Protist, Vol. 167, 185–204, April 2016
http://www.elsevier.de/protis
Published online date 17 February 2016

Protist

ORIGINAL PAPER

Leucocytozoon Diversity and Possible Vectors in the Neotropical highlands of Colombia

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Submitted September 29, 2015; Accepted February 2, 2016
Monitoring Editor: Frank Seeber

Studies of the lowland avifauna in the Neotropical Region have shown a paucity of *Leucocytozoon* species. However, surveys conducted in the Colombian highlands revealed a great diversity of these parasites infecting resident birds. To further investigate the relationship between *Leucocytozoon* diversity, the potential vectors, and altitudinal distribution, birds from 41 families were sampled at low and high elevations in Colombia. Blood samples were screened by microscopy, and a fragment of cytochrome *b* was amplified from *Leucocytozoon*-positive samples. The complete mitochondrial genome was also obtained for each morphospecies of *Leucocytozoon*. *Leucocytozoon* species were detected in resident birds, with various degrees of host specificity, at elevations from 2,400 to 3,950 meters above sea level, where five new host-parasite associations were discovered. Phylogenetic analysis based on the cytochrome b fragment suggested that two nominal taxa, *L. fringillinarum* and *L. majoris*, are species complexes. Blood sources of Simuliidae revealed generalist-feeding habits that included avian and mammalian hosts. Molecular analysis of parasites in black flies indicated a close relationship with the parasites found in birds. Our investigation provides further evidence that the distribution and transmission of *Leucocytozoon* species in the Neotropics are influenced by elevation, with the highest prevalence between 2,400 and 3,200 m asl.

Key words: *Leucocytozoon* diversity; host-feeding sources; Neotropical Andes; black flies; elevation; cytochrome b.

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http://dx.doi.org/10.1016/j.protis.2016.02.002
1434-4610/

Introduction

Leucocytozoon species are vector-borne parasites that infect a broad range of avian taxa worldwide (Jones et al. 2005; Valkiūnas 2005). Previous studies, however, have reported a low prevalence of these parasite species in the Neotropical lowlands (Belo et al. 2011; Bennett et al. 1980; Durrant et al. 2006; Galindo and Sousa 1966; Valkiūnas et al. 2003). This pattern has been explained by the lack of suitable vectors and the low parasite load (parasitemia) in migratory birds (Valkiūnas et al. 2003). Merino et al. (2008) suggested that the distribution of *Leucocytozoon* species in Chile follows a latitudinal gradient, with the highest prevalence at high latitudes. This pattern was partially explained by the presence of mountainous areas and the distribution of suitable vectors. Studies at high elevations in the Neotropical Region also have revealed these parasites in resident birds (Forrester et al. 2001; Galen and Witt 2014; Harrigan et al. 2014; Lotta et al. 2013, 2015; Matta et al. 2014a; Merino et al. 2008; Rodríguez et al. 2009; Smith and Ramey 2015).

Forty-one species of *Leucocytozoon* are recognized (Jones et al. 2005; Lotta et al. 2015; Matta et al. 2014a; Valkiūnas 2005; Valkiūnas et al. 2010). Species in wild bird populations have been characterized recently by molecular tools, providing information such as lineage distributions, evolutionary history, and host ranges. However, the life cycles of only a third of the described species have been studied, some of which are associated with high mortality in poultry (Valkiūnas 2005). However, the pathogenic effects of *Leucocytozoon* infection in wild birds have not been fully characterized. Studies on this issue have shown detrimental effects of blood parasites by affecting the reproductive success and parental care of the infected hosts; there are even reports of *Leucocytozoon* associated mortality on heavily infected birds (Forrester and Greiner 2008; Herman et al. 1975; Merino et al. 2000).

Black flies (family Simuliidae) have been implicated as the vectors of *Leucocytozoon* parasites worldwide (Skidmore 1931). The exception is *Leucocytozoon* (*Akiba*) *caulleryi*, which is transmitted by a biting midge (family Ceratopogonidae), *Culicoides arakawae* (Akiba 1960). Twenty-six genera of black flies are found in the world, of which 10 inhabit the Neotropical Region. The identity of possible Neotropical vectors is still unknown. However, *Leucocytozoon* infections in resident birds of the South American highlands suggest that these ecosystems have suitable biotic and abiotic conditions to sustain active transmission (Matta et al. 2014a).

We summarize recent information about the distribution of *Leucocytozoon* spp. in South America and provide new information regarding their possible vectors in the Colombian highland ecosystem. Our goals were 1) to morphologically and molecularly characterize the *Leucocytozoon* species in Colombia, 2) to infer the phylogenetic relationships of the parasite lineages in avian and simuliid hosts, and 3) to analyze *Simulium* and *Gigantodax* species in Colombia as possible vectors.

Results

Bird Sampling

Samples from 2,379 birds (17 orders, 41 families, and 273 species) were screened microscopically for haemosporidian parasites (Table A.1). Elevation showed a significant association with *Leucocytozon* transmission (logistic regression: estimate coefficient 6.2x10-4, ST error 1.1x10-4, $P < 0.001$; one-way ANOVA: Sum Sq 598.26, F 8.6099, $P < 0.05$). In particular, infected birds were more commonly found at high elevations (> 2,400 m above sea level, asl). Furthermore, these were the only hemoparasites detected by microscopy at 3,950 m asl. We also detected five new host-parasite records (Table 1). At least five morphotypes of *Leucocytozoon* were consistent with *L. fringillinarum*, *L. majoris*, *L. dubreuilli*, *L. quynzae*, and *L. pterotenuis* (Fig. 1). Other potential species could not be identified due to low intensity of infections.

Overall, *Leucocytozoon* prevalence was 4.6% across all 13 localities. However, *Leucocytozoon* prevalence in the highlands was 6.4% (110 of 1,727 resident birds of 34 species) (Table 1). Passeriformes (1,388 individuals, 80.3% of samples) and Apodiformes (299 individuals, 17.3% of samples) were the most commonly sampled orders (Table 1 and 2) and also the orders most commonly infected (Table 2). In particular, Turdidae (110 individuals, 7.9%), Thraupidae (406 individuals, 29.2%), and Emberizidae (269 individuals, 19.4%) were the passerine families with higher *Leucocytozoon* prevalence (Table 2). Overall, the intensities of these infections were low, ranging from 0.001 to 0.36%.

The prevalence of single infections was 56.4% (62/110). In 18.2% of the cases (20/110), two or three *Leucocytozoon* morphospecies were observed. Prevalence of co-infections of

Table 1. Cytochrome *b* (*cytb*) lineages obtained from *Leucocytozoon* species and avian hosts in Colombia.

