from (*Passerella iliaca* (accession number G418712 in the Collection of the International Reference Centre for Avian Haematozoa [IRCAH] of the Queensland Museum, Australia) and *Z. capensis* (G462484 and G428314, samples from Venezuela and Chile, respectively). For comparative purposes, we also used blood films with *H. coatneyi* from the blood of *Z. capensis* and *Arremon brunneinucha* (accessions numbers GERPH 02937 and GERPH 08045, respectively) deposited in the biological collection Grupo de studio Relación Parásito Hospedero [GERPH].

Table 1
Sampling sites in Colombia and Ecuador. The sites' coordinates, elevation, number of bird sampled, number of *Zonotrichia capensis* infected with *Haemoproteus* (*Parahaemoproteus*) *erythrogravidus* and parasite lineages are provided.

Site	Coordinates	Elevation (m asl[a])	Number of bird species sampled/total number of individuals sampled	Number of *Zonotrichia capensis* infected/sampled	*Haemoproteus eritrogravis* lineage
Bogotá D.C. (Colombia)					
Campus Universidad Nacional de Colombia	04°38′N; 74°05′W	2560	45/427	19/76	ZOCAP01(n = 5), ZOCAP14(n = 3)
Gimnasio Colombo Británico School	04°48′N; 74°04′W	2560	3/4	1/2	ZOCAP01
Chingaza National Natural Park (Colombia)					
Palacio Forest	04°41′N; 73°50′W	2900	54/287	1/18	ZOCAP01
Monterredondo	04°37′N; 73°43′W	3100	29/126	0/8	
Los Nevados National Natural Park (Colombia)					
Otún Lagoon	04°46′N; 75°24′W	3950	27/446	0/22	
El Bosque station	04°43′N; 75°27′W	3300	56/367	0/66	
Ucumarí Regional Natural Park (Colombia)					
La Pastora	04°42′N; 75°29′W	2400	65/244	2/60	ZOCAP14
El Cedral	04°42′N; 75°32′W	2100	60/163	1/33	ZOCAP14
Wldlife Sanctuary Otún Quimbaya (Colombia)					
SFF Otun Quimbaya	4°43′N; 75°34′W	1850	42/125	1/28	ZOCAP14
Podocarpus National Park (Ecuador)					
Rumi Wilco	04°25′S; 79°21′W	1500	29/127	0/0	
El Bosque	04°22′S; 79°19′W	2000	57/414	0/0	
Cerro Toledo	04°36′S; 79°14′W	2500	57/188	1/1	ZOCAP01
Cerro Toledo	04°35′S; 79°11′W	3000	51/176	0/0	

[a] Meters above sea level.

2.4. DNA extraction, PCR amplification, and sequencing of the parasite cytochrome b fragment and the mitochondrial genome (mtDNA)

Total genomic DNA was extracted with a standard ammonium acetate or phenol-chloroform method (Sambrook et al., 1989) for samples diagnosed as positive by microscopy. First, a fragment of 1034 bp of the *cytochrome b* gene (*cyt b*) was amplified using the Pacheco et al. (2011b) protocol as modified in Mantilla et al. (2013). If amplification was unsuccessful using this protocol, a fragment of 479 bp of *cyt b* of the parasite was amplified using the nested PCR protocol described by Waldenström et al. (2004). The success of amplification was evaluated by running 2 μl of the final amplification products in a 2% agarose gel stained with ethidium bromide or GelRed™ DNA Stain (Biotium). Every reaction was accompanied by negative and positive controls and amplification products were precipitated with ammonium acetate and 95% ethanol (Bensch et al., 2000). Bi-directional sequencing of amplification products was conducted using 3730xl DNA Analyzer (Applied Biosystems, Foster City, USA).

In addition to the *cyt b* gene, two complete mitochondrial genomes (mtDNA) from *Haemoproteus* lineages were amplified, cloned and sequenced from a Rufous-Collared Sparrow and a Chestnut-capped Brush finch (*A. brunneinucha*). The morphospecies were corroborated by microscopy as *Haemoproteus erythrogravidus* (lineage ZOCAP01) and *H. coatneyi* (linage ARBRU02), respectively. Both bird samples were diagnosed by microscopy as single infections; however, sub-microscopic mixed infections could still be a problem. Thus, in order to avoid potential mixed infections, PCR products were amplified and cloned as followed: the primers forward 5′ GA GGA TTC TCT CCA CAC TTC AAT TCG TAC TTC and reverse 5′ CAG GAA AAT WAT AGA CCG AAC CTT GGA CTC were used to amplify 5871 base pairs of mtDNA genome with TaKaRa LA TaqTM Polymerase (TaKaRa Mirus Bio Inc., Shiga, Japan) as described by Pacheco et al. (2011a). 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 s at 94 °C and 7 min at 68 °C, followed by a final extension of 10 min at 72 °C. Following manufactory directions, two independent PCR products (bands of approximately 6 kb) were excised from the gel, purified using QIAquick® Gel extraction kit (Qiagen, GmbH, Hilden, Germany), and cloned in the pGEM®-T Easy Vector systems (Promega, Madison, USA). For at least three clones, both strands were sequenced using an Applied Biosystems 3730 capillary sequencer. There were no inconsistencies among the clones. The mtDNA genome sequences were submitted to GenBank under accession numbers KT698209 for *H. erythrogravidus* and KT698210 for *H. coatneyi*.

2.5. Phylogenetic analysis of the cytochrome b fragment and mitochondrial genome (mtDNA)

In the case of *cyt b*, sequences were edited using Sequencer 4.1.4 (Gene Codes, USA), and aligned in BioEdit version 7.0.5.3 (Hall 1999). The final alignment included 38 sequences of 488 nucleotides from different morphospecies reported for the Americas in Valkiūnas (2005) and the MalAvi database (Bensch et al., 2009) (last accessed 21 July 2015). Other lineages with 99% sequence similarity (in a MegaBLAST search) with the new species were included, although they had not been identified to morphospecies. A phylogenetic hypothesis was reconstructed using Bayesian Inference by MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). We selected the substitution model using the corrected Akaike Information Criterion implemented in jModeltest 2.1.3 (Darriba et al., 2012), which was the Transitional Model (Posada 2008), including a proportion invariable sites = 0.365 and substitution rate changing among sites according to a gamma distribution with alpha = 0.691 (TIM2 + I+ Γ). Two independent runs of one million generations were conducted with four chains, sampling every hundred generation. Convergence was assessed using average standard deviation of split frequencies between the two runs below 0.01 and were graphically analyzed using Tracer version 1.5 (Rambaut and Drummond 2006). In total, 25% of the trees were discarded as burn-in step and 15,000 trees were used to construct a 50% majority rule consensus tree. The phylogeny was visualized using FigTree v1.3.1 (Rambaut 2006).

The phylogenetic relationships were also inferred on the complete mtDNA genome by using MrBayes v3.1.2 with the default priors (Ronquist and Huelsenbeck 2003). This analysis included the 22 genome sequences available in GenBank for haemosporidian parasites isolated from lizards and birds. The alignment (6004 bp including gaps) was made using ClustalX v2.0.12 and Muscle as implemented in SeaView v4.3.5 (Gouy et al., 2010) with manual editing. The analysis considered four categories (*cytochrome oxidase* 1, *cytochrome oxidase* 3, *cytochrome b*, and noncoding regions) that were used as separate partitions (Pacheco et al., 2011a). *Leucocytozoon* genus were included as outgroup (Borner et al., 2015). A General Time-Reversible model (GTR + I + Γ) was the best model that fit to our mtDNA genome data. Bayesian support for the nodes was inferred in MrBayes using 6×10^6 Markov Chain Monte Carlo (MCMC) steps, and after convergence was reached (posterior probability < 0.01, potential scale reduction factor between 1.00 and 1.02), we discarded 25% of the samples as burn-in (Ronquist and Huelsenbeck 2003). Then, the sequence divergence between *Haemoproteus* species (including the one described here) was calculated on both alignments (*cyt b* and mtDNA) using a Kimura two-parameter model of substitution as implemented in MEGA v.6.05 (Tamura et al., 2013).

3. Results

In total, 3092 bird individuals belonging to 289 species were sampled; of these, 314 (10.1%) individuals were *Z. capensis*. Only this bird species was found to be infected by the newly described *Haemoproteus* parasite (see description below) (Supplementary Table S1 in the online version at DOI: 10.1016/j.actatropica.2016.02.025). Microscopic examination of blood smears revealed the presence of this parasite in 26 of 314 individuals (apparent prevalence = 8.28%; Table 1); five individuals were co-infected with *H. coatneyi* (15/314, 4.77%). A fragment of *cyt b* was amplified from seven individual birds with single infection, as determined by microscopic examination of blood films. Two lineages were obtained: ZOCAP01 and ZOCAP14 (GenBank accessions KF537315 and KF537329, respectively).

