

Disentangling *Leucocytozoon* parasite diversity in the neotropics: Descriptions of two new species and shortcomings of molecular diagnostics for leucocytozoids



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ABSTRACT

Avian communities from South America harbor an extraordinary diversity of *Leucocytozoon* species (Haemosporida, Leucocytozoidae). Here, of 890 birds sampled, 10 (1.2%) were infected with *Leucocytozoon* parasites. Among them, two new species were discovered and described. *Leucocytozoon grallaridae* sp. nov. and *Leucocytozoon neotropicalis* sp. nov. were found in non-migratory highland passeriforms belonging to the Grallaridae and Cotingidae, respectively. They both possess gametocytes in fusiform host cells. However, due to combining microscopic examination and molecular detection, it was revealed that these parasites were present in co-infections with other *Leucocytozoon* species, which gametocytes develop in roundish host cells, therefore exhibiting two highly distant parasite lineages isolated from the same samples. Remarkably, the lineages obtained by cloning the mtDNA genomes were not captured by the classic nested PCR, which amplifies a short fragment of cytochrome *b* gene. Phylogenetic analyses revealed that the lineages obtained by the classic nested PCR clustered with parasites possessing gametocytes in roundish host cells, while the lineages obtained by the mtDNA genome PCR protocol were closely related to *Leucocytozoon* parasites possessing gametocytes in fusiform host cells. These findings suggest problems with the sensitivity of the molecular protocols commonly used to detect *Leucocytozoon* species. A detailed analysis of the primers used in the classic nested PCR revealed a match with DNA sequences from those parasites that possess gametocytes in roundish host cells (i.e., *Leucocytozoon fringillinarum*), while they differ with the orthologous regions in the mtDNA genomes isolated from the samples containing the two new species. Since these are mixed infections, none of the lineages detected in this study can be assigned accurately to the new *Leucocytozoon* morphospecies that develops in fusiform host cells. However, phylogenetic analyses allowed us to hypothesize their most probable associations. This study highlights the need for developing detection methods to assess the diversity of *Leucocytozoon* parasites accurately.

1. Introduction

The Andes in South America are recognized as hotspots for avian endemism. This region includes approximately 133 different ecosystems (Morrone, 2001; Josse et al., 2009) with more than 2000 avian species reported, including nearly 600 endemic species (Myers et al., 2000; Herzog and Kattan, 2011). Such host species richness seems to

drive a high diversity of avian haemosporidian parasites that is just starting to be characterized using microscopy and Polymerase Chain Reaction (PCR)-based detection methods (i.e. Merino et al., 2008; Rodríguez et al., 2009; Jones et al., 2013; Mantilla et al., 2013, 2016; Matta et al., 2014; Galen and Witt, 2014; Harrigan et al., 2014; González et al., 2014, 2015; Marzal et al., 2015; Lotta et al., 2016; Moens et al., 2016; Moens and Pérez-Tris, 2016; Cadena-Ortiz et al.,

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2018; de Aguilar et al., 2018; Gil-Vargas and Sedano-Cruz, 2019).

Molecular techniques present significant advantages for parasite detection, particularly in samples with very low parasitemia (sub-microscopic infections). Many molecular protocols targeting to different molecular markers such as the rRNA (Richard et al., 2002), nuclear sequences (Bensch et al., 2004), the apicoplast (Caseinolytic protease C-Clpc) (Martinsen et al., 2008), and mitochondrial genes (Cytochrome b -cytb, Cytochrome oxidase subunit I and III – *cox1*, *cox3*) (Escalante et al., 1998; Bensch et al., 2000; Perkins and Schall, 2002; Hellgren et al., 2004; Pacheco et al., 2018b) have been used with diagnosis purpose. Currently, most of the avian haemosporidian inventories rely mainly on molecular detection. Unfortunately, most of the sequences of haemosporidian, including *Leucocytozoon*, reported in the Neotropics remain without being associated with a morphospecies because few studies applied both microscopic and molecular diagnosis in parallel. That is an obstacle for a reliable estimate of parasite species diversity.