Family	Species	N_{PCR}/N_{Mic}	Morphospecies and lineage name (MalAvi, GenBank Accession N°)
COTINGIDAE	*Ampelion rubrocristatus*[h]	0/1	*Leucocytozoon* sp.
EMBERIZIDAE	*Arremon brunneinucha*[b, h]	*2/5*	*L. fringillinarum* (L_MYFUM01, KF717047), *Leucocytozoon* sp. (L_ATBRU01, KF717051).
	Arremon torquatus[h]	1/3	*Leucocytozoon* sp. (L_ METYR01, KF699312)
	Atlapetes albinucha[a]	3/4	*L. fringillinarum* (L_MYFUM01, KF717047), *Leucocytozoon* sp. (L_ATALB01, KF717063; L_ATALB02, KF717064)
	Atlapetes pallidinucha[h]	2/4	*Leucocytozoon* sp. (L_ATPA_01, KF717065) *L. fringillinarum* (L_MYFUM01, KF717047)
	Atlapetes schistaceus[h]	2/2	*L. fringillinarum* (L_MYFUM01, KF717047), *Leucocytozoon* sp. (L_ATSCHI01, KF717067)
	Hemispingus atropileus[e]	1/1	*Leucocytozoon* sp. (L_HEATR01, KF717052)
	Hemispingus superciliaris[e]	5/5	*Leucocytozoon* sp. (L_HEATR01, KF717066) *L. fringillinarum*. (L_MYFUM01, KF717047; L_HESUP02, KF717058), *L. majoris* (L_HESUP01,KF962962; TROAED02, KF717060),
	Hemispingus verticalis[b]	1/3	*Leucocytozoon* sp. (L_HEVE01, KF717062)
	Zonotrichia capensis[c]	2/6	*Leucocytozoon* sp. (L_ZOCAP07, KF717054; TROAED02, KF717060)
FURNARIIDAE	*Margarornis squamiger*[b]	0/1	*Leucocytozoon* sp.
GRALLARIIDAE	*Grallaria quitensis*[h]	1/2	*Leucocytozoon* sp. (L_GRQUI_01, KM272251)
	Grallaria ruficapilla[h]	1/1	*Leucocytozoon pterotenuis* (L_GRRUF_01, KM272250)
PARULIDAE	*Dendroica discolor*[d]	0/1	*Leucocytozoon* sp.
	Dendroica fusca[a]	0/1	*Leucocytozoon* sp.
	Myioborus ornatus[a]	1/1	*Leucocytozoon* sp. (L_MYORN01, KF717059)
THRAUPIDAE	*Anisognathus igniventris*[h]	0/3	*Leucocytozoon* sp.
	Anisognathus lacrymosus[h]	1/1	*Leucocytozoon* sp. (L_ANILA01, KF962961)
	Buthraupis montana[b,h]	1/4	*L. fringillinarum* (L_BUTMO01, KF717048)
	Catamenia inornata[h]	2/2	*Leucocytozoon* sp. (L_ZOCAP07, KF717054; L_CAINO01, KF717050)
	Diglossa albilatera[a]	2/1	*L. fringillinarum* (L_MYFUM01, KF717047) *Leucocytozoon* sp. (L_HEVE01, KF717062)
	Diglossa cyanea[b,h]	1/5	*Leucocytozoon* sp. (L_DICYA01, KF717066)
	Dubusia taeniata[b,h]	1/4	*Leucocytozoon* sp. (L_DUTAE_01, KF717061)

Table 1 (*Continued*)

Family	Species	N_{PCR}/N_{Mic}	Morphospecies and lineage name (MalAvi, GenBank Accession N°)
	Iridosornis rufivertex[b]	0/1	*Leucocytozoon* sp.
	Phrygilus unicolor[h]	0/1	*Leucocytozoon* sp.
TITYRIDAE	*Pachyramphus versicolor*[a]	1/1	*Leucocytozoon* sp. (L_PAVER01, KF717057)
TROCHILIDAE	*Coeligena helianthea*[g]	1/1	*L. quynzae* (L_COHEL01, KF309189)
	Heliangelus amesthysticollis[g]	1/1	*L. quynzae* (L_HEAME01, KF309188)
	Metallura tyrianthina[e,g]	2/3	*L. quynzae* (L_HEAME01, KF309188), *Leucocytozoon* sp. (L_METYR01, KF699312)
	Opisthoprora euryptera[b]	0/1	*L. quynzae*
TURDIDAE	*Turdus fuscater*[b, f,h]	11/31	*Leucocytozoon* sp. (L_TFUS08, KF717053; L_TFUS09, KF717055; L_TFUS10, KF717056; L_TFUS07, KF699311; L_TFUS12, KF699310) (*L. dubreuili*; *L. fringillinarum* (L_TFUS04, JQ815435); *L. majoris* (L_TFUS_11, KF699313)
	Turdus serranus[b]	0/2	*L. fringillinarum*, *L. dubreuili*
TYRANNIDAE	*Mecocerculus leucophrys*[b,h]	1/6	*Leucocytozoon* sp. (L_ METYR01, KF699312)
	Myiotheretes fumigatus[h]	1/1	*Leucocytozoon* sp. (L_MYFUM01, KF717047)
	Phyllomyias nigrocapillus[h]	1/1	*Leucocytozoon* sp.*1* (L_PHNIG01, KF717049)

Numbers of captured individuals are provided in Table A.1. N_{Mic}: Number of individuals positive by microscopy. N_{PCR}: Number of positive samples detected and processed by PCR. [a]New host-parasite relationships for Colombia; host-parasite relationships previously reported by [b](Rodríguez et al. 2009), [c](Merino et al. 2008), [d](Basto et al. 2006), [e]Witt & McNew (Unpublished data) [f](Lotta et al. 2013), [g](Matta et al. 2014a), [h](Lotta et al. 2015).

Leucocytozoon spp. with other blood hemoparasites (25.4%, 28/110) was as follows: *Haemoproteus* 10.0%, *Plasmodium* 8.2%, microfilariae 1.8%; triple infections with *Haemoproteus* and microfilariae 1.8%, *Plasmodium* and microfilariae 1.8%, *Plasmodium* and *Trypanosoma* 0.9%, and microfilariae and *Trypanosoma* 0.9%.

Species and Distributions of Black Flies

In our study, 26 species of simuliids were collected, 16 belonging to the genus *Simulium* and 10 to *Gigantodax* (adults and immatures). Three *Gigantodax* species and four *Simulium* species were shared between Chingaza NNP and Los Nevados NNP (Table 3). The distributions of all simuliid species found in the sampled areas, except for the *S. townsendi* complex, *S. dinellii*, and *S.* (*Ectemnaspis*) sp2, match the distributional range of *Leucocytozoon*; most of the species were concentrated between 2,400 and 3,200 m asl (Fig. 2), where the parasites had their highest prevalence. Also, the simuliid species from Lake Otún, were restricted to the paramo ecosystem.

Dissections, Blood-meal Identifications, and Molecular Detection of *Leucocytozoon* spp. in Black Flies

All slide preparations from the 501 dissected adults (33.98%) of *Simulium* sp., *S. cormonsi*, *S. muiscorum*, *S. furcillatum*, *S. ignescens* complex, and *S. bicoloratum* were negative by microscopy for haemosporidian parasites (Table 3). Adults of *Gigantodax* spp. were captured in low numbers (2 to 24 individuals) and were not processed by the same methodology.

To determine the blood sources of black flies, 991 individuals of 11 species were processed. Despite no visual evidence of a blood meal in 67 pools