4. Description

Haemoproteus (***Parahaemoproteus***) ***erythrogravidus*** sp. nov. (Fig. 1, Table 2).

All blood stages develop in mature erythrocytes.

Young gametocytes (Fig. 1A and B): earliest forms seen anywhere in the cytoplasm of infected erythrocytes, but more frequently recorded in a position polar to erythrocyte nuclei Gametocytes surpass the length of the host cell nucleus and adhere to the erythrocyte nucleus (Fig. 1A), a characteristic feature in the development of this species. As parasites develop, gametocytes always assume dumbbell-like shapes with thickenings on their ends, which do not adhere to erythrocyte membrane (Fig. 1B). The cytoplasm is barely visible in young forms (Fig. 1A) and is prominent and coarsely-granulated in advanced forms (Fig. 1B). Pigment granules are small (<0.5 μm), roundish, golden-brown, and are scattered in the cytoplasm (Fig. 1A and B, Table 2). The outline of growing gametocytes is even or slightly wavy (Fig. 1A and B).

Macrogametocytes (Fig. 1C–L): fully-grown gametocytes are small; their maximum length is 12 μm. Position in erythrocytes, features of growth, and morphological characteristics of the gametocytes are similar to those in *H. coatneyi*, which were described by Burry-Caines and Bennett (1992) and Valkiūnas (2005). However, mature gametocytes of the *H. erythrogravidus* extend to the ends of erythrocytes and at least 70% of them markedly deform the host cells by causing readily visible balloon-like protrusions, which are located in the non-invaded cytoplasmic region of the erythrocytes. Infected erythrocytes resemble a 'gravid' state (see arrows in Fig. 1H, J, and M). These protrusions often remain in erythrocytes with fully-grown gametocytes, and they resemble empty spaces surrounded by the erythrocyte envelope in the parasitized cells (Fig. 1M), although the protrusions were not visible in some parasitized erythrocytes (Fig. 1L). Gametocytes grow along nuclei of infected erythrocytes, closely touching erythrocyte nuclei from early stages of development; they do not or only slightly displace nuclei laterally. The ends of the macrogametocytes only slightly enclose the nucleus. The central part of gametocyte pellicle is constricted (Fig. 1C–K), conferring dumbbell-like appearance to the parasite. Dumbbell-shaped gametocytes predominate among growing gametocytes; they are closely associated with the host cell nucleus. Advanced dumbbell-shaped gametocytes touch the envelope of erythrocytes by their ends (Fig. 1C–K). Approximately 60% of the growing dumbbell-shaped gametocytes are markedly atten-

Table 2
Morphometric parameters of host cells and mature gametocytes of *Haemoproteus* (*Parahaemoproteus*) *erythrogravidus* (lineage ZOCAP01, Genbank accession no. KF537315) from the blood of *Zonotrichia capensis*.

Feature	Measurement
Uninfected erythrocyte	N = 25
Length	10.3–11.7 (10.9 ± 0.4)
Width	5.8–6.5 (6.1 ± 0.2)
Area	41.8–56.3 (47.9 ± 3.7)
Uninfected erythrocyte nucleus	N = 25
Length	4.7–5.6 (5.1 ± 0.1)
Width	1.9–2.4 (2.1 ± 0.1)
Area	7.5–9.1 (8.2 ± 0.5)
Macrogametocytes	N = 25
Infected erythrocyte	
Length	10.3–11.7 (11.1 ± 0.5)
Width	5.4–7.2 (6.4 ± 0.7)
Area	49.6–56.7 (52.7 ± 2.1)
Infected erythrocyte nucleus	N = 25
Length	4.1–5 (4.6 ± 0.2)
Width	2–2.2 (2.1 ± 0.05)
Area	6.4–8 (7.4 ± 0.5)
Gametocyte	N = 25
Length	10.3–12 (11.1 ± 0.6)
Width	2.3–7.2 (4.1 ± 1.7)
Area	34.3–40 (36.9 ± 1.8)
Gametocyte nucleus	N = 25
Length	1.4–3.1 (2.2 ± 0.4)
Width	1–2.2 (1.6 ± 0.39)
Area	2.3–4.5 (3.6 ± 0.7)
No. of pigment granules	12–17 (13.5 ± 1.6)
NDR	0.5–0.8 (0.6 ± 0.1)
Microgametocytes	
Infected erythrocyte	N = 11
Length	13.8–16.9 (15.3 ± 1)
Width	6.8–8.4 (7.6 ± 0.6)
Area	36.7–45.9(40.6 ± 2.9)
Infected erythrocyte nucleus	N = 11
Length	6.2–8.2 (6.9 ± 0.6)
Width	2.2–3.1 (2.6 ± 0.3)
Area	15.4–21.7(18 ± 2.1)
Gametocyte	N = 11
Length	13.8–6.9 (15.3 ± 1)
Width	0.7–3.4 (2.2 ± 0.7)
Area	38.5–48.9 (44.8 ± 3.7)
Gametocyte nucleus	N = 11
Length	8.4–12.1 (10.4 ± 1.2)
Width	0.7–3.3 (2.1 ± 0.7)
Area	21.3–30.6 (27.4 ± 2.7)
No. of pigment granules	8–15(10 ± 1.2)
NDR	0.7–1.2 (0.9 ± 0.1)

All measurements are given in micrometres. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

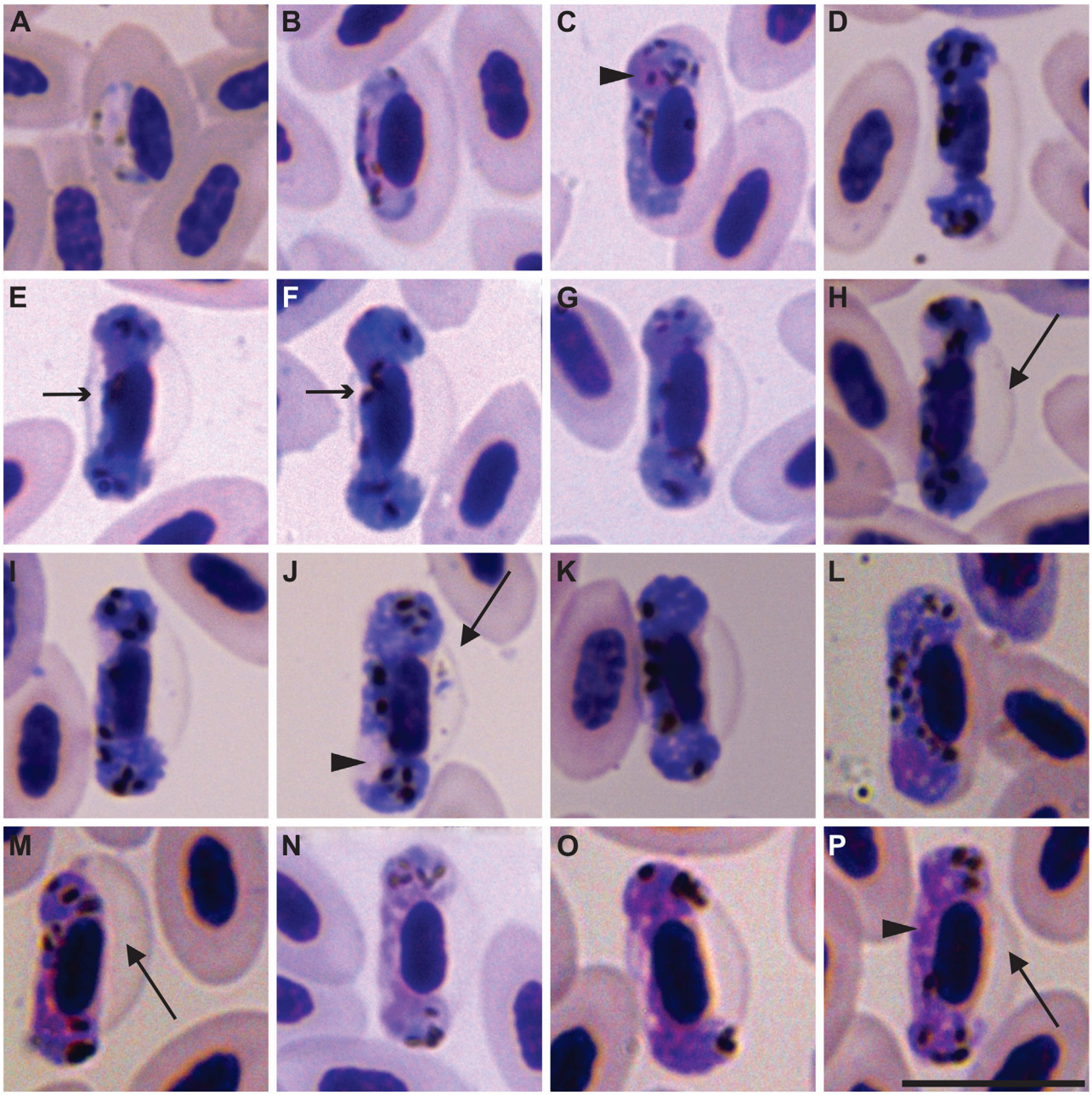

Fig. 1. *Haemoproteus* (*Parahaemoproteus*) *erythrogravidus* sp. nov. (lineage ZOCAP01) from *Zonotrichia capensis*. A, B: young gametocytes, C–M: macrogametocytes, N–P microgametocytes. Long triangle arrows—protrusions of infected erythrocyte envelope. Long simple arrows—unfilled spaces between dumbbell-shaped gametocytes and erythrocyte envelope. Triangle arrowheads—parasite nuclei. Giemsa-stained thin blood films. Scale bar = 10 μm.