PCR-based methods commonly overlook the presence of co-infections (Bernotienė et al., 2016; Pacheco et al., 2018a). The two main reasons are 1) the primers may have a higher affinity for one of the parasites in the sample, and 2) there may be an uneven amount of the template for each of the species or lineages in the co-infected samples (Perez-Tris and Bensch, 2005; Bernotienė et al., 2016; Pacheco et al., 2018a). Nevertheless, there are many reported cases where the amplification fails to detect haemosporidian parasite DNA in samples with an evident high intensity of parasitemia (Zehindjiev et al., 2012; Schaer et al., 2015; Bernotienė et al., 2016), indicating that other variables are also involved in the PCR performance in co-infections (Pacheco et al., 2018a). Indeed, co-infections of avian haemoparasites are common in natural populations (Perez-Tris and Bensch, 2005; Van Rooyen et al., 2013; Bernotienė et al., 2016; Pacheco et al., 2018a), making this problem far more complicated (Bernotienė et al., 2016; Ciloglu et al., 2018; Pacheco et al., 2018a). Although recent publications have provided alternatives to overcome the issue of mixed infections (Perez-Tris and Bensch, 2005; Bernotienė et al., 2016; Pacheco et al., 2018a), the molecular detection of lineages belonging to the same genus in the same sample and their linkage to certain morphospecies remains challenging. For example, in the case of birds sampled in Colombian mountain ranges, co-infections of *Leucocytozoon* species with parasites of other genera have been detected in 25.4% of samples. Furthermore, in 18.2% of the co-infections two or more species of *Leucocytozoon* were observed in the same blood film (Lotta et al., 2016).

In this study, new leucocytozoid species were found in two non-migratory species of passerines (Undulated Antpitta, *Grallaria squamigera* and Green and black Fruiteater, *Pipreola riefferii*) from highland ecosystems of Colombia. These parasites were described using both microscopic and molecular diagnosis. Importantly, both bird species were co-infected with different lineages of *Leucocytozoon* species. Using morphological and phylogenetic analyses, a possible linkage was proposed between the new morphospecies under description and other lineages amplified from co-infections. Furthermore, we also discussed current problems in molecular diagnosis of *Leucocytozoon* parasites.

2. Materials and methods

2.1. Study sites

During this study, 840 birds belonging to 139 species were caught using mist nets in the central and eastern Andean mountain ranges of Colombia (Table 1). In the central mountain chain (Cordillera Central), 686 birds belonging to 118 species were captured during April, July, August, and December of 2015 and January of 2016 (Table 1). Sample sites included three distinct life zones (Cuatrecasas, 1958): (i) Sub-andean Forest (SF) (1800–2600 m above sea level (masl)); represented by the Fauna and Flora Sanctuary Otún Quimbaya (FFS), El Cedral station and Ucumari Natural Regional Park (NRP); (ii) the Andean Forest (AF) (2900–3500 masl) as found in the locality of El Bosque, and (iii) the

Table 1

Birds captured in Los Nevados National Natural Park, and Palacio forest at Chingaza NNP in this study.