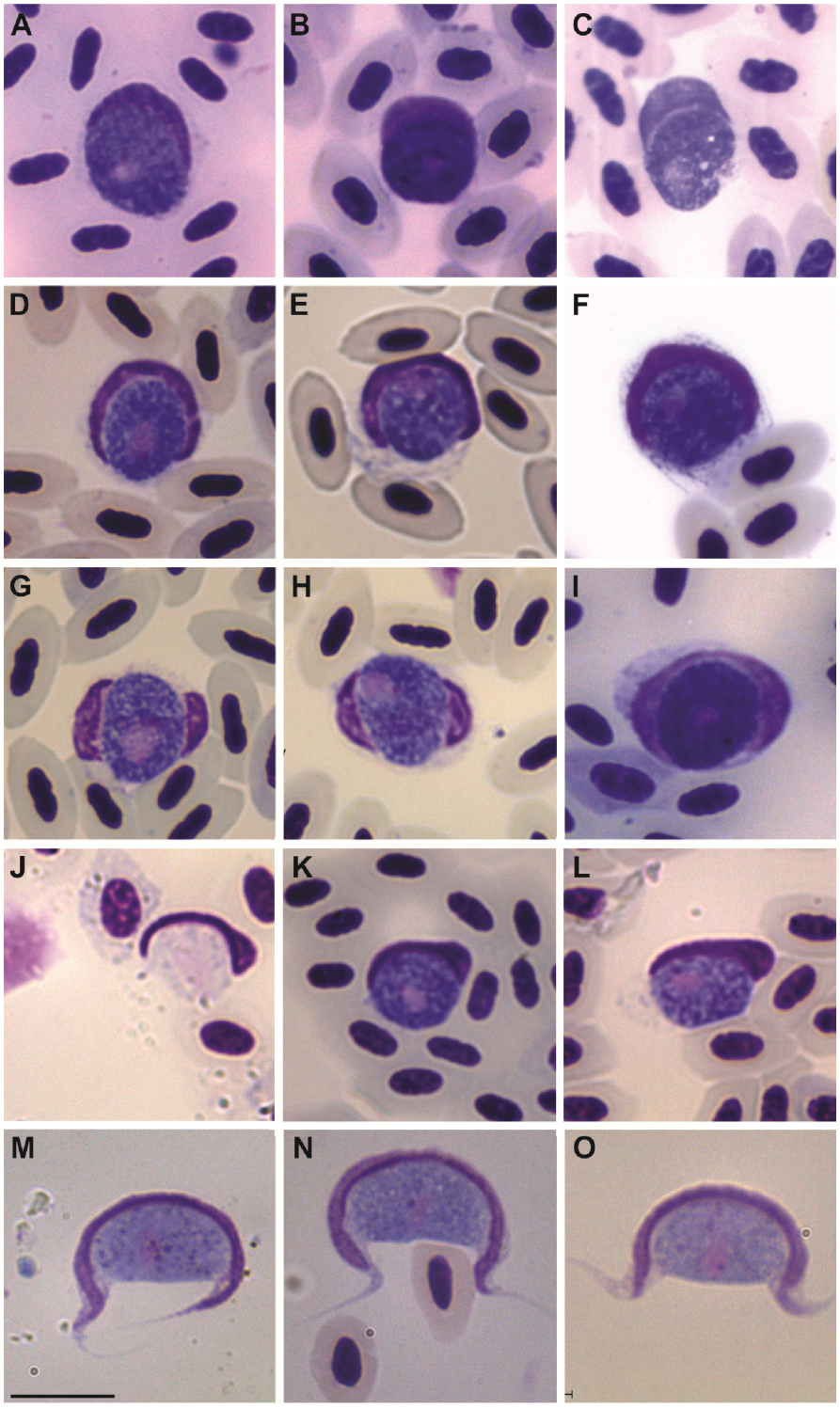

Figure 1. Morphospecies of *Leucocytozoon* found in Colombia. Gametocytes of *Leucocytozoon fringillinarum* from *Turdus fuscater* and *Dendroica discolor* (**A-C**), *L. majoris* from *Catamenia inornata*, *Arremon brunneinucha*, and *Turdus fuscater* (**D-F**), *L. dubreuili* from *Catamenia inornata*, *Turdus fuscater*, and *Atlapetes albinucha* (**G-I**), *L. quynzae* from *Heliangelus amethysticollis* (**J-L**), and gametocytes of *L. pterotenuis* (**M-O**). Giemsa-stained thin blood films. Scale bar: 10 μm.

Table 2. *Leucocytozoon* infection in birds sampled in highlands and lowlands of Colombia. Percentages were calculated over the total of individuals captured for each family. Percentages of captured individuals per family are also presented.

Order/Family	Birds captured (%)	Birds captured in lowlands (below 2100 m asl)	Birds captured in highlands (above 2100 m asl)	Birds infected (%)
Anseriformes				
Anatidae	1 (0.04%)		1	0 (0%)
Apodiformes				
Trochilidae	334 (14.04%)	35	299	6 (1.80%)
Caprimulgiformes				
Caprimulgidae	3 (0.13%)	1	2	0 (0%)
Charadriiformes				
Scolopacidae	1 (0.04%)		1	0 (0%)
Ciconiiformes				
Ardeidae	1 (0.04%)		1	0 (0%)
Columbiformes				
Columbidae	37 (1.56%)	23	14	0 (0%)
Coraciiformes				
Alcedinidae	14 (0.59%)		14	0 (0%)
Momotidae	4 (0.17%)	2	2	0 (0%)
Falconiformes				
Accipitridae	2 (0.08%)	1	1	0 (0%)
Galliformes				
Odontophoridae	3 (0.13%)	3		0 (0%)
Gruiformes				
Rallidae	3 (0.13%)		3	0 (0%)
Passeriformes				
Cardinalidae	26 (1.09%)	15	11	0 (0%)
Corvidae	7 (0.29%)	2	5	0 (0%)
Cotingidae	1 (0.04%)		1	1 (100%)
Cuculidae	3 (0.13%)	1	2	0 (0%)
Emberizidae	290 (12.19%)	21	269	33 (11.38%)
Fringillidae	9 (0.38%)	6	3	0 (0%)
Furnariidae	94 (3.95%)	11	83	1 (1.06%)
Grallariidae	3 (0.13%)		3	3 (100%)
Hirundinidae	100 (4.20%)	6	94	0 (0%)
Icteridae	24 (1.01%)	4	20	0 (0%)
Mimidae	2 (0.08%)	2		0 (0%)
Parulidae	108 (4.54%)	5	103	3 (2.78%)
Pipridae	102 (4.29%)	102		0 (0%)
Rhinocryptidae	2 (0.08%)		2	0 (0%)
Thamnophilidae	3 (0.13%)	3		0 (0%)
Thraupidae	554 (23,29%)	148	406	22 (3.97%)
Tityridae	9 (0.38%)	8	1	1 (11.11%)
Troglodytidae	89 (3.74%)	17	72	0(0%)
Turdidae	147 (6.18%)	37	110	33 (22.45%)
Tyrannidae	347 (14.58%)	162	185	8 (2.30%)
Vireonidae	25 (1.05%)	12	13	0 (0%)
Piciformes				
Galbulidae	3 (0.13%)	3		0 (0%)
Picidae	8 (0.37%)	1	7	0 (0%)
Ramphastidae	7 (0.29%)	1	6	0 (0%)

Table 2 (*Continued*)

Order/Family	Birds captured (%)	Birds captured in lowlands (below 2100 m asl)	Birds captured in highlands (above 2100 m asl)	Birds infected (%)
Psittaciformes				
Psittacidae	6 (0,25%)	4	2	0 (0%)
Strigiformes				
Strigidae	2 (0.08%)	2		0 (0%)
Tinamiformes				
Tinamidae	1 (0.04%)		1	0 (0%)
Trogoniformes				
Trogonidae	4 (0.17%)		4	0 (0%)

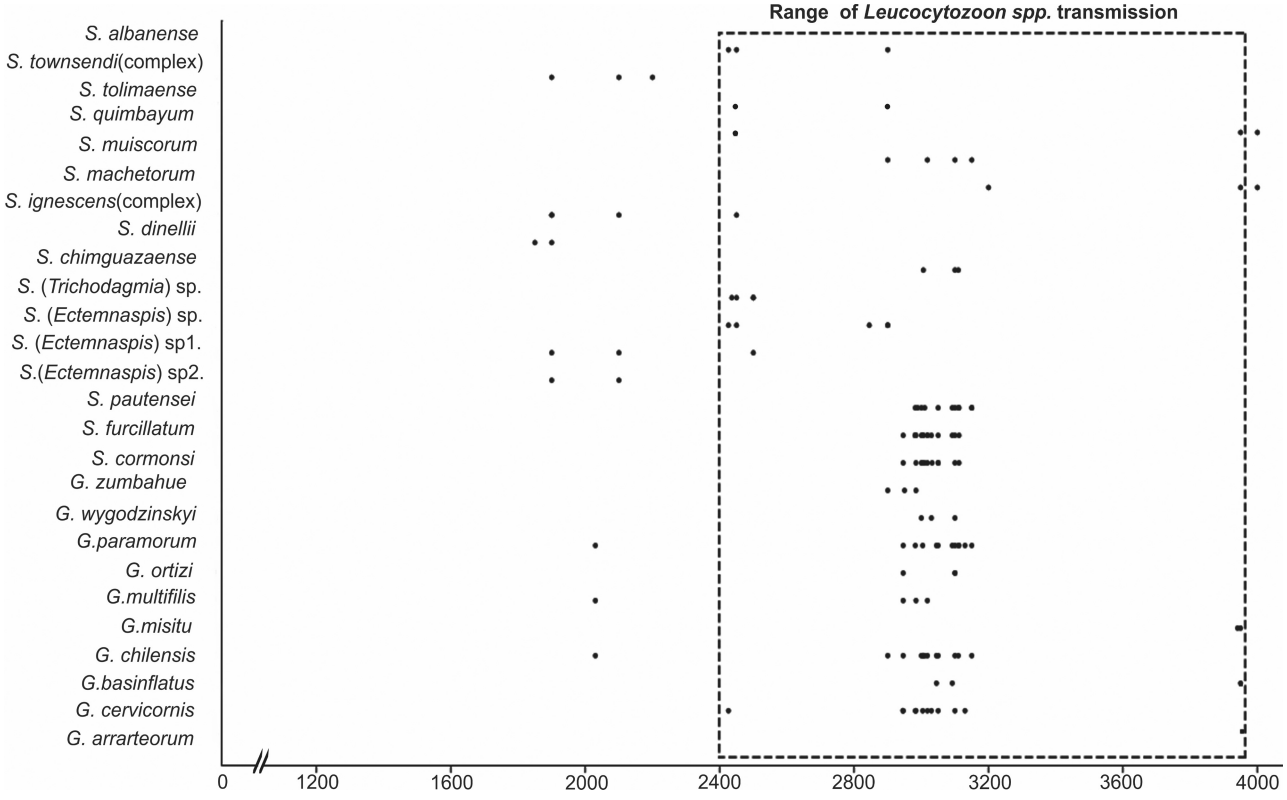

Figure 2. Elevational distribution of Simuliidae species collected and *Leucocytozoon* spp. The abscissa shows the elevation (m asl) and the ordinate depicts the species of Simuliidae (pupae) collected (Simuliidae pupae were only sought at elevations above 1,900 m asl). Range of elevational distribution of *Leucocytozoon* species is represented by a square (dotted line).