uated in width, which is less than 1 μm (see arrows in Fig. 1E,F) a characteristic feature of this species development. Mature gametocytes fill up the poles of erythrocytes (Fig. 1F–L). Parasiteís nucleus is compact, prominent, variable in shape and subterminal in position (Fig. 1C, J, and P). There is no visible nucleolus. Pigment granules are randomly scattered throughout the cytoplasm, roundish or oval in form, approximately 13 per gametocyte on average, predominantly of medium size (0.5–1.0 μm) and often form groups of two or more granules (Fig. 1H–L). The cytoplasm is blue, heterogeneous in appearance, lacking visible volutin granules and possesses vacuoles in mature gametocytes (Fig. 1L). The outline of macrogametocytes is generally even, but may occasionally become wavy or slightly ameboid.

Microgametocytes (Fig. 1N–P): general configuration as for macrogametocytes with usual haemosporidian sexually dimorphic characters. Fully-grown microgametocytes are larger than macrogametocytes; the maximum length of fully-grown microgametocytes is 16.9 μm (Table 2 The nucleus of microgametocytes is granular and their cytoplasm possesses small yet readily visible vacuoles. The ratio of micro- to macrogametocytes in the type material was 1:5.

4.1. Remarks

H. erythrogravidus sp. nov. is particularly similar to *H. coatneyi*. Because both parasitize the same or closely-related avian hosts (see Valkiūnas (2005) and Supplementary Table S1 in the online version at DOI: 10.1016/j.actatropica.2016.02.025), the new species was carefully compared with type materials of *H. coatneyi* in *P. iliaca* (Fig. 2A–D) and *Z. capensis* (Fig. 2E–P). The new

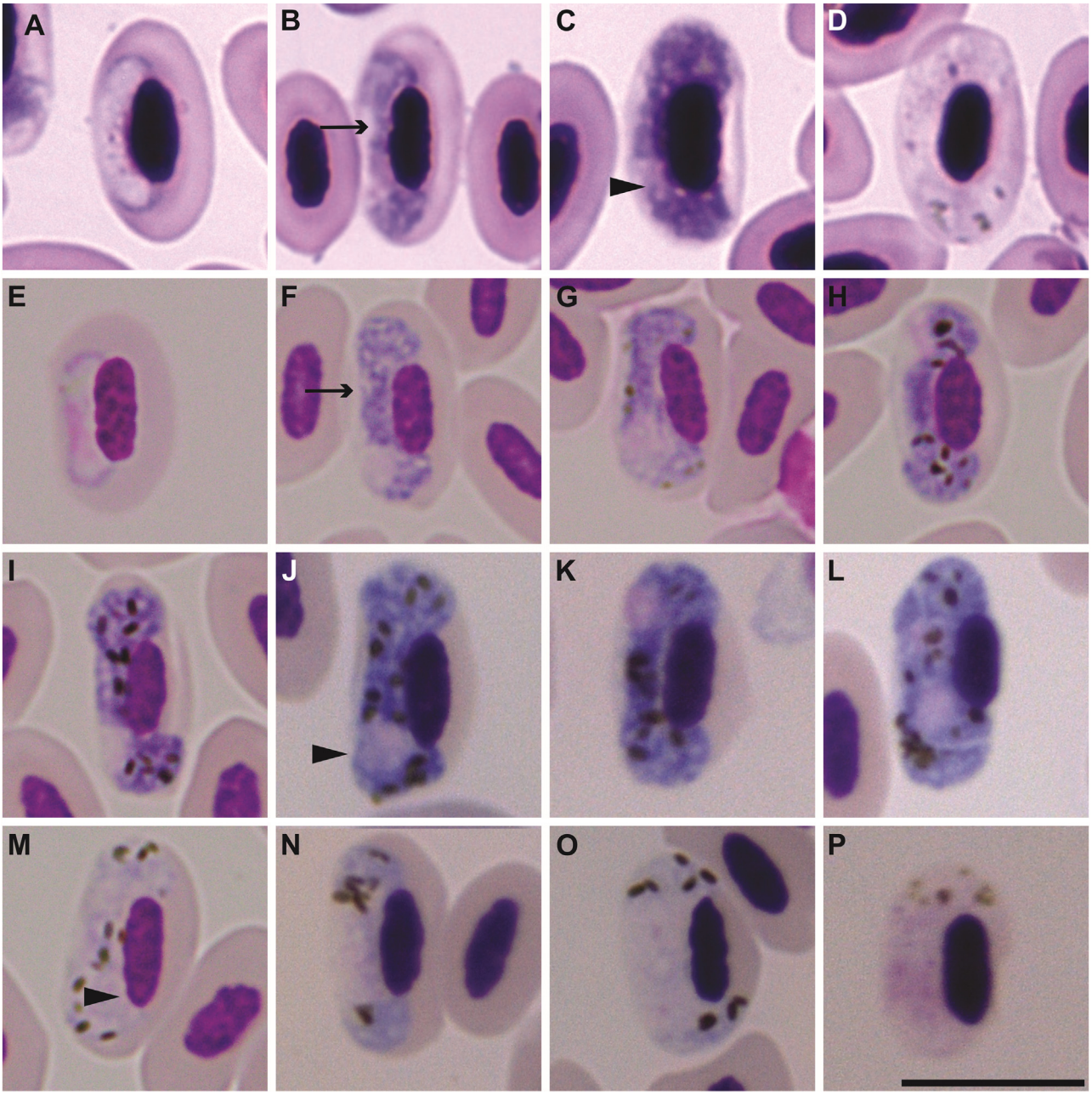

Fig. 2. *Haemoproteus* (*Parahaemoproteus*) *coatneyi* from the blood of *Passerella iliaca* and *Zonotrichia capensis*. The images are from the parahapantotype material (accession G418712 *P. iliaca*) deposited in the International Reference Centre for Avian Haematozoa (A-D) and from this study (accession GERPH 02937 *Z. capensis*) (lineage ZOCAP13, E-P). A, E: young gametocytes. B–C, F-L: macrogametocytes. D, M–P: microgrametocyte, Long simple arrows—unfilled spaces between dumbbell-shaped gametocytes and erythrocyte envelope. Triangle arrowheads—parasite nuclei. Giemsa-stained thin blood films. Scale bar = 10 μm.

species was described from a single infection, as confirmed by both morphological and genetic data, and *H. erythrogravidus* can be distinguished from *H. coatneyi* based on two key characteristics: (1) the development of marked protrusion in the envelope of infected erythrocytes ('gravid' morphology; Fig. 1H, J, and M), and (2) the markedly attenuated width of growing dumbbell-shaped macro- and microgametocytes (Fig. 1E, F, and O). Both these features are not characteristic of *H. coatneyi* (compare Fig. 1D–K, N–P and Fig. 2B–D and F–P). Based on a 479 bp fragment of the by *cyt b* gene, the mean genetic distance between *H. erythrogravidus* (lineages ZOCAP01 and ZOCAP14) and *H. coatneyi* (lineage ARBRU01) is between 0.7% and 0.9%, and between 1.9% and 2.1% with ZOCAP13 (*H. coatneyi* from *Z. capensis* captured in the type locality of *H. erythrogravidus*) (Supplementary Table S2 in the online version at DOI: 10.1016/j.actatropica.2016.02.025).