	Life zone	Altitudinal range	N° cap (N° infected) per chain of mountains	
			Eastern	Central
Anseriformes				
Anatidae				
<i>Anas flavirostris</i>	AF	2900–3500	1(0)	
Apodiformes				
Trochilidae				
<i>Adelomyia melanogenys</i>	SAF	1800–2600		5(0)
<i>Agelaiocercus kingii</i>	SAF	1800–2600		1(0)
<i>Amazilia franciae</i>	SAF	1800–2600		4(0)
<i>Boissonneaua flavescens</i>	AF	2900–3500		1(0)
<i>Boissonneaua flavescens</i>	SAF	1800–2600		2(0)
<i>Coeligena</i>	SAF	1800–2600		7(0)
<i>Coeligena helianthea</i>	AF	2900–3500	1(0)	
<i>Coeligena torquata</i>	SAF	1800–2600		3(0)
<i>Coeligena torquata</i>	AF	2900–3500		1(0)
<i>Colibri coruscans</i>	AF	2900–3500		3(0)
<i>Colibri thalassinus</i>	SAF	1800–2600		3(0)
<i>Doryfera ludovicae</i>	SAF	1800–2600		2(0)
<i>Ensifera</i>	AF	2900–3500	1(0)	3(0)
<i>Eriocnemis cupreiventris</i>	AF	2900–3500	2(0)	
<i>Eriocnemis derbyi</i>	AF	2900–3500		14(0)
<i>Eriocnemis derbyi</i>	SAF	1800–2600		1(0)
<i>Helianthus</i>	AF	2900–3500	1(0)	
<i>amethysticollis</i>				
<i>Helianthus exortis</i>	AF	2900–3500	1(0)	7(0)
<i>Helianthus exortis</i>	SAF	1800–2600		5(1)
<i>Lafresnaya lafresnaya</i>	AF	2900–3500	6(0)	4(0)
<i>Metallura tyrianthina</i>	AF	2900–3500		3(0)
<i>Metallura tyrianthina</i>	P	3900–4100		2(0)
<i>Phaethornis guy</i>	SAF	1800–2600		4(0)
<i>Phaethornis</i>	SAF	1800–2600		3(0)
<i>symmatophorus</i>				
<i>Schistes geoffroyi</i>	SAF	1800–2600		1(0)
Columbiformes				
Columbidae				
<i>Zenaida auriculata</i>	SAF	1800–2600		2(0)
Charadriiformes				
Charadriidae				
<i>Vanellus chilensis</i>	SAF	1800–2600		2(0)
Falconiformes				
Accipitridae				
<i>Rupornis magnirostris</i>	SAF	1800–2600		1(0)
Passeriformes				
Cinclidae				
<i>Cinclus leucocephalus</i>	AF	2900–3500	3(0)	
Corvidae				
<i>Cyanolyca viridicyanus</i>	SAF	1800–2600		2(0)
<i>Cyanocorax yncas</i>	SAF	1800–2600		3(0)
Cotingidae				
<i>Pipreola riefferii</i> [§]	AF	2900–3500		1(1)
Dendrocolaptidae				
<i>Pseudocolaptes boissonneautii</i>	AF	2900–3500	1(0)	
Emberizidae				
<i>Arremon brunneinucha</i>	SAF	1800–2600		4(0)
<i>Arremon brunneinucha</i>	AF	2900–3500	2(1)	
<i>Arremon torquatus</i>	AF	2900–3500	4(0)	
<i>Atlapetes albinucha</i>	SAF	1800–2600		6(0)
<i>Atlapetes albinucha</i>	AF	2900–3500	2(0)	
<i>Atlapetes pallidinucha</i>	AF	2900–3500	7(1)	
<i>Atlapetes schistaceus</i>	AF	2900–3500	6(1)	
<i>Zonotrichia capensis</i>	SAF	1800–2600		93(0)
<i>Zonotrichia capensis</i>	P	3900–4100		6(0)

(continued on next page)

Table 1 (continued)

	Life zone	Altitudinal range	N° cap (N° infected) per chain of mountains	
			Eastern	Central