of *Simulium* spp., four bird *cytb* sequences were obtained, from *Grallaria* sp. (2), *Basileuterus nigrocristatus* (1), and *Atlapetes pileatus* (1). From the 67 individuals processed separately, one mammal source was amplified. For *Gigantodax*, in 60.0% of 5 pools analyzed, one sequence of mammal sources was detected. Mixed blood sources were not found. We did not detect host blood in the *S. townsendi* complex, *S. pautense*, *S. tolimaense*, *S. tunja*, or *G. chilensis*. The 10 *Gigantodax* species found in the sampling areas had bifid tarsal claws, whereas all *Simulium* species had claws with a sub-basal tooth. The typical correlation of mammal feeding with claws bearing a sub-basal tooth

Table 3. Females of Simuliidae collected in Colombia, with their blood sources, claw morphology, and *Leucocytozoon* lineages amplified.

Simuliidae species	N_{ip}	N_{Mic}		N	N_{PCR}/N_{pool}		Blood source (%)	Claw morphology	*Leucocytozoon* lineage
		Sg	Mg		T	Ab			
Simulium sp.*	np	np	np	50	0/-	0/-		ST	
*Simulium furcillatum** [2,3,4]	11	0	0	14	0/-	0/-	*Grallaria* sp. (7.1%) *Equus caballus* (7.1%)	ST	
*Simulium cormonsi** [3,4]	10	0	0	50	0/14	0/14	*Grallaria ruficapilla* (16.6%)	ST	
Simulium muiscorum[2,4,5]	61	0	0	190	1/14	0/14	*Atlapetes pileatus* (2.4%)	ST	One lineage in salivary glands (L_SIMUI_01, KT247889), closely related (99%) to KR052948, KF314777,EF153656 and KF767435.
*Simulium pautense** [3,4]	np	np	np	2	0/-	0/-		ST	
Simulium townsendi (complex)* [1,3]	np	np	np	1	0/-	0/-		ST	
Simulium tolimaense[2]	np	np	np	12	0/2	0/2		ST	
Simulium bicoloratum[2,3]	133	0	0	266	0/18	1/18		ST	One lineage in midgut (L_SIBIC_01, KT247890) closely related (96%) to KR052948 KF314777
Simulium ignescens (complex) [1,2,3,5]	295	0	0	365	0/19	0/19	*Basileuterus nigrocristatus* (2.9%)	ST	
*Gigantodax chilensis**[3,4,5]	np	np	np	2	0/-	0/-		BC	

Gigantodax misitu[6]	np	np	np	39	0/5	2/5	*Homo sapiens* (75%)	BC	Two lineages detected in midgut: one identical to *Leucocytozoon* sp. (L_HEVE01, KF717062), and one (L_GIMIS_01, KT247891) related (99%) to JQ988120

Percentages of blood sources were calculated as the number of pools positive in each different source over the total groups per species. N_{ip}: Number of processed individuals by microscopy, $N_{:}$ Number of individuals processed for molecular analysis, Number of positive N_{Mic}: individuals detected by microscopy, Number of positive N_{PCR}: pools detected by PCR, N_{pool}: Number of pools processed by PCR, np: not processed by microscopy, ST: Sub-basal tooth, BC: Bifid claw, Sg: Salivary glands, Mg: Midgut, T: Thorax, Ab: Abdomen. *Individuals processed one by one with both methodologies. Sampled localities: [1] El Cedral, [2] Ucumarí Natural Regional Park (NRP), [3] Bosque Palacio, [4] Estación Monterredondo, [5] El Bosque, and [6] Laguna del Otún. Species collected only as larvae or pupae in the locality indicated: [1, 2]*Simulium albanense*, [4]*Simulium chimguazaense*, [1]*Simulium dinellii*, [6]*Simulium machetorum*, [6]*Simulium quimbayium*, [1,2,3]*Simulium pifanoi*, [1,5]*Simulium roquemayu*, [6]*Gigantodax arrarteorum*, [1,4,6]*Gigantodax basinflatus*, [1,2,3,4]*Gigantodax cervicornis*, [3]*Gigantodax multifilis*, [3,4]*Gigantodax ortizi*, [3,4]*Gigantodax paramorum*, [3]*Gigantodax wygodzinskyi*, [3,5]*Gigantodax zumbahuae*.

(Crosskey 1990) was not strong in our study (Table 3).

Four different lineages of *Leucocytozoon cytb* were amplified from salivary glands or midgut pools of black flies. Of 72 pools and 69 individuals, one amplification produced a lineage from the salivary glands of *S. muiscorum*, and three of 72 pools and 69 individuals produced three different lineages from midgut pools of *G. misitu*, *S. bicoloratum*, *S. cormonsi*, and *S. muiscorum* (Table 3). The sequences in black flies were closely related to the lineages in resident birds of Colombia (Table 3, Fig. 3). One of them (Lineage KF717062), from the midgut of *Gigantodax misitu*, was identical to an isolate from *Hemispingus verticalis*. The remaining three sequences have not been recorded previously (Table 3, Supplementary Material Fig. A.3).

Phylogenetic Relationships

The phylogeny estimated from a 476-bp *cytb* fragment exhibited low support for many nodes (Fig. 3). As previously reported (Lotta et al. 2015), the deepest node of the *Leucocytozoon* clade that includes the majority of passerine and non-passerine lineages is still unresolved. Our phylogenetic analysis revealed that *Leucocytozoon* lineages identified as *L. majoris* and *L. fringillinarum* in passerine birds were not monophyletic, as evidenced by their distribution throughout the phylogenetic tree. The average genetic divergence between lineages within these morphotypes was 7-10% for *L. fringillinarum s.l.* and 4% for *L. majoris s.l.* (Fig. 3, Table 4).

Although the lineages detected in the Turdidae were not a monophyletic group, they belonged to two well-supported clades (Fig. 3, clades A and B). These species were not found in any other avian family. The *Leucocytozoon* lineages infecting Apodiformes were not part of a monophyletic group. However, a major clade contained several lineages found in these birds (Clade C), whereas other lineages were in different clades together with passerine parasites (Fig. 3). For example, the lineage METHYR01 (KF699312) was isolated from a hummingbird (*Metallura tyrianthina*, Fig. 3) and two passerine birds (*Mecocerculus leucophrys* and *Arremon torquatus*) (Table 1); in all these cases, gametocytes were observed in the blood smears. Nine lineages in our study were identical to those found in the Ecuadorian and Peruvian Andes (Supplementary Material Table A.2). Some clades included lineages from different bird families, suggesting different degrees of host specificity (Supplementary Material Table A.2, Fig. A.3). Birds sampled in the two geographically distant mountain

Table 4. Genetic distances (percentages) calculated using Kimura 2-parameter model of substitutions, between some cytochrome *b* lineages of *Leucocytozoon* spp. used in Figure 3. *Haemoproteus columbae* lineage was used as outgroup.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	KF717064. L_ATALB02														
2	KT247889. L_SIMUI_01	0.71													
3	KT247890. L_SIBIC_01	3.84	4.09												
4	FJ168564.	1.92	1.18	4.34											
5	KF717056. L_TFUS_10	5.01	5.61	8.95	6.14										
6	KF699313. L_TFUS_11	4.33	4.85	8.16	5.45	0.63									
7	KF717047. L_MYOFU_01	8.92	9.49	13.02	9.87	8.93	8.22								
8	KT247891. L_GIMIS_01	9.76	10.57	14.15	9.75	10.03	9.22	1.66							
9	FJ168563	9.17	9.77	13.02	9.16	8.69	8.45	3.88	4.60						
10	KF309188. L_HELIAM_01	9.16	10.03	13.58	9.16	8.45	7.75	5.90	6.87	6.82					
11	KM272250. L_GRQUI_01	9.87	10.83	14.42	9.87	9.64	8.93	7.28	8.43	8.22	5.23				
12	AB299369	18.62	19.57	23.25	18.36	18.57	18.86	19.12	18.92	18.55	17.24	18.34			
13	FJ168562	23.13	25.00	28.64	22.26	22.57	22.26	21.91	23.61	21.94	21.07	22.51	21.28		
14	AB302215	16.71	16.77	20.68	17.24	16.71	16.45	18.04	18.55	17.24	16.71	16.45	17.56	15.5	