It worth mentioning that the examination of two parahapanto types of *H. coatneyi* (IRCAH G462484 and G428314) (IRCAH) revealed presence of a co-infection by *H. erythrogravidus* and *H. coatneyi* that was overlooked during original deposition of this material in IRCAH. In other words, both these species parasitize *Z. capensis*, and we recognised this for the first time in this study. It is important to note that *H. erythrogravidus* as a valid taxon is corroborated by the fact that: (1) we demonstrated that single infections of *H. coatneyi* in *Z. capensis* are morphologically similar to the hapaton type of *H. coatneyi* from *P. iliaca* and both are distinct from *H. erythrogravidus* in *Z. capensis*, indicating that gravid morphology is absent from host cells parasitized by *H. coatneyi* in both

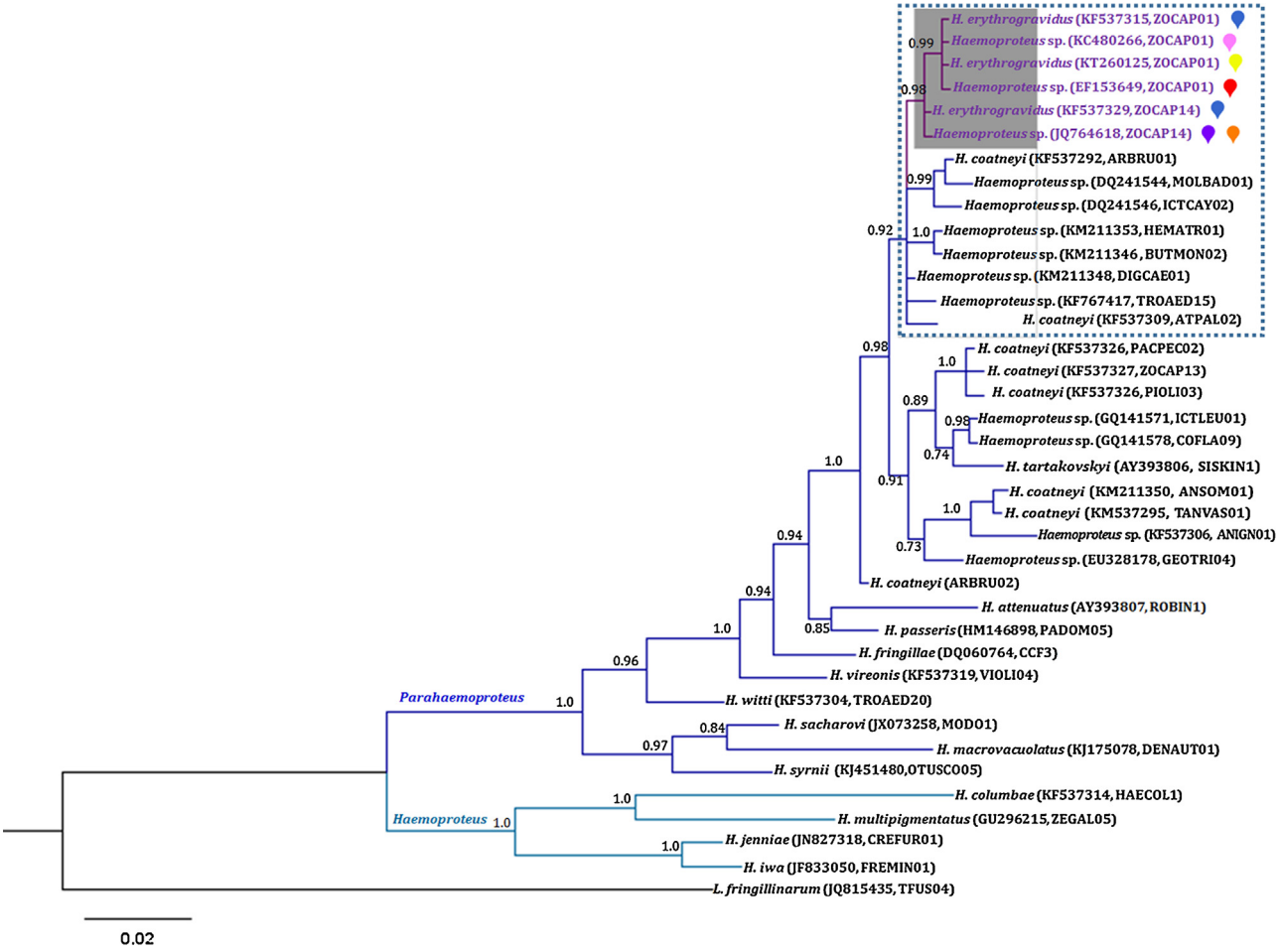

Fig. 3. Bayesian phylogeny based on 488 bp of partial *cytochrome b* gene sequences of morphologically identified *Haemoproteus* spp. (21 lineages) and unidentified (13 lineages) species reported in America; among them were 10 lineages with 99% nucleotide similarity with the lineages of ZOCAP01 and ZOCAP14 of *Haemoproteus* (*Parahaemoproteus*) *coatneyi*. The lineage TFUS04 of *Leucocytozoon fringillinarum* was used as outgroup. Light blue colour indicates lineages of *Haemoproteus* subgenus, and dark blue colour indicates lineages of *Parahaemoproteus* subgenus. *Haemoproteus* (*Parahaemoproteus*) *erythrogravidus* lineages are shown in purple and are marked with a grey square. GenBank accession numbers, MalAvi lineage codes are provided before the parasite species names. Posterior probabilities lower than 0.70 are omitted. The branch lengths are drawn proportionally to the amount of change; the scale bars represent the nucleotide substitution rates. The colour spaces correspond to sites where this parasite has been reported by molecular or microscopical studies (blue = Colombia, pink and orange = Perú (different areas), yellow = Ecuador, red = Chile and purple = Venezuela). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

these hosts, and (2) we demonstrated there are clear molecular differences between *H. erythrogravidus* and *H. coatneyi* (see Fig. 3). Further studies are needed to determine how often these parasites are present in co-infection in *Z. capensis* and other bird species.

It is important to note that the slightly visible protrusions in envelope of infected erythrocytes are occasionally visible in *Haemoproteus* (*Parahaemoproteus*) *fringillae* infection (see Fig. 64, 8 in Valkiūnas (2005)). However, such protrusions have been observed only at final stages of gametocyte development in the *H. fringillae* infection, and the protrusions are considerably less prominent in comparison to *H. erythrogravidus* infections. Additionally, these parasites are clearly different genetically. Mean genetic distance between *H. erythrogravidus* and *H. fringillae* (lineage Ccf3) is between 4.3% to 4.5% based on a fragment of 479 bp of *cyt b* gene (Supplementary Table S2 in the online version at DOI: 10.1016/j.actatropica.2016.02.025).

Markedly attenuated width of growing dumbbell-shaped gametocytes was described in *Haemoproteus* (*Parahaemoproteus*) *attenuates* (Valkiūnas, 2005). However, this feature is observed only in microgametocytes of the latter parasite. The genetic distance between *H. erythrogravidus* (lineages ZOCAP01 and ZOCAP14) and *H. attenuates* (the lineage ROBIN1) is between 4.3% and 4.6% based on a fragment of 479 bp of *cyt b* gene (Supplmentary Table S2 in the online version at DOI: 10.1016/j.actatropica.2016.02.025).

4.2. Taxonomic summary

Type host: Rufous-collared Sparrow *Z. capensis* (Muller, 1776) (Emberizidae, Passeriformes).

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

Type specimens: Hapantotype (accession numbers GERPH-03276 in the collection Grupo de Estudio Relación Parásito Hospedero (GERPH), Department of Biology, Universidad Nacional de Colombia, Bogotá, Colombia; intensity of infection is 0.5%; collected by Juan S. Mantilla on 29 October 2011 at the campus of Universidad Nacional de Colombia; lineage ZOCAP01 in MalAvi, GenBank KF537315). Parahapantotypes (accession numbers: GERPH-03355, GERPH-05859, GERPH-06054, GERPH-03278) were deposited in the biological collection GERPH at Universidad Nacional de Colombia, Bogotá, Colombia. Digital images of blood stages of the newly described parasite from the type preparations are available on request from GERPH.

DNA sequences: Complete mitochondrial lineage (5871 bp, GenBank no. KT698209, from the hapantotype sample, lin-

eage ZOCAP01). Mitochondrial cytochrome *b* lineages ZOCAP01 (1056 bp., GenBank no. KF537315, from the hapantotype sample) and ZOCAP14 (964 bp. GenBank, accession numbers KF537329, from a parahapantotype sample).

Site of infection: Mature erythrocytes, no other data.

Prevalence: In the type locality, 19 out of 427 individuals of all bird species sampled were infected (the overall prevalence is 4.4%), but the prevalence was 19 out of 76 (26.4%) in the type host. No infections were observed among 351 bird individuals other than *Z. capensis*.

Distribution: This parasite species was found at five sites in Colombia (Appendix A in Supplementary material) and at one site in Ecuador. Altitudes of these sites vary between 1850 and 2900 m asl. This parasite likely is widespread in Americas (see Discussion).

Additional hosts: Additional hosts are unknown.

Etymology: The species name '*erythrogravidus*' is derived from the words 'erythrocyte' and 'gravid'; it reflects the main diagnostic character of this parasite, namely the marked deformation of infected erythrocytes by gametocytes. The shape of this deformation resembles a 'gravid' state (Fig. 1E–K, M, see the feature indicated with a long triangle arrow).