<i>Zonotrichia capensis</i>	AF	2900–3500	17(0)	30(0)
Fringillidae				
<i>Astragalinus psaltria</i>	AF	2900–3500		2(0)
<i>Astragalinus psaltria</i>	SAF	1800–2600		4(0)
<i>Euphonia lanirostris</i>	SAF	1800–2600		3(0)
<i>Euphonia xanthogaster</i>	SAF	1800–2600		4(0)
<i>Saltator atripennis</i>	SAF	1800–2600		1(0)
Furnariidae				
<i>Cinclodes excelsior</i>	P	3900–4100		6(0)
<i>Dendrocincla tyrannina</i>	AF	2900–3500		1(0)
<i>Dendrocincla tyrannina</i>	SAF	1800–2600		2(0)
<i>Hellmayrea gularis</i>	AF	2900–3500	1(0)	
<i>Leptasthenura andicola</i>	P	3900–4100		6(0)
<i>Lepidocolaptes</i>	SAF	1800–2600		1(0)
<i>lacrymiger</i>				
<i>Margarornis squamiger</i>	AF	2900–3500	12(0)	
<i>Premnoplex brunescens</i>	AF	2900–3500		1(0)
<i>Premnoplex brunescens</i>	SAF	1800–2600		2(0)
<i>Synallaxis azarae</i>	SAF	1800–2600		10(0)
<i>Syndactyla subalaris</i>	SAF	1800–2600		1(0)
<i>Xiphocolaptes</i>	SAF	1800–2600		3(0)
<i>promeropirhynchus</i>				
Grallariidae				
<i>Grallaria squamigera</i> *	AF	2900–3500	1(1)	
Hirundinidae				
<i>Orochelidon murina</i>	AF	2900–3500	2(0)	7(0)
<i>Orochelidon murina</i>	P	3900–4100		5(0)
<i>Pygochelidon</i>	SAF	1800–2600		14(0)
<i>cyanoleuca</i>				
<i>Pyroderus scutatus</i>	SAF	1800–2600		4(0)
<i>Stelgidopteryx ruficollis</i>	SAF	1800–2600		2(0)
Icteridae				
<i>Molothrus bonariensis</i>	SAF	1800–2600		2(0)
Momotidae				
<i>Momotus momota</i>	SAF	1800–2600		1(0)
Parulidae				
<i>Basileuterus tristriatus</i>	SAF	1800–2600		2(0)
<i>Cardellina canadensis</i>	SAF	1800–2600		8(1)
<i>Myioborus sp.</i>	AF	2900–3500	1(0)	
<i>Myioborus miniatus</i>	SAF	1800–2600		6(0)
<i>Myioborus ornatus</i>	AF	2900–3500	4(0)	6(0)
<i>Myiothlypis coronata</i>	AF	2900–3500		1(0)
<i>Myiothlypis coronata</i>	SAF	1800–2600		5(0)
<i>Myiothlypis nigrocrissatus</i>	AF	2900–3500	13(0)	1(0)
<i>Setophaga fusca</i>	SAF	1800–2600		1(0)
Rhinocryptidae				
<i>Scytalopus infasciatus</i>	AF	2900–3500	1(0)	
<i>Scytalopus micropterus</i>	AF	2900–3500	2(0)	
Thraupidae				
<i>Anisognathus igniventris</i>	AF	2900–3500	3(0)	
<i>Anisognathus lacrymosus</i>	AF	2900–3500		5(0)
<i>Buthraupis montana</i>	AF	2900–3500		1(1)
<i>Catamblyrhynchus diadema</i>	AF	2900–3500	3(0)	
<i>Catamenia homochroa</i>	P	3900–4100		12(0)
<i>Catamenia inornata</i>	P	3900–4100		18(0)
<i>Conirostrum rufum</i>	AF	2900–3500	1(0)	
<i>Diglossa albilatera</i>	AF	2900–3500	2(0)	3(0)
<i>Diglossa albilatera</i>	SAF	1800–2600		1(0)
<i>Diglossa cyanea</i>	AF	2900–3500	7(0)	18(0)
<i>Diglossa cyanea</i>	SAF	1800–2600		3(0)
<i>Diglossa humeralis</i>	P	3900–4100		9(0)