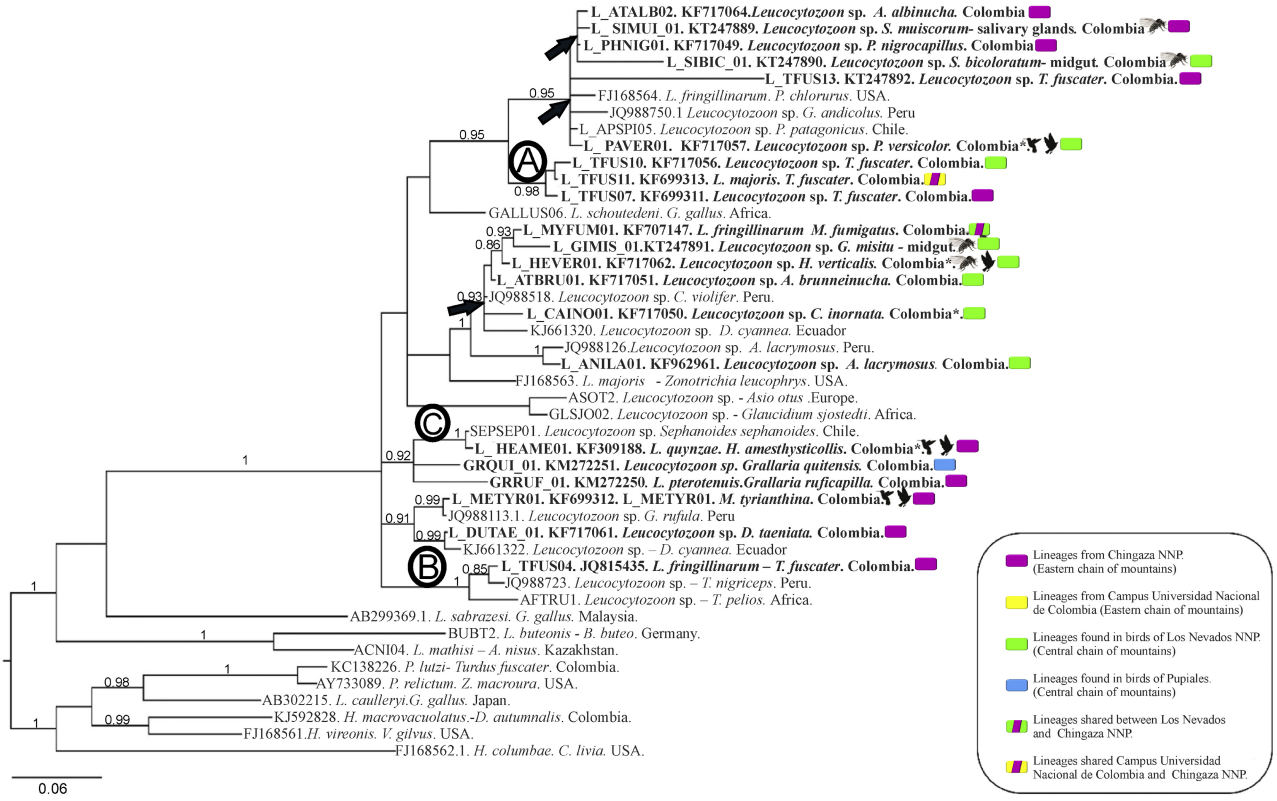

Figure 3. Bayesian phylogeny constructed with *Leucocytozoon cytb* fragments (476 bp). Posterior probability values greater than 80% are shown. Identical sequences found in other South American countries are marked with an asterisk. Lineages shared between different host orders are indicated by ( ) for Apodiformes and ( ) for Passeriformes. Parasite lineages in Turdidae are clustered in two clades marked A and B, respectively. Unresolved nodes are indicated with arrows. Lineages found in simuliids are also shown ( ).

ranges (Chingaza and Los Nevados NNP) shared some parasite lineages (Fig. 3).

Figure 4 depicts phylogenies using the complete mitochondrial genomes with and without *L.* (*Akiba*) *caulleryi*. The NCBI codes for each mitochondrial genome were included in the figure as references. Two well-supported clades of *Leucocytozoon* species shared a common ancestor. In one clade, *Leucocytozoon* species from birds of the family Grallariidae (KM610045 and KM610046) appeared as the sister group of *L. sabrazesi* (AB299369) isolated from a Malaysian chicken. In the other clade, *L. fringillinarum* and *L. majoris* plus *L. quynzae* were sister taxa. In both phylogenies (*cytb* genes and mtDNA genomes), *L. caulleryi* was not part of the *Leucocytozoon* clade. Indeed, in the complete mitochondrial genome phylogeny (Fig. 4), *L. caulleryi* appeared within a clade that included *Haemoproteus* (*Parahaemoproteus*), whereas in the *cytb* fragment analysis it was near *Plasmodium* species (Fig. 3). When *L. caulleryi* was not included in these analyses, the tree topology resembled those previously obtained by Lotta et al. (2015).

Discussion

The Andean Mountains are the only known Neotropical ecosystem where *Leucocytozoon* transmission occurs (Lotta et al. 2015; Matta et al. 2014a). These highlands are characterized by a high level of species richness and endemism (Josse et al. 2009; Van der Hammen and Cleef 1986), providing a great diversity of vertebrate hosts, vectors, and habitats. New host-parasite-vector associations have been discovered in the Andes (Lotta et al. 2015; Mantilla et al. 2013a, b; Walther et al. 2014).

At least five *Leucocytozoon* morphotypes have been found in the Colombian highlands. Studies of *Leucocytozoon* and other haemosporidians