4.3. Phylogenetic relationships of parasites

The phylogenies based on partial *cyt b* sequences and the mtDNA genomes are given in Figs. 3 and 4, respectively. The phylogeny based on partial *cyt b* sequences (Fig. 3) showed a well-supported clade formed by the newly described *Haemoproteus* species, two lineages of *H. coatneyi*, and six *Haemoproteus* lineages without morphological identification (Fig. 3). These species are grouped in the clade containing species of *Parahaemoproteus* subgenus (Fig. 3). *Haemoproteus erythrogravidus* consists of two lineages with a genetic distance of 0.2% between them (Supplementary Table S2 in the online version at DOI: 10.1016/j.actatropica.2016.02.025). We demonstrated that the *cyt b* lineages ZOCAP01 and ZOPCAP14 correspond to the morphospecies *H. erythrogravidus*. We identified these lineages in *Z. capensis* in Colombia and Ecuador, but previous studies had also detected identical *cyt b* sequences in birds at other locations. ZOCAP01 was detected in *Z. capensis* in Perú (GenBank accession number KC480266; Jones et al., 2013) and *Z. capensis* in Chile and Perú (GenBank EF153649; Merino et al., 2008; Marzal et al., 2015), whereas ZOCAP14 was detected in *Chlorospingus ophthalmicus* (Common bush tanager) in Venezuela (Mijares et al., 2012). Additionally, similar lineages with genetic distance of 0.5% and 1% (3 and 5 different nucleotides respectively) were found in 17 species from 6 families and 4 countries (Table 3, Fig. 3). However, none these studies demonstrated presence of gametocytes in the PCR-positive birds.

The mtDNA phylogenetic analysis (Fig. 4) shows that species of the subgenus *Parahaemoproteus* form a monophyletic group sharing a common ancestor with *Plasmodium* species. However, relationship of *Parahaemoproteus* and *Haemoproteus* parasites remain unresolved because of the limited taxon sampling for the parasites of the latter subgenus in this study. The mtDNA genome phylogenetic analysis (Fig. 4) indicates that *H. coatneyi* (lineage ARBRU01) is the closest species to *H. erythrogravidus* (lineage ZOCAP01) in this dataset. Indeed, the genetic divergence between the complete mtDNA genome of *H. erythrogravidus* and *H. coatneyi* was 0.9% (0.009 ± 0.0012). The average pairwise genetic divergence between other well-known *Haemoproteus* species (with morphological descriptions) was: 4.3% (0.0429 ± 0.0028) for *H. erythrogravidus* and *H. vireonis* (FJ168561) and 6% (0.0599 ± 0.0030) for *H. erythrogravidus* and *H. macrovacuolatus* (KJ499987). As a comparison, the genetic distance estimated between sister taxa of *Plasmodium* parasites was 1.8% for *P. lutzi* and *P. relictum* (AY733089).

5. Discussion

Many bird species were sampled in South America, but only *Z. capensis* was infected by *H. erythrogravidus* (Supplementary Table S1 in the online version at DOI: 10.1016/j.actatropica.2016.02.025), suggesting that this parasite might be specific for this bird species. Additionally, morphological confirmation of presence of gametocytes (the final stage of haemosporidian development in vertebrate hosts) in the blood is only available from the Colombian and Ecuadorian samples (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.actatropica.2016.02.025). However, even though the genetic lineages retrieved from birds sampled in Perú lack of morphological confirmation of presence of gametocytes, the available molecular evidence suggests that this parasite occurs in other populations of *Z. capensis*, and demonstrates the presence of parasite transmission at this latitude and at elevations between 130 m and 1894 m asl (Jones et al., 2013; Marzal et al., 2015). Furthermore, a *cyt b* lineage identical to one of the *H. erythrogravidus* lineages identified in this study was also documented in *C. ophthalmicus* (Mijares et al., 2012), however it is not clear whether this is a susceptible host or if it merely represents an abortive infection.

Several studies have discussed the use of a percentage of divergence in the *cyt b* gene to delimit species in the absence of other criteria (Križanauskienė et al., 2006; Outlaw and Ricklefs 2014). *Haemoproteus* lineages differing of over 5% are typically morphologically distinct, but there are numerous well-established examples of species that differ less than as 1% in the *cyt b* gene, but are readily distinguishable morphologically. For example, *Haemoproteus pallidus* and *Haemoproteus minutus* differ only at 7 positions of 1070 bp in the complete *cyt b* gene (Hellgren et al., 2007). In addition, based on host and geographic distribution of three morphologically undescribed *Haemoproteus* lineages infecting birds in the Lesser Antilles that differ at 0.3% in *cyt b* gene, and combined with sequencing of three additional loci, it was suggested that these should be recognized as good phylogenetic species (Outlaw and Ricklefs 2014). Furthermore, some *Haemoproteus* parasite lineages have been reported to differ only by one base pair, showing strong host structuration and suggesting possible speciation due to adaptation to particular avian host species (Reullier et al., 2006). Likewise a parasite group formed by similar morphology and *cyt b* lineages, which showed a different temporary patterns of transmission (Pérez-Tris and Bensch, 2005; Pérez-Rodríguez et al., 2015), were demonstrated to be different genetically isolated groups (Pérez-Tris et al., 2007), we think that our study adds *H. erythrogravidus* and *H. coatneyi* to the same set of data from the Neotropics.

It is interesting to note that similar *Haemoproteus* lineages were also reported in numerous bird species belonging to the families Troglodytidae, Thraupidae, Trochilidae, Icteridae and Turdidae at many sites in South America (Table 3, see Durrant et al., 2006; Galen and Witt 2014; Harrigan et al., 2014). However, these findings were not supported by observation of parasite gametocytes. Whereas the lack of morphological confirmation makes it impossible to completely discard abortive *Haemoproteus* infections in these birds (Valkiūnas et al., 2013, 2014; Valkiūnas, 2015); reproducible observations of lineages by PCR in bird species across geographic areas may indicate active transmission or other important ecological phenomenon. In particular, reports from the house wren (*Troglodytes aedon*) in Perú indicate that this species is infected by parasite lineages that are similar to *H. erythrogravidus* (Galen and Witt, 2014). However, in Colombia (the type locality of the new species), *T. aedon* has been extensively sampled, and examined both microscopically and by PCR-based tools, but has not been found