Table 1 (continued)

	Life zone	Altitudinal range	N° cap (N° infected) per chain of mountains	
			Eastern	Central
<i>Diglossa humeralis</i>	AF	2900–3500		11(0)
<i>Diglossa sittoides</i>	SAF	1800–2600		6(0)
<i>Euphonia lanirostris</i>	SAF	1800–2600		3(0)
<i>Euphonia xanthogaster</i>	SAF	1800–2600		4(0)
<i>Hemispingus atropileus</i>	AF	2900–3500	4(0)	2(0)
<i>Hemispingus frontalis</i>	AF	2900–3500		1(0)
<i>Hemispingus sp.</i>	AF	2900–3500		1(0)
<i>Hemispingus supercilialis</i>	SAF	1800–2600		1(1)
<i>Hemispingus supercilialis</i>	AF	2900–3500	3(0)	1(0)
<i>Hemispingus verticalis</i>	AF	2900–3500	1(0)	
<i>Phrygilus unicolor</i>	P	3900–4100		64(0)
<i>Pipraeidea melanonota</i>	SAF	1800–2600		5(0)
<i>Saltator atripennis</i>	SAF	1800–2600		3(0)
<i>Saltator maximus</i>	SAF	1800–2600		1(0)
<i>Sericossypha albocristata</i>	SAF	1800–2600		5(0)
<i>Sporophila nigricollis</i>	SAF	1800–2600		14(0)
<i>Tangara arthus</i>	SAF	1800–2600		1(0)
<i>Tangara cyanicollis</i>	SAF	1800–2600		1(0)
<i>Tangara gyrola</i>	SAF	1800–2600		2(0)
<i>Tangara heinei</i>	SAF	1800–2600		1(0)
<i>Tangara labradorides</i>	SAF	1800–2600		1(0)
<i>Tangara nigroviridis</i>	SAF	1800–2600		2(0)
<i>Tangara vassorii</i>	AF	2900–3500		6(1)
<i>Tangara vitriolina</i>	SAF	1800–2600		6(0)
<i>Thraupis episcopus</i>	SAF	1800–2600		4(0)
<i>Thraupis palmarum</i>	SAF	1800–2600		2(0)
<i>Tiaris olivaceus</i>	SAF	1800–2600		2(0)
Troglodytidae				
<i>Henicorhina leucophrys</i>	SAF	1800–2600		7(0)
<i>Henicorhina leucophrys</i>	AF	2900–3500	3(0)	
<i>Pheugopedius genibarbis</i>	SAF	1800–2600		3(0)
<i>Pheugopedius mystacalis</i>	SAF	1800–2600		3(0)
<i>Troglodytes aedon</i>	P	3900–4100		2(0)
<i>Troglodytes aedon</i>	AF	2900–3500		2(0)
<i>Troglodytes aedon</i>	SAF	1800–2600		8(0)
<i>Troglodytes solstitialis</i>	SAF	1800–2600		1(0)
Turdidae				
<i>Catharus aurantirostris</i>	SAF	1800–2600		1(0)
<i>Catharus ustulatus</i>	SAF	1800–2600		1(0)
<i>Myadestes ralloides</i>	SAF	1800–2600		1(0)
<i>Turdus fuscater</i>	P	3900–4100	2(0)	2(0)
<i>Turdus fuscater</i>	AF	2900–3500		5(0)
<i>Turdus fuscater</i>	SAF	1800–2600		1(0)
<i>Turdus ignobilis</i>	SAF	1800–2600		14(0)
Tyrannidae				
<i>Contopus fumigatus</i>	SAF	1800–2600		1(0)
<i>Elaenia albiceps</i>	SAF	1800–2600		1(0)
<i>Elaenia frantzii</i>	SAF	1800–2600		1(0)
<i>Empidonax alhorum</i>	SAF	1800–2600		1(0)
<i>Empidonax sp.</i>	SAF	1800–2600		1(0)
<i>Mecocerculus leucophrys</i>	AF	2900–3500	3(0)	7(0)
<i>Mecocerculus leucophrys</i>	SAF	1800–2600		1(0)
<i>Mecocerculus</i>	AF	2900–3500	12(0)	
<i>stictopterus</i>				
<i>Mionectes olivaceus</i>	SAF	1800–2600		1(0)
<i>Mionectes striaticolis</i>	SAF	1800–2600		11(0)
<i>Mionectes striaticolis</i>	AF	2900–3500	2(0)	
<i>Myiarchus tuberculifer</i>	SAF	1800–2600		1(0)
<i>Myiophobus sp.</i>	SAF	1800–2600		1(0)
<i>Myiotheretes striaticollis</i>	AF	2900–3500	1(0)	
<i>Nephelomyias pulcher</i>	SAF	1800–2600		1(0)
<i>Ochthoeca</i>	AF	2900–3500	3(0)	3(0)
<i>cinnamomeiventris</i>				
<i>Ochthoeca diadema</i>	SAF	1800–2600		2(0)
<i>Ochthoeca diadema</i>	AF	2900–3500	4(0)	
<i>Ochthoeca fumicolor</i>	P	3900–4100		5(0)
<i>Ochthoeca rufipectoralis</i>	AF	2900–3500		1(0)

(continued on next page)

Table 1 (continued)

	Life zone	Altitudinal range	N° cap (N° infected) per chain of mountains	
			Eastern	Central
<i>Phyllomyias nigrocapillus</i>	SAF	1800–2600		1(0)
<i>Phyllomyias nigrocapillus</i>	AF	2900–3500	1(0)	
<i>Phylloscartes poecilotis</i>	SAF	1800–2600		2(0)
<i>Pyrrhomyias cinnamomeus</i>	SAF	1800–2600		4(0)
<i>Sayornis nigricans</i>	SAF	1800–2600		9(0)
<i>Serpophaga cinerea</i>	SAF	1800–2600		4(0)
<i>Sicalis flaveola</i>	SAF	1800–2600		3(0)
<i>Tyrannus melancholicus</i>	SAF	1800–2600		2(0)
<i>Uromyias agilis</i>	AF	2900–3500	6(0)	
<i>Uromyias agilis</i>	SAF	1800–2600		1(0)
<i>Zimmerius viridiflavus</i>	SAF	1800–2600		12(0)
Vireonidae				
<i>Cyclarhis nigrrostris</i>	SAF	1800–2600		1(0)
Piciformes				
Ramphastidae				
<i>Aulacorhynchus haematopygus</i>	SAF	1800–2600		1(0)
Picidae				
<i>Picumnus olivaceus</i>	SAF	1800–2600		1(0)
Trogoniformes				
Trogonidae				
<i>Trogon collaris</i>	SAF	1800–2600		1(0)
<i>Trogon personatus</i>	AF	2900–3500		1(0)
Total per locality			154(4)	686(6)
<i>Leucocytozoon</i> sp. prevalence			2.6%	0.9%
<i>Leucocytozoon</i> sp. nov. prevalence			0.6%	0.14%
<i>Leucocytozoon</i> sp. overall prevalence				1.19%
Total				840(10)

The number of birds capture per species followed of the occurrence of *Leucocytozoon* sp. (in parenthesis), is given. ^aHosts infected with *L. neotropicalis* sp. nov., * hosts infected with *L. grallariae* sp. nov. Life zone as follow Sub-Andean Forest (SAF); Andenan Forest (AF); Paramo (P); and altitudinal range data is also provided.