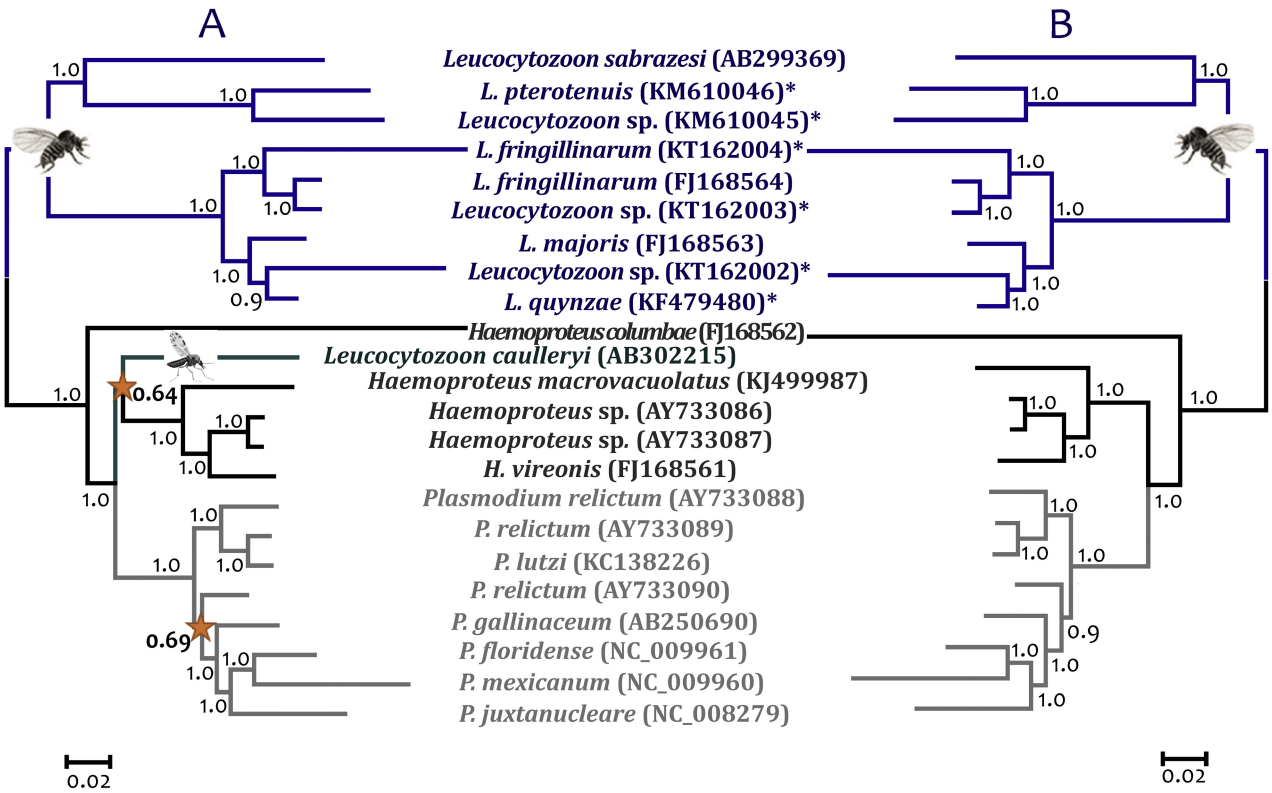

Figure 4. Bayesian phylogenetic hypothesis, using complete mitochondrial genomes (5,485 bp. excluding gaps) of *Leucocytozoon* (*L.*) species, including (**A**) and excluding *L.* (*Akiba*) *caulleryi* (**B**). The values above branches are posterior probabilities. Lineages found in Colombia are labeled with a red asterisk (*).

have been conducted traditionally by microscopy (Bennett et al. 1991; Valkiūnas 2005); however, molecular approaches for the detection of hemoparasites are more sensitive than microscopic examination when birds harbor light parasitemias (Durrant et al. 2006; Richard et al. 2002; Valkiūnas et al. 2003; Valkiūnas et al. 2009). Indeed, molecular studies have revealed greater diversity of these parasites, including cryptic species (Hellgren 2005; Ishak et al. 2008; Krone et al. 2008; Martinsen et al. 2006; Sehgal et al. 2006). We did not perform PCR amplification in all samples collected, only those positive by microscopy where morphological examination could be carried out. Thus, we possibly missed infections with low parasitemia. Nevertheless, the high genetic divergence that we found in *L. fringillinarum* and *L. majoris* suggests that these *Leucocytozoon* morphotypes are complexes of cryptic species emphasizing the value of molecular methods in the characterization of parasite biodiversity.

Our *cytb* phylogeny reveals intraspecific variation for each of these two morphotypes, similar to the distances reported by Sehgal et al. (2006) for *L. mattisi* and *L. buteonis* previously included in the *L. toddi* morphospecies. According to these authors, the levels of *cytb* sequence divergence that delineate species of *Leucocytozoon* are unknown, and *Leucocytozoon* spp. might have rates of evolution different from those of other haemosporidian parasites. Moreover, morphotypes of *Leucocytozoon* (*L. fringillinarum* and *L. majoris*) included in the phylogenetic analysis have been identified by different researchers around the world (e.g. FJ168564, FJ168563 in Perkins (2008)), potentially leading to erroneous correlations between nucleotide sequences and morphotypes. To test the hypothesis of cryptic species, the number of markers, including nuclear DNA markers, should be increased.

Phylogenetic relationships estimated with the *cytb* fragment, using sequences reported throughout South America, show a close relationship among these *Leucocytozoon* lineages, albeit with low nodal support. The lack of phylogenetic signal in the *cytb* fragment suggests that many of these lineages could be polymorphisms in the same species (Reeves et al. 2015) distributed across a wide range

of hosts and locations. In contrast, several lineages are exclusively in species of Turdidae. Nonetheless, limited evidence indicates host specificity of *Leucocytozoon* spp. below the host-order level. Specificity at the family level has been demonstrated for *L. simondi*, *L. caulleryi*, and *L. smithi* (Forrester and Greiner 2008). The lineages found in the Turdidae might be another case of family specificity.

Leucocytozoon (*Akiba*) *caulleryi* falls outside the *Leucocytozoon* clade in both phylogenies. This species of *Leucocytozoon* has several unique characteristics that set it apart from others members of the genus. In particular, the complete mtDNA phylogeny shows that *L. caulleryi* shares a recent common ancestor with the subgenus *Parahaemoproteus*. This subgenus includes species transmitted by *Culicoides*. Earlier studies of the sporogony of *Haemoproteus* and *Leucocytozoon* in *Culicoides* and black flies found developmental similarities for these parasites (Fallis and Bennett 1961). Additional data from nuclear loci are needed to evaluate the taxonomic status of this species and to unveil processes that could explain the mitochondrial DNA phylogeny.

Multiple infections in Colombian samples were common; the most prevalent coinfection was *Leucocytozoon-Haemoproteus* (39.3%). Multiple parasite infections, including co-infections with other species of *Leucocytozoon*, are frequent in natural populations (Pérez-Tris and Bensch 2005; Van Rooyen et al. 2013a). Although coinfections with *Haemoproteus* and *Leucocytozoon* have been recorded with fatal outcomes in owls (Evans and Otter 1998), the high percentage of coinfections in our study suggests that the combinations we observed might be less virulent.

Leucocytozoon (*Leucocytozoon*) parasites complete sporogonic development in different simuliid species (Desser and Yang 1973; Fallis and Bennett 1961; Fallis et al. 1976; Forrester and Greiner 2008). In Colombia, the genera *Gigantodax* and *Simulium* are represented by 17 species and 50 species, respectively (Adler and Crosskey 2015; Diaz et al. 2015; Esquivel et al. 2015; Mantilla et al. 2013c, 2014; Moncada et al. 2013). *Gigantodax* is distributed from Arizona in North America to Tierra de Fuego in South America and has its greatest richness along the Andean Mountains (Adler and Crosskey 2015; Coscarón and Coscarón-Arias 1995; Wygodzinsky and Coscarón 1989). *Simulium* is the only genus in the Colombian lowlands (Wygodzinsky and Coscarón 1973). The elevational distribution of *Gigantodax* ranges from 1,500 to 4,700 m asl (Wygodzinsky and Coscarón 1989), whereas *Simulium* is found from sea level to more than 4,000 m, with some species restricted to the Andean Mountains (Coscarón and Coscarón-Arias 2007; Mantilla et al. 2013c; Muñoz de Hoyos and Miranda-Esquivel 1997). Available evidence indicates that the Simuliidae originated in Pangea, with subsequent divergence of the Prosimuliini and Simuliini (including *Gigantodax* and *Simulium*) after the separation of Laurasia and Gondwana (Adler et al. 2010). Part of the Andean biota is hypothesized to have originated in Patagonia and gradually spread to the northern Andes (Morrone 2001). As we found for the *Leucocytozoon* lineages, some *Simulium* and *Gigantodax* species are shared with Ecuador, Peru, and Chile (Adler and Crosskey 2015; Coscarón and Coscarón-Arias 2007). Distributional histories of the vectors could help explain why *Leucocytozoon* transmission in South America occurs only in the highlands, contrasting with the situation in Africa where transmission occurs in the lowlands and highlands. Africa has numerous ornithophilic taxa, and therefore numerous potential vectors, distributed over a wide elevational range (Crosskey 1990). Although Colombia is a wintering area for migratory birds from the Nearctic Region where *Leucocytozoon* prevalence is high (Bennett and Squires-Parsons 1992), the *Leucocytozoon* lineages in South America are closer to other lineages reported from the continent (Matta et al. 2014a), than they are to lineages in the Nearctic Region.

Studies in the Nearctic Region often report hundreds or thousands of black flies collected in the field (Crosskey 1990; Adler et al. 2004), in contrast to the limited numbers of simuliids trapped in Neotropical habitats. These marked differences might be explained, in part, by the seasonality at higher latitudes (Atkinson 1988). The numbers of black flies that emerge from northern rivers can be in the billions (Malmqvist et al. 2004), and many northern vectors are univoltine (Adler et al. 2004). In contrast, in the absence of marked seasonality in the Neotropics, simuliids are typically multivoltine, with smaller numbers emerging throughout the year (Crosskey 1990).