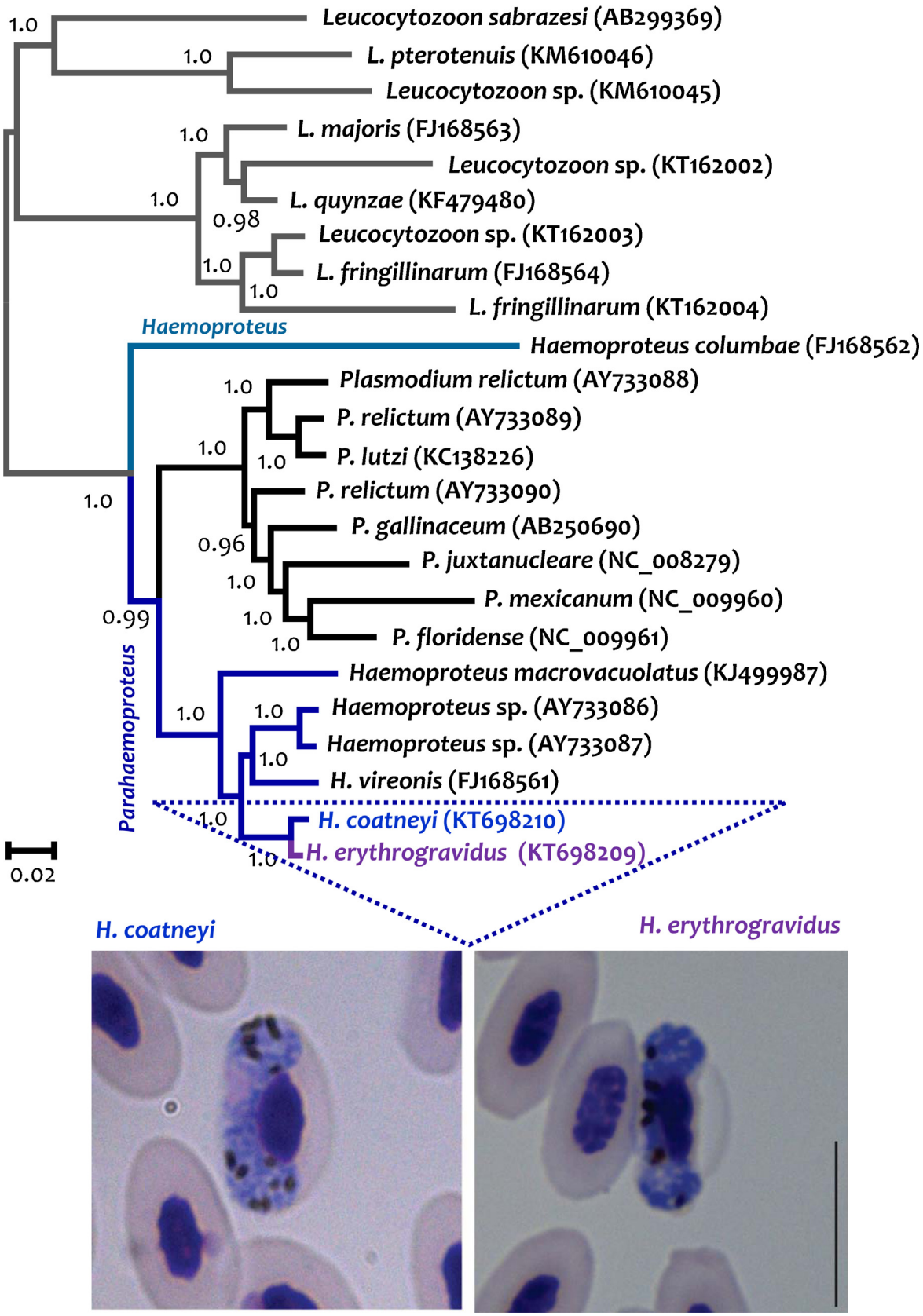

Fig. 4. Bayesian phylogenetic hypothesis constructed using complete mitochondrial genomes of *Haemoproteus* spp. The values above branches are posterior probabilities (see methods). *Leucocytozoon* species were included as outgroup.

Table 3
Cytochrome b lineages of *Haemoproteus* parasites, which showed genetic distance between 0.5% and 1% with the lineages ZOCAP01or ZOCAP14 of *Haemoproteus* (*Parahaemoproteus*) *erythrogravidus.*

Lineage	Host species	Host family	Country	Reference
KM211353 *H.* sp. HEMATRO01	*Hemispingus atropileus*	Thraupidae	Colombia	González et al. (2015)
KM211346 *H.* sp. BUTMON02	*Buthraupis montana*	Thraupidae	Colombia	González et al. (2015)
KF767417 *H.* sp. TROAED15	*Troglodytes aedon*	Trogrodytidae	Peru	Galen and Witt (2014)
	Diglossa cyanea	Thraupidae	Ecuador	Harrigan et al. (2014)
	Phaethornis guy	Trochilidae	Ecuador	
	Phaethornis superciliosus			
	Phaethornis syrmatophorus			
	Adelomyia melanogenys			
	Phaethornis baroni			
	Phaethornis malaris			
	Phaethornis yaruqui			
	Phaethornis striigularis			
DQ241546 *H.* sp. ICTCAY02	*Icterus cayanensis*	Icteridae	Uruguay	Durrant et al. (2006)
	Turdus amaurochalinus	Turdidae		
KF537292 *H. coatneyi* ARBRU01	*Arremon brunneinucha*	Emberizidae	Colombia	González et al. (2015)
KF537309 *H. coatneyi* ATPAL02	*Atlapetes pallidinucha*	Emberizidae	Colombia	González et al. (2015)
KM211348 *H.* sp. DIGCAE01	*Buthraupis montana*	Thraupidae	Colombia	González et al. (2015)
	Diglossopis caerulescens			
PA287 *H. coatneyi*	*Arremon brunneinucha*	Emberizidae	Colombia	unpublished

infected with *H. erythrogravidus*. Although there could be ecological difference between Perú and Colombia that explain this pattern, it may indicate possible abortive development of *H. erythrogravidus* in *T. aedon* in Perú. The frequency and ecological importance of such abortive infections warrant additional studies. It is also worth noting that similar lineages reported from other Emberizidae species in Colombia (Table 3) were identified morphologically as *H. coatneyi*. However, judging by the scattered distribution of *H. coatneyi* in our phylogeny (Fig. 3), this morphospecies likely is a complex of several cryptic species (González et al., 2015); that warrants further investigation.

H. coatneyi is a common parasite in birds of the order Passeriformes and is widely distributed throughout the Holarctic and Neotropical regions (Valkiūnas, 2005). This parasite species has been found infecting migratory birds *Piranga rubra*, *Piranga olivacea* and *Dendroica fusca*. It is a common species at the type locality of *H. erythrogravidus*. In *Z. capensis*, *H. coatneyi* was reported in 37.5% of sampled birds, and on some cases it was documented in co-infection with *H. erythrogravidus* (4.7%). The continued presence of both parasite species during the four years of sampling in Bogotá and the marked territoriality shown by *Z. capensis* at this area (Chapman 1940) corroborate that both parasites are locally transmitted at this study site. The occurrence of these two parasite species not only in the same geographic area but within the same host could be the result of sympatric speciation, secondary contact between originally isolated host populations with divergent parasite lineages, or secondary acquisition via host switching from an unidentified alternative host as it has been proposed for other avian (Pérez-Tris et al., 2007; Ricklefs 2010) and mammalian parasites (Pacheco et al., 2013; Muehlenbein et al., 2015). Differentiating among these alternative hypothesis require additional investigations.

The *cyt b* lineages ZOCAP01 and ZOCAP14 of *H. erythrogravidus* were isolated from *Z. capensis* sampled in Ecuador, Colombia, Chile and Perú (Merino et al., 2008; Jones et al., 2013; Marzal et al., 2015) and from *C. ophthalmicus* sampled in Venezuela (without microscopical confirmation) indicating at least the local transmission of the parasite (Mijares et al., 2012). These results suggest the possible distribution of this parasite throughout the Andean range. The absence of previous reports for this parasite species in studies conducted in South America could be associated to its morphological similarity with *H. coatneyi*, low parasitemia, or the scarcity of studies by microscopical confirmation carried out on Andean ecosystems.

There is one molecular report of the lineage ZOCAP01 from the southern region of South America; this lineage is identical to *H. erythrogravidus*, and it was isolated from *Z. capensis* (Merino et al., 2008). The southern populations of this species in South America are latitudinal migrants to northern regions (even more than 30° in latitude) (Handford 1985). Thus, it is possible that these individuals become infected during their seasonal migrations, and the true areas of transmission are primarily in the tropics. On the other hand, there are some lineages similar to ZOCAP01 and ZOCAP14 of *H. erythrogravidus* reported from the central regions of the South America. However, such molecular evidence should be confirmed by morphological studies to verify if they truly correspond to *H. erythrogravidus*; observation of gametocytes of the parasite is necessary. That is essential to discard of possible cases of abortive development of the parasite, and to add to the knowledge on its possible additional hosts, pathology, vectors and geographical range.

Vectors of *H. erythrogravidus* remain unknown. We attributed the new species to the subgenus *Parahaemoproteus* because its *cyt b* lineages cluster with lineages of *Parahaemoproteus* parasites, whose vectors were identified experimentally and belong to *Culicoides* biting midges (Ceratopogonidae) as is the case for: *Haemoproteus* (*Parahaemoproteus*) *tartakovskyi*, *Haemoproteus* (*P.*) *passeris*, *Haemoproteus* (*P.*) *syrnii* (Valkiūnas, 2005; Bukauskaitė et al., 2015). The following species of *Parahaemoproteus* were also proved to be transmitted by *Culicoides* biting midges: *Haemoproteus* (*P.*) *balmorali*, *Haemoproteus* (*P.*) *belopolskyi*, *Haemoproteus* (*P.*) *fringillae*, *Haemoproteus* (*P.*) *lanii*, *Haemoproteus* (*P.*) *minutus*, *Haemoproteus* (*P.*) *parabelopolskyi*, *Haemoproteus* (*P.*) *noctuae* (Valkiūnas, 2005; Žiegytė et al., 2014; Bukauskaitė et al., 2015). Recent experimental studies complemented with molecular phylogenetic analysis show that phylogenies based on partial *cyt b* gene indicate parasite-vector relationships, and can be used in predicting possible parasite-vector relationships and planning research on avian *Haemoproteus* spp. vectors in the wild (Bukauskaitė et al., 2015). Our phylogenetic analysis therefore suggests that *H. erythrogravidus* likely is transmitted by biting midges (Fig. 3), since only *Culicoides* species have been reported as vectors of *Haemoproteus* (*Parahaemoproteus*) parasites (Valkiūnas, 2005; Atkinson 2008). Approximately 32 species of *Culicoides* have been reported in the Americas between Mexico and Argentina (Spinelli et al., 2009), thus there are many possible vector species overlapping with the distribution of *H. erythrogravidus*. Close to the type locality in Bogotá, Colombia, only *Culicoides suarezi* has been reported (De

Rodriguez and Wirth, 1986) which is a probable vector at the type locality of this parasite.