Paramo ecosystem (P) (3900–4100 masl) as represented by the Otún Lake. In the eastern mountain range (*Cordillera Oriental*) 154 birds, belonging to 42 species were captured during January of 2015 and December 2016 (Table 1) at the *Bosque Palacio de Chingaza* (NNP), which is an Andean Forest (2900–3500 masl) life zone.

2.2. Sampling and blood film examination

Birds were identified according to the taxonomic lists of the South American Classification Committee (SACC) (Remsen et al., 2012). Blood samples were collected by brachial or tarsal vein puncture or toenail clipping (last method for the tiny hummingbirds). For each bird, three thin smears were made, and 50 µl of blood were stored in an EDTA-anticoagulant solution or SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0). Blood films were air dried immediately in the field, and then fixed with absolute methanol and stained with 30% Giemsa solution in the laboratory according to Valkiūnas (2005). The smears were double-blind scanned by microscopic examination using an Olympus BX43 microscope, and digital images were captured with an Olympus DP27 digital camera, processed with cellSens software standard 1.13 (Olympus, Tokyo, Japan). For the morphological characterization of parasites, more than 100 images were taken, and the best images for morphometrical measurements were selected using ImageJ (Schneider et al., 2012), following the recommendations of Valkiūnas et al. (2010). The intensity of parasitemia was determined by an actual counting of the number of infected cells per 10,000

erythrocytes (Muñoz et al., 1999). A Student's t-test implemented in XLSTAT (Addinsoft, 2017) was used to determine statistical significance between the mean values of parasite morphometric measurements. A P-value of 0.05 or less was considered significant.

2.3. DNA extraction, PCR amplification and sequencing of cytochrome b gene and DNA mitochondrial genome

DNA extractions were carried out using a standard phenol–chloroform protocol (Sambrook et al., 1989). Cytochrome b gene (*cytb*) amplifications were done by using a nested PCR protocol (Hellgren et al., 2004). Purifications of PCR products were performed with ethanol and ammonium acetate protocol according to Bensch et al. (2000) and then visualized on a 1.5% agarose gel. All purified PCR products were subsequently sequenced in both senses using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Five independent amplifications were carried out for all available samples infected with the new *Leucocytozoon* species and made careful visual inspection of the electropherograms to confirm that the lineage sequences obtained were not chimeric products as a result of mixed infections. The *cytb* sequences obtained in this study were submitted to GenBank under accession numbers MH909275 (L_GRSQU_02) and MH909276 (L_PIRIE_02).

In addition to the *cytb* gene, two *Leucocytozoon* mitochondrial genomes (mtDNA) from one Undulated Antpitta (*Grallaria squamigera*, Grallariidae) and one Green-and-black Fruiteater (*Pipreola riefferii*, Cotingidae) were amplified, cloned and sequenced. PCR products were amplified with TaKaRa LA Taq™ Polymerase (TaKaRa Mirus Bio Inc., Shiga, Japan) as described by (Pacheco et al., 2011, 2018b) using 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. Then, a nested PCR was performed using the internal oligos forward 5' TTT CATCCTAAATCTCGTAAC 3'/reverse 5' GACCGAACCTTGACTCTT 3'. PCR amplifications for both PCR (outer and inner) were carried out in a 50 µl volume using 20 ng of total genomic DNA. The PCR conditions were as follow: 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 the manufacturer's directions, six independent PCR products (bands of approximately 6 kb) were excised from the gel, purified using QIAquick® Gel extraction kit (Qiagen, GmbH, Hilden, Germany), and four of them were cloned in the pGEM®-T Easy Vector systems (Promega, Madison, WI, USA) and two were directly sequenced. For at least three clones from each independent PCR and two PCR products, we sequenced both strands using an Applied Biosystems 3730 capillary sequencer. There were no inconsistencies among the clones and between the direct sequencing of the PCR products and clones. We submitted the mtDNA genome sequences to GenBank under accession numbers MK103894 and MK103895. In addition, following the above-mentioned methodologies, new DNA extractions and amplifications of the parasite *cytb* gene were performed using samples infected with *Leucocytozoon pterotenuis* (Blood film: GERPH07966- Blood sample: UNAL:GERPH:PA262), and a *Leucocytozoon* sp. (Blood film: GERPH-07737- Blood sample:UNAL:GERPH:AN18). These two parasites were previously detected and described by us in other species of the Grallariidae (*Grallaria ruficapilla* and *Grallaria quitensis* respectively; as *Leucocytozoon pterotenuis* (Lotta et al., 2015).