The low number of insects naturally infected with *Leucocytozoon* species in our study reflects the situation for other vector-borne pathogens, such as human malaria (Alavi et al. 2003; Sinden et al. 2004). The presence of mammals in the study area (e.g., cattle and deer) might limit the contact between the vector and infected birds (Keesing et al. 2010). Blood meals identified in our captured simuliids suggest rather generalized feeding habits. The detection of *Leucocytozoon* sp. in the midguts of *S. cormonsi* and *S. muiscorum*

(which were captured feeding on mammals), provides evidence of a previous blood meal on infected birds; previous studies have shown that *Leucocytozoon* species can develop in simuliids that feed on mammals (Desser and Yang 1973).

A key factor that determines haemosporidian distribution and community composition is elevation (González et al. 2014; LaPointe et al. 2010; Van Riper et al. 1986; Van Rooyen et al. 2013a, b). In our study, the highest prevalence was detected at 3,100 m asl in the páramo ecosystem in Chingaza NNP (0°-14 °C annually). Studies of the sporogonic cycle of *Leucocytozoon simondi* have revealed that the *in vitro* exflagellation of this parasite rarely occurs below 15 °C (Roller and Desser 1973). Nevertheless, blood feedings of simuliids *in vivo* take more than one minute, allowing time for the parasite to become exflagellated (Roller and Desser 1973). Although low temperatures (<15 °C) affect sporogonic development in *L.* (*A.*) *caulleryi* and other haemosporidians (LaPointe et al. 2010), *L.* (*Leucocytozoon*) species are adapted to development and transmission below 15 °C (Valkiūnas 2005). Thus, temperature should not be an ecological limitation to the transmission of these parasites in the Neotropical highlands.

Molecular tools have led to the discovery of a remarkable diversity of potential vectors of haemosporidians (Njabo et al. 2011; Santiago-Alarcon et al. 2012). Dissections in which sporozoites are seen in salivary glands provide a reliable indication of the effective development of the parasite in the insect. Even in the absence of patent parasitic forms in the dissections, these insects might be good vector candidates of the parasite, but not necessarily competent vectors. Knowledge is limited, however, regarding the susceptibility of simuliid species to parasite infections and the range of temperatures at which Neotropical *Leucocytozoon* lineages can develop sporozoites in potential vectors. Additional research on vector biology, as well as experimental infections, are needed to better understand the transmission dynamics of *Leucocytozoon* species in the Neotropical Region.

Methods

Ethical statement: The methods used in this study were not invasive and were approved by the "Comité de Bioetica of Departamento de Ciencias para la Salud Animal", Facultad de Medicina Veterinaria y de Zootecnia, Universidad Nacional de Colombia (Permit number CBE-FMVZ-016). Birds were captured and handled in a manner to minimize their stress. All birds were released after the samples were collected. The 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), the "Corporación autonoma regional de Risaralda CARDER" and "Autoridad Nacional de Licencias Ambientales, ANLA" (file 4120E1104774 of 2011, file 4120E183893 of 2011, and resolution 0688 of 2012 modified by resolution 0787 of 2013). Access to genetic resources was authorized by contract No. 98 of March 26, 2014 of Ministerio de Ambiente y Desarrollo Sostenible.

Study sites: The study was carried out at three lowland sites (Yamato Foundation, La Macarena National Natural Park (NNP), and Villavicencio Botanical Garden) and six highland sites (Campus Universidad Nacional de Colombia, Chingaza NNP, Pupiales, El Cedral, Ucumarí Natural Regional Park (NRP), and Los Nevados NNP) (Table 5). In Chingaza NNP, four localities were sampled: Bosque Palacio, Estación Monteredondo, Bosque de Encenillo, and Valle de los Fraylejones. In Los Nevados NNP, two sites were sampled: El Bosque and Laguna del Otún.

Bird sampling: Birds were captured by mist nets. In the lowlands, sampling was performed from 1999 to 2000, and in the highlands from 2002 to 2003 and from 2009 to 2014. Blood samples were collected by brachial vein puncture or toenail clipping. For each bird, three thin smears were prepared and approximately 50 μl of blood were stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for molecular analyses. Smears were air dried, fixed with absolute methanol, and stained with Giemsa. The smears were double-blind scanned and the intensity of infection was calculated by counting the number of infected cells/10,000 erythrocytes (Muñoz et al. 1999).

Elevation and *Leucocytozoon* infection status: Association between parasite infection and elevation was assessed by a General Linear Model (GLM), with the infection status by *Leucocytozoon* spp. of each sampled individual (presence or absence) as the response variable, and elevation (m above sea level) of the sampled areas as the predictor variable in a logistic regression model for the second approach. Sample areas were divided in two groups: the first group included sampled areas between 0 and 2,000 m asl, and the second group included areas located between 2,000 and 4,000 m asl. The the difference in the prevalence between the groups was tested by ANOVA of one factor. These analyses were carried out using the R statistical program (v. 3.2.2) (R Development Core Team 2008; Wickham 2009).

Blood DNA extraction and molecular analysis of *Leucocytozoon*: Only DNA from avian blood samples determined by microscopy as positive for *Leucocytozoon* was extracted using the standard phenol: chloroform protocol (Sambrook et al. 1989). Molecular characterization was carried out using the mitochondrial cytochrome *b* gene (*cytb*) and the complete mitochondrial genome (mtDNA). Molecular detection using the *cytb* gene was made by nested PCR (Hellgren et al. 2004). DNA from uninfected birds was included as negative controls, and samples from an infected great thrush (*Turdus fuscater*, GenBank Number accession KF699310) were used as positive controls in all PCR assays. PCR products were purified with ammonium acetate and 96% ethanol (Bensch et al. 2000) and sequenced on a 3730xl DNA Analyzer. The sequences were edited manually and aligned using MEGA v5.05 software (Tamura et al. 2011). Co-infections were amplified more than once, until sequences with single peaks were obtained. These sequences were compared with lineages whose morphotype had been clearly determined. Samples with co-infections for which a unique single peak could not be obtained were discarded.

Table 5. Localities sampled for *Leucocytozoon* and Simuliidae in Colombia.

Locality	Coordinates	Elevation (m asl)	Ecosystem
Yamato Foundation*[r]	4° 31′ N; 71° 31′ W	130	Dry savanna and gallery forests. Median annual temperature: 28.7 °C. Annual average rainfall: 2,000 mm.[1]
La Macarena National Natural Park (NNP)*[r]	2° 28′ N; 73° 47′ W	400-500	Grasslands and savanna. Median annual temperature: 24 °C. Annual average rainfall: 2,973 mm.[2]
Villavicencio Botanical Garden*[r]	4° 0.9′ N; 73° 39′ W	640	Secondary tropical rainforest. Median annual temperature: 26 °C. Annual average rainfall: 3,000 mm.[1]
El Cedral*[†]	4° 42′ N; 75° 32′ W	1900-2100	High Andean forests, paddocks, and herbaceous vegetation. Temperature range: 12-18 °C. Annual average rainfall: 2,724 mm.[3]
Ucumarí Natural Regional Park (NRP) *[†d]	4° 42′ N; 75° 29′ W	2400	High Andean forests. Temperature range: 11.8-16.6 °C. Annual average rainfall: 2,220 mm. [3,4]
Campus Universidad Nacional de Colombia[*b]	4° 38′ N; 74° 5′ W	2560	Urban area with vegetation patches. Median annual temperature: 15 °C; daily variation: 10 to 20 °C. Annual average rainfall: 1,788 mm.[5]
Chingaza National Natural Park (NNP)			
Bosque Palacio*[†b]	4° 41′ N; 73° 50′ W	2900	
Estación Monterredondo*[†b]	4° 37′ N; 73° 43′ W	3100	Paramo and high Andean forests. Temperature range: 12-18 °C, but may be under 0 °C. Annual average rainfall: 1,900 mm.[6]
Bosque de Encenillo*	4° 36′ N; 73° 43′ W	3155	
Valle de los Fraylejones*	4° 32′ N; 73° 46′ W	3203	
Pupiales*	0° 54′ N, 77° 39′W	3014	Paramo, montane dry and wet forests. Annual median rainfall: 942.85 mm. Average temperature: 12 °C.[7]
Los Nevados National Natural Park (NNP)			
El Bosque*[†b]	4° 43′ N; 75° 27′ W	3200-3300	Paramo and high Andean forest remnants in rocky sheltered areas and paddocks. Median annual temperature: 6 °C. Annual average rainfall: 980 mm.[8,9]
Laguna del Otún*[†b]	4° 46′ N, 75° 24′ W	3950-4100	

Localities where *birds or [†]insects were collected. Localities sampled in [r]rainy season, [d]dry season, and [b]both seasons. Descriptions of environmental conditions of sampling areas are available in the following references: [1](Rodríguez and Matta 2001); [2](Basto et al. 2006); [3](Lentijo and Kattan 2005); [4](Vásquez and Serrano 2009); [5](Department of Geosciences Universidad Nacional de Colombia 2011); [6](Vargas-Rios and Pedraza 2004); [7](Cadena et al. 2012); [8](Rivera 2001), [9](Gil-Ospina et al. 2010).