Acknowledgments

The authors wish to thank to three anonymous referees for their comments which greatly helped to improve the manuscript, Universidad Nacional de Loja in Ecuador, Chingaza National Natural Park Los Nevados National Park, Wildlife Sanctuary Otún Quimbaya administrative staff in Colombia, and students and administrative staff of Gimnasio Colombo Británico School in Bogotá. The students belonging to the GERPH group, Diego Cueva from the Ornithological group of Universidad Nacional (GOUN) for their field assistance. We thank Dr. Mal Bryant and Robert Adlard of the Queensland Museum for the loan of material from the IRCAH collection. This study was supported by El Departamento Administrativo de Ciencias, Tecnología e Innovación COLCIENCIAS (contracts Nos. 556-2014, 0737-2013 and 359-2011), ECOPETROL S.A (contract No. 138-2009), Gestión de Proyectos Estudiantiles of the Dirección de Bienestar of Universidad Nacional de Colombia-Bogotá (Project UGP164), Jardín Botánico José Celestino Mutis Bogotá program Estímulos a la investigación 'Thomas van der Hammen' (contract No. 1100-2012) and Ministry of Economy and Competitiveness of Spain (MINECO, EEBB-I-12-05798 and CGL2013-41642-P/BOS). The funding source had no role on study design, data collection and analysis, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2016.02.025.

References

Žiegytė, R., Palinauskas, V., Bernotienė, R., Iezhova, T.A., Valkiūnas, G., 2014. *Haemoproteus* minutus and *Haemoproteus belopolskyi* (Haemoproteidae): complete sporogony in the biting midge *Culicoides impunctatus* (Ceratopogonidae), with implications on epidemiology of haemoproteosis. Exp. Parasitol. 145, 74–79.

Atkinson, C.T., 2008. Haemoproteus. In: Atkinson, C.T., Thomas, N.J., Hunter, B.C. (Eds.), Parasitic Diseases of Wild Bird. Wiley-Blackwell, Ames, Iowa.

Bennett, G.F., de Souza, O., 1980. Blood parasites of some birds from São paulo state Brazil. Mem. Inst. Oswaldo Cruz 75, 117–134, http://dx.doi.org/10.1590/S0074-02761980000100012.

Bennett, G.F., Bishop, M.A., Peirce, M.A., 1993. Checklist of the avian species of Plasmodium (Apicomplexa) and their distribution by avian family and Wallacean life zones. Syst. Parasitol. 26, 171–179, http://dx.doi.org/10.1007/BF00009724.

Bensch, S., Stjernman, M., Hasselquist, D., et al., 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc. R. Soc. B: Biol. Sci. 267, 1583–1589, http://dx.doi.org/10.1098/rspb.2000.1181.

Bensch, S., Pérez-Tris, J., Waldenström, J., Hellgren, O., 2004. Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation? Evolution 58, 1617–1621, http://dx.doi.org/10.1111/j0014-3820.2004.tb01742.x.

Bensch, S., Hellgren, O., Pérez-Tris, J., 2009. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial *cytochrome b* lineages. Mol. Ecol. Resour. 9, 1353–1358, http://dx.doi.org/10.1111/j.1755-0998.2009.02692.x.

BirdLife International, (accessed 25.05.15) http://www.birdlife.org/datazone/species/factsheet/22721079/additional.

Borner, J., Pick, C., Thiede, J., et al., 2015. Phylogeny of haemosporidian blood parasites revealed by a multi-gene approach. Mol. Phylogenet. Evol. 94A, 221–231.

Bukauskaitė, D., Žiegytė, R., Palinauskas, V., et al., 2015. Biting midges (Culicoides Diptera) transmit Haemoproteus parasites of owls: evidence from sporogony and molecular phylogeny. Parasit Vectors, http://dx.doi.org/10.1186/s13071-015-0910-6.

Burry-Caines, J.R., Bennett, G.F., 1992. The haemoproteidae (Apicomplexa: Haemosporina) of the avian families fringillidae and emberizidae s.l. Can. J. Zool. 70, 1149–1160, http://dx.doi.org/10.1139/z92-161.

Chapman, F.M., 1940. The post-glacial history of *Zonotrichia capensis*. Bull. Am. Mus. Nat. Hist. 77, 381–438.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772, http://dx.doi.org/10.1038/nmeth.2109.

De Rodriguez, M.C., Wirth, W.W., 1986. A new species of man-biting *Culicoides* from the high andes of Colombia (Diptera: Ceratopogonidae). Fla. Entomol. 69 (311), http://dx.doi.org/10.2307/3494934.

Dimitrov, D., Zehtindjiev, P., Bensch, S., et al., 2014. Two new species of *Haemoproteus Kruse* 1890 (Haemosporida, Haemoproteidae) from European birds, with emphasis on DNA barcoding for detection of haemosporidians in wildlife. Syst. Parasitol. 87, 135–151, http://dx.doi.org/10.1007/s11230-013-9464-1.

Durrant, K.L., Beadell, J.S., Ishtiaq, F., et al., 2006. Avian hematozoa in South America: a comparison of temperate and tropical zones. Ornithol. Monogr. 60, 98–111.

Fotheringham, J.R., 1995. Differences in singing behavior between rufous-collared sparrows in Costa Rica and Northwestern Argentina. Condor 97, 821–826, http://dx.doi.org/10.2307/1369193.

Galen, S.C., Witt, C.C., 2014. Diverse avian malaria and other haemosporidian parasites in Andean house wrens: evidence for regional co-diversification by host-switching. J. Avian Biol. 45, 374–386, http://dx.doi.org/10.1111/jav.00375.

Garnham, P.C.C., 1966. Malaria Parasites and Other Haemosporidia. Blackwell, Oxford.

González, A.D., Matta, N.E., Ellis, V.A., 2014. Mixed species flock, nest height, and elevation partially explain avian haemoparasite prevalence in Colombia. PLoS One 9, e100695, http://dx.doi.org/10.1371/journal.pone.0100695.

González, A.D., Lotta, I.A., García, L.F., et al., 2015. Avian haemosporidians from Neotropical highlands: evidence from morphological and molecular data. Parasitol. Int. 64, 48–59, http://dx.doi.org/10.1016/j.parint.2015.01.007.

Gouy, M., Guindon, S., Gascuel, O., 2010. Sea View version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27, 221–224, http://dx.doi.org/10.1093/molbev/msp259.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Handford, P., 1985. Morphological relationships among subspecies of the Rufous-collared Sparrow *Zonotrichia capensis*. Can. J. Zool. 63, 2383–2388, http://dx.doi.org/10.1139/z85-352.

Harrigan, R.J., Sedano, R., Chasar, A.C., et al., 2014. New host and lineage diversity of avian haemosporidia in the northern Andes. Evol. Appl. 7, 799–811, http://dx.doi.org/10.1111/eva.12176.

Hellgren, O., Križanauskiene, A., Valkiūnas, G., Bensch, S., 2007. Diversity and phylogeny of mitochondrial cytochrome B Lineages from six morphospecies of avian *Haemoproteus* (haemosporida: Haemoproteidae). J. Parasitol. 93 (4), 889–896.

Iezhova, T.A., Dodge, M., Sehgal, R.N.M., et al., 2011. New avian *Haemoproteus* species (Haemosporida: haemoproteidae) from African birds, with a critique of the use of host taxonomic information in hemoproteid classification. J. Parasitol. 97, 682–694, http://dx.doi.org/10.1645/GE-2709.1.

Jones, M.R., Cheviron, Z.A., Carling, M.D., 2013. Spatial patterns of avian malaria prevalence in *Zonotrichia capensis* on the western slope of the Peruvian Andes. J. Parasitol. 99, 903–905, http://dx.doi.org/10.1645/12-147.1.

Križanauskienė, A., Hellgren, O., Kosarev, V., 2006. Variation in host specificity between species of avian Haemosporidian parasites: evidence from parasite morphology and *cytochrome b* gene sequences. J. Parasitol. 92, 1319–1324.

Lacorte, G.A., Félix, G.M.F., Pinheiro, R.R.B., 2013. Exploring the diversity and distribution of Neotropical avian malaria parasites a molecular survey from Southeast Brazil. PLoS One 8, e57770, http://dx.doi.org/10.1371/journal.pone.0057770.

Lougheed, S.C., Handford, P., 1992. Vocal dialects and the structure of geographic variation in morphological and allozymic characters in the Rufous-collared Sparrow *Zonotrichia capensis*. Evolution 46, 1443, http://dx.doi.org/10.2307/2409948.

Mantilla, J.S., González, A.D., Valkiūnas, G., et al., 2013. Description and molecular characterization of *Plasmodium* (*Novyella*) *unalis* sp. nov. from the Great Thrush (*Turdus fuscater*) in highland of Colombia. Parasitol. Res. 112, 4193–4204, http://dx.doi.org/10.1007/s00436-013-3611-0.

Marzal, A., Lope, F., Navarro, C., Møller, A.P., 2005. Malarial parasites decrease reproductive success: an experimental study in a passerine bird. Oecologia 142, 541–545, http://dx.doi.org/10.1007/s00442-004-1757-2.