2.3.1. Phylogenetic analysis

First, phylogenetic relationships of the new *Leucocytozoon* species were estimated from an alignment using partial *cytb* gene sequences (476 base pairs (bp)). This alignment, constructed in MEGA 7 (Kumar et al., 2016) and aligned with Clustal Omega tool (McWilliam et al., 2013), included 88 lineages of parasites from passerine and non-passerine of South American birds and lineages belonging to unidentified morphospecies that had been deposited in the GenBank (Benson et al., 2015) and MalAvi database (Bensch et al., 2009), as well as the new

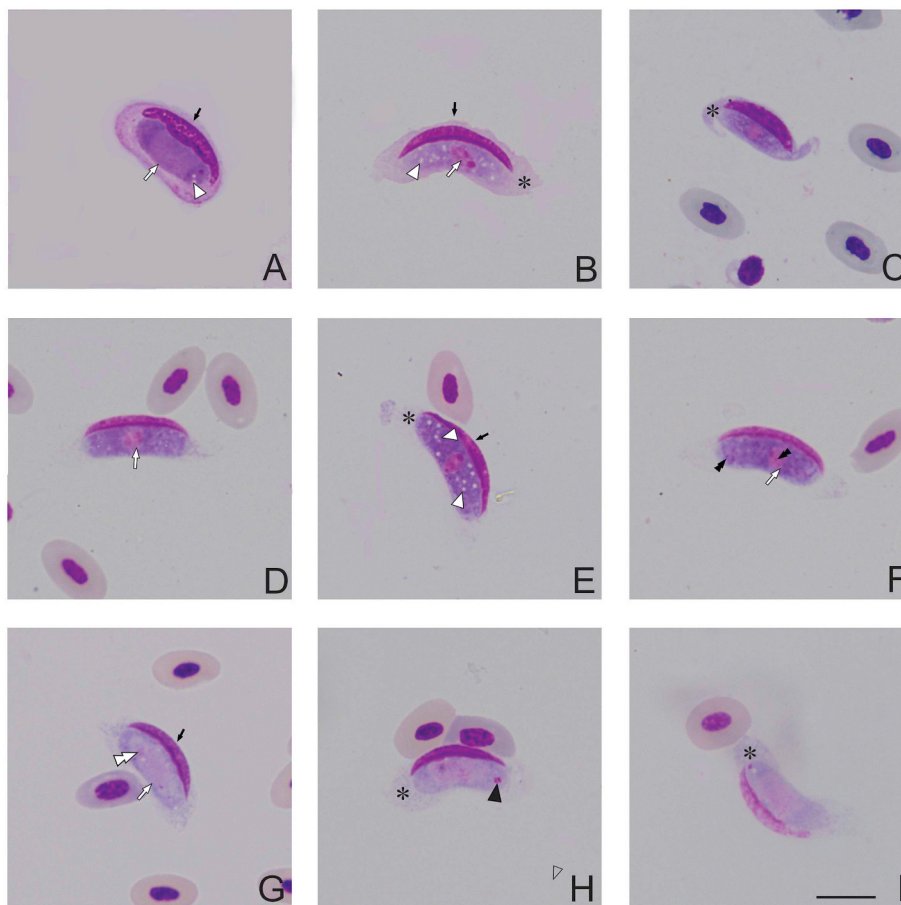


Fig. 1. *Leucocytozoon grallariae* sp. nov. from Undulated Antpitta (*Grallaria squamigera*) captured at Palacio forest in the Chingaza National Natural Park (NNP), Colombia. Immature gametocytes (A–C), macrogametocytes (D–F) and microgametocytes (G–I) in fusiform host cells. Nucleus of host cells (black arrows) possessing mature gametocytes assumes a slender waning moon shape (D–I). Parasite nuclei are indicated by white arrows (↖) and parasite nucleolus – by double white arrow tips (↗). Host cell cytoplasm is distorted by developing parasites forming a thin rim that develops into the cytoplasmic processes (asterisk *). In mature gametocytes, vacuoles are indicated by white arrow tips (↖). Volutin granules are indicated by double black arrow tips (↗) and azurophilic granule by black arrow tips (↘). Giemsa-stained thin blood films. Scale bar = 10 µm.

sequences reported in this study (Supplementary Table S1). A second alignment was done with only 28 *cytb* partial sequences (476 bp) including the partial *cytb* sequences available and the *cytb* from the mtDNA genomes obtained in this study. Finally, an alignment was done using 26 mtDNA genomes (5487 bp excluding gaps) in order to show the phylogenetic relationship between the new parasites that we found, and the ones reported previously. It is important to note that for both birds infected with the new *Leucocytozoon* sp., no inconsistencies between the mtDNA genomes clones and mtDNA genomes obtained by direct sequencing of PCR products were found.

In all the cases we performed phylogenetic reconstructions using Bayesian methods implemented on MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003), through the CIPRES portal (Miller et al., 2010). For partial *cytb* sequences and mtDNA genomes, phylogenetic relationships were estimated under the General Time-Reversible model (GTR + Γ + I), which was the best model that fit these data, according to the corrected Akaike information criterion implemented on jModelTest 2.1.1 (Darriba et al., 2012). Two independent Markov Chain Monte Carlo (MCMC) simulations were conducted simultaneously for 5×10^6 generations, sampled every 100 generations. After discarding 25% of the trees as a burn-in period, a majority rule consensus phylogeny was obtained from 75,000 trees. Then, the phylogeny was visualized and edited using FigTree v1.3.1 (Rambaut, 2006). We estimated genetic distances between lineages using a Kimura two-parameter model of substitution, implemented in MEGA 7.0 (Kumar et al., 2016).

2.4. Analysis of primers

Affinity of the primers proposed by Hellgren et al. (2004) with the *cytb* gene sequences obtained from *Leucocytozoon* parasites was evaluated by aligning the oligonucleotides with lineages of parasite species

with gametocytes developing in roundish host cells, like *Leucocytozoon fringillinarum* and *L. dubreuilii* (Perkins, 2008; Pacheco et al., 2018b) and lineages obtained from those parasites, which gametocytes develop in fusiform host cells, such as *L. pterotenuis* (Lotta et al., 2015) and the two species described in this study.

2.5. Ethical statement

Samples were collected using a non-invasive methodology, approved by the “Comité de Bioética of Departamento de Ciencias para la Salud Animal,” Facultad de Medicina Veterinaria y de Zootecnia, Universidad Nacional de Colombia (Permit number CBE-FMVZ-016). Bird capture and manipulation were done in a way that reduced stress caused by these activities. Once the blood samples were taken, the birds were released. Fieldwork were conducted under authorization of “Unidad Administrativa Especial del Sistema de Parques Nacionales Naturales de Colombia UAESPNN - Subdirección técnica” and “Autoridad Nacional de Licencias Ambientales, ANLA” (file 4120E183893 of 2011, resolution 0787 of 2013, and resolution 255 of 2014).

3. Results

3.1. Prevalence of infection and description of parasites

Leucocytozoon infections were detected in 10 birds of the 840 sampled (1.2%) by microscopic examination of blood smears. Molecular characterization was made only on the positive samples by microscopic examinations. Whilst gametocytes of *Leucocytozoon quynzae*, *L. fringillinarum* and *Leucocytozoon* sp. were observed in eight individuals belonging to Emberizidae, Parulidae, Thraupidae and Trochilidae (Table 1), two new morphologically readily distinct species of

Leucocytozoon were found in widely distributed South American native non-migrating passerine bird species (McMullan et al., 2011; BirdLife International, 2017a, 2017b): one Undulated Antpitta (*Grallaria squamigera*) and one Green and black Fruiteater (*Pipreola riefferii*) (Table 1).

Undulated Antpitta (Grallaridae) is distributed in the Andean mountain ranges above 2000 masl in Neotropical humid forests and scrublands of Bolivia, Peru, Ecuador, Colombia and Venezuela (BirdLife International, 2017a). In general, Antpitta birds are elusive, rarely seen and demanding in their capture and sampling. Green and black Fruiteaters (Cotingidae) are arboreal birds that inhabit tropical moist montane forests above 1000 to 3300 masl from Peru to Venezuela (BirdLife International, 2017b).

3.2. Description of parasites

3.2.1. *Leucocytozoon (Leucocytozoon) grallariae* sp. nov

Young gametocytes