To complement previous phylogenetic studies on *Leucocytozoon* spp. from Colombia, additional complete mitochondrial genomes (mtDNA) were sequenced. In particular, we obtained sequencing data from two great thrushes (*Turdus fuscater*: *L. fringilinarum* and *Leucocytozoon* sp.) and one chestnut-capped brush finch (*Arremon brunneinucha: Leucocytozoon* sp.) that were diagnosed as single infections. PCR was performed using primers 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). To avoid potentially mixed infections, the PCR products were cloned. PCR and cloning protocols were described by Pacheco et al. (2011a, b). For at least three clones, both strands were sequenced using an Applied Biosystems 3730 capillary sequencer. The mtDNA genome alignment was made using ClustalX v2.0.12 and Muscle, as implemented in SeaView v4.3.5 (Gouy et al. 2010) with manual editing; it included the 20 mitochondrial genomes available in GenBank for haemosporidians isolated from lizards and birds, (Table A.4, Bastien et al. 2014; Beadell and Fleischer 2005; Lotta et al. 2015; Levin et al. 2012; Mantilla et al. 2013b; Matta et al. 2014a, b; Omori et al. 2007, 2008). The mtDNA alignment was divided into four categories and each gene (cytochrome oxidase I, cytochrome oxidase III, and cytochrome *b*) was used as a separate partition plus the noncoding regions (Pacheco et al. 2011a). The partial *cytb* gene and the mtDNA genome sequences were submitted to GenBank under accession nos. KT162002, KT162003, and KT162004, respectively.

Phylogenetic relationships: A phylogenetic analysis of *Leucocytozoon* spp. was made with a fragment of the *cytb* gene (476 nucleotides) from 45 individuals as follows: GenBank (16), MalAvi (8), and Colombia (21). Phylogenetic reconstruction using Bayesian inference was made with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). The model selected by jModeltest 2.1.1 (Darriba et al. 2012), as the best of 88 models according to the Akaike information criterion, was a General Time-Reversible model including invariable sites and variation among sites (GTR+I+Γ). Two independent runs of 5×10^6 generations were conducted with six chains, sampling every 100 generations. In all, 25% of the trees were discarded as burn-in; in total, 22,500 trees were used to construct the majority rule consensus tree. The phylogeny was edited using FigTree v1.3.1 (Rambaut 2006). Genetic distances between lineages were calculated using the Kimura two-parameter model of substitution, implemented in MEGA v5.05 software (Tamura et al. 2011).

Phylogenetic relationships were also estimated with the complete mtDNA genome by using MrBayes v3.1.2 with the default priors (Ronquist and Huelsenbeck 2003). A General Time-Reversible model (GTR+I+Γ) was also the best model that fit these data. Bayesian support for the nodes was inferred in MrBayes using $6x10^6$ Markov Chain Monte Carlo (MCMC) steps. After convergence was reached, we discarded 25% of the samples as burn-in. In both cases (*cytb* and mtDNA genomes), convergence is reached when 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).

Simuliid collection, maintenance, and identification: Adults of *Simulium* and *Gigantodax* species were collected at high elevations (2,100-3,950 m asl) in El Cedral station, Ucumarí NRP, Chinagaza NNP, and Los Nevados NNP (Table 4). No adults were collected at Campus Universidad Nacional de Colombia (2,560 m asl). Insects were collected by Centers for Disease Control (CDC) miniature UV light traps located in ecotones, on banks of rivers, and in forest patches. Aspiration directly from bird and mammal baits and Bennett traps (Anderson and DeFoliart 1961; Service 1981) were also used. After collection, the insects were maintained in dark containers with 10% sucrose solution before dissections. A subset of insects was dissected in the field, using an AmScope Portable Stereo Microscope 10x (California, USA), but the majority of insects were preserved for molecular analyses.

Simuliid pupae were collected, with forceps, from substrates (e.g., stones and trailing vegetation) in streams near the sampled localities to determine the species and their distributions. Adults and pupae were identified morphologically using identification keys (Bueno et al. 1979; Coscarón and Coscarón-Arias 2007). Tarsal claw structure of female flies, especially the presence of a lobe (bifid claw) or sub-basal tooth, also was recorded. Presence-absence data for simuliid species were used in the analyses of elevational distribution.

Dissections, blood-meal identifications, and molecular detection of *Leucocytozoon* spp. in black flies: Female flies were anesthetized and the legs and wings were removed. Midguts were extracted by cutting the terminal segments of the abdomen and pulling out the contents, which were separated onto a new slide with saline solution and stained with 2% mercurochrome. Salivary glands were dissected by pulling off the head with a needle in a drop of saline solution and were disrupted to release sporozoites. The gland preparations were fixed in absolute methanol and stained with Giemsa. Some midguts and salivary glands were not stained; instead, they were stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for molecular analysis. All procedures followed the recommendations of Valkiūnas (2005). Wings, legs, ovaries, and remaining portions of the body were deposited as vouchers in the biological collection GERPH of the Biology Department of Universidad Nacional de Colombia (GERPH accession numbers IN 00041 - IN 00145).

DNA was extracted using the standard phenol-chloroform protocol from midguts and salivary glands that were previously stored in SET buffer (Sambrook et al. 1989). The thoraxes and abdomens of some individuals that were not dissected in the field were processed separately with the same methodology (Hellgren et al. 2008).

To determine the blood sources, 950 individuals of *Simulium* spp. (883 individuals in 67 pools, and 67 individuals separately) and 41 individuals of *Gigantodax* spp. (39 individuals in 5 pools, and 2 individuals separately) were analyzed. Amplifications of *cytb* from vertebrate hosts were performed, first using the primers L14841 and H15149 when a 305-bp fragment was expected (Kocher et al. 1989). Then, to detect avian- or mammalian-derived blood meals, independent PCR with *cytb*-specific primers for birds, Avian-3 and Avian-8 (Sawabe et al. 2010), or mammals (Molaei et al. 2006) was performed on the positive samples for vertebrate blood. Products obtained from these last two reactions had a size of 530 bp and 770 bp, respectively. Amplified fragments were sequenced and compared with the GenBank database to determine the host-blood sources.

DNA extracted from midguts and salivary glands was also used to amplify a fragment of 476 bp of *Leucoytozoon cytb* in pools of midguts or salivary glands from 15 or fewer individuals. Amplifications were made using nested PCR and primers reported by Hellgren et al. (2004). *Leucocytozoon* lineages obtained from positive amplifications were compared with those in public databases (GenBank and MalAvi) (Fig. A.5).

The sequences reported in this paper have been deposited in the GenBank database (accession nos. KT162002 - KT162004 (Fig. 5), KF717048, KF717050, KF717052 - KF717067, KF962962 (Table 1), KT247889, KT247890, KT247891 (Table 2)).

Acknowledgements

The authors thank the students of the Host-Parasite Relationship Research Group: Avian Haemoparasites Model for field assistance. We thank the staff of Unidad Administrativa Especial del Sistema de Parques Nacionales Naturales for supporting field logistics. This study was supported by El Departamento Administrativo de Ciencias, Tecnología e Innovación COLCIENCIAS (contract No. 556-2014, project numbers 110152128340 and 110165944139), ECOPETROL S.A contract. no. 138, Jardín Botánico José Celestino Mutis Bogotá program "Thomas van der Hammen", and Division de Investigación Bogotá-Universidad Nacional de Colombia, project N° 18714. The funding sources had no role in the 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.protis.2016.02.002.

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