Marzal, A., García-Longoria, L., Cárdenas Callirgos, J.M., Sehgal, R.N., 2015. Invasive avian malaria as an emerging parasitic disease in native birds of Perú. Biol. Invasions 17, 39–45, http://dx.doi.org/10.1007/s10530-014-0718-x.

Matta, N.E., Pacheco, M.A., Escalante, A.A., et al., 2014. Description and molecular characterization of *Haemoproteus macrovacuolatus* n. sp. (Haemosporida, Haemoproteidae), a morphologically unique blood parasite of black-bellied whistling duck (*Dendrocygna autumnalis*) from South America. Parasitol. Res. 113, 2991–3000, http://dx.doi.org/10.1007/s00436-014-3961-2.

Merino, S., Moreno, J., Vásquez, R.A., et al., 2008. Haematozoa in forest birds from southern Chile: latitudinal gradients in prevalence and parasite lineage richness. Austral Ecol. 33, 329–340, http://dx.doi.org/10.1111/j.1442-9993.2008.01820.x.

Mijares, A., Rosales, R., Silva-Iturriza, A., 2012. Hemosporidian parasites in forest birds from Venezuela: genetic lineage analyses. Avian Dis. 56, 583–588, http://dx.doi.org/10.1637/10058-011312-ResNote.1.

Muehlenbein, M.P., Pacheco, M.A., Taylor, J.E., et al., 2015. Accelerated diversification of nonhuman primate malarias in southeast Asia: adaptive

radiation or geographic speciation? Mol. Biol. Evol. 32, 422–439, http://dx.doi.org/10.1093/molbev/msu310.
Munro, H.J., Martin, P.R., Moore, I.T., Bonier, F., 2009. Blood parasites in adult and nestling birds in the Ecuadorian Andes. Ornitol. Neotrop. 20, 461–465.
Olias, P., Wegelin, M., Zenker, W., 2011. Avian malaria deaths in parrots Europe. Emerg. Infect. Dis. 17, 950–952, http://dx.doi.org/10.3201/eid1705.101618.
Outlaw, D.C., Ricklefs, R.E., 2014. Species limits in avian malaria parasites (Haemosporida): how to move forward in the molecular era. Parasitology 141, 1223–1232.
Pérez-Rodríguez, A., De la Hera, I., Bensch, S., Pérez-Tris, J., 2015. Evolution of seasonal transmission patterns in avian blood-borne parasites. Int. J. Parasitol. 45, 605–611.
Pérez-Tris, J., Bensch, S., 2005. Dispersal increases local transmission of avian malarial parasites. Ecol. Lett. 8, 838–845.
Pérez-Tris, J., Hellgren, O., Križanauskienė, A., 2007. Within-host speciation of malaria parasites. PLoS One 2, e235, http://dx.doi.org/10.1371/journal.pone.0000235.
Pacheco, M.A., Battistuzzi, F.U., Junge, R.E., 2011a. Timing the origin of human malarias: the lemur puzzle. BMC Evol. Biol. 11, 299, http://dx.doi.org/10.1186/1471-2148-11-299.
Pacheco, M.A., Escalante, A.A., Garner, M.M., et al., 2011b. Haemosporidian infection in captive masked bobwhite quail (*Colinus virginianus ridgwayi*), an endangered subspecies of the northern bobwhite quail. Vet. Parasitol. 182, 113–120, http://dx.doi.org/10.1016/j.vetpar.2011.06.006.
Pacheco, M.A., Cranfield, M., Cameron, K., Escalante, A.A., 2013. Malarial parasite diversity in chimpanzees: the value of comparative approaches to ascertain the evolution of *Plasmodium falciparum* antigens. Malar. J. 12, 328, http://dx.doi.org/10.1186/1475-2875-12-328.
Perkins, S.L., 2014. Malaria's many mates: past, present, and future of the systematics of the order haemosporida. J. Parasitol. 100, 11–25, http://dx.doi.org/10.1645/13-362.1.
Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256, http://dx.doi.org/10.1093/molbev/msn083.
Rambaut, A., Drummond, A., 2006. Tracer v1.5 (accessed 09.06.13.) http://beast.bio.ed.ac.uk/Tracer.
Rambaut, A., 2006. FigTree Tree Figure Drawing Toll Version 1.3.1. Institute of Evolutionary Biology. University of Edinburgh (accessed 31.01.13.) http://tree.bio.ed.ac.uk/software/figtree/.
Remsen, J.V., Areta, J.I., Cadena, C.D., et al., 2014. A Classification of the Bird Species of South America American Ornithologists' Union Version (accessed 22.06.14.) http://www.museum.lsu.edu/?Remsen/SACCBaseline.html.
Reullier, J., Pérez-Tris, J., Bensch, S., Secondi, J., 2006. Diversity, distribution and exchange of blood parasites meeting at an avian moving contact zone. Mol. Ecol. 15, 753–763.
Ricklefs, R.E., Fallon, S.M., Bermingham, E., 2004. Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. Syst. Biol. 53, 111–119, http://dx.doi.org/10.1080/10635150490264987.
Ricklefs, R.E., 2010. Host–pathogen coevolution, secondary sympatry and species diversification. Philos. Trans. R. Soc. B: Biol. Sci. 365, 1139–1147, http://dx.doi.org/10.1098/rstb.2009.0279.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574, http://dx.doi.org/10.1093/bioinformatics/btg180.
Sambrook, J., Fritsch, E., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.
Santiago-Alarcon, D., Outlaw, D.C., Ricklefs, R.E., Parker, P.G., 2010. Phylogenetic relationships of haemosporidian parasites in New World Columbiformes, with emphasis on the endemic Galapagos dove. Int. J. Parasitol. 40, 463–470, http://dx.doi.org/10.1016/j.ijpara.2009.10.003.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675, http://dx.doi.org/10.1038/nmeth.2089.
Shurulinkov, P., Chakarov, N., Daskalova, G., 2012. Blood parasites, body condition, and wing length in two subspecies of yellow wagtail (*Motacilla flava*) during migration. Parasitol. Res. 110, 2043–2051, http://dx.doi.org/10.1007/s00436-011-2733-5.
Spinelli, G.R., Santamaría, E., Cabrera, O.L., et al., 2009. Five new species of Culicoides Latreille described from Colombia, yielding a new species list and country records (Diptera: Ceratopogonidae). Mem. Inst. Oswaldo Cruz 104, 81–92.
Tamura, K., Stecher, G., Peterson, D., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729, http://dx.doi.org/10.1093/molbev/mst197.
Valkiūnas, G., Iezhova, T.A., 2004. Detrimental effects of *Haemoproteus* infections on the survival of biting midge *Culicoides impunctatus* (Diptera: Ceratopogonidae). J. Parasitol. 90, 194–196, http://dx.doi.org/10.1645/GE-3206RN.
Valkiūnas, G., Kazlauskien, R., Bernotien, R., 2013. Abortive long-lasting sporogony of two *Haemoproteus* species (Haemosporida, Haemoproteidae) in the mosquito Ochlerotatus cantans, with perspectives on haemosporidian vector research. Parasitol. Res. 112, 2159–2169, http://dx.doi.org/10.1007/s00436-013-3375-6.
Valkiūnas, G., Palinauskas, V., Ilgūnas, M., Bukauskaite, D., Dimitrov, D., Bernotienė, R., Zehtindjiev, P., Ilieva, M., Iezhova, T.A., 2014. Molecular characterization of five widespread avian haemosporidian parasites (Haemosporida), with perspectives on the PCR-based detection of haemosporidians in wildlife. Parasitol. Res. 112, 2251–2263, http://dx.doi.org/10.1007/s00436-014-3880-2.
Valkiūnas, G., 2005. Avian Malaria Parasites and Other Haemosporidia. CRC Press, Boca Raton.
Valkiūnas, G., 2015. Haemoproteus. In: Mehlhorn, H. (Ed.), Encyclopedia of Parasitology. , 4th ed. Springer, New York.
Waldenström, J., Bensch, S., Hasselquist, D., Ostman, O., 2004. A new nester polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. J. Parasitol. 90, 189–191, http://dx.doi.org/10.1645/GE-3136RN.
Walther, E.L., Valkiūnas, G., González, A.D., 2014. Description, molecular characterization, and patterns of distribution of a widespread New World avian malaria parasite (Haemosporida: Plasmodiidae) *Plasmodium* (Novyella) *homopolare* sp. nov. Parasitol. Res., http://dx.doi.org/10.1007/s00436-014-3995-5.
Young, B.E., Garvin, M.C., McDonald, D.B., 1993. Blood parasites in birds from monteverde Costa Rica. J. Wildl. Dis. 29, 555–560, http://dx.doi.org/10.7589/0090-3558-29.4.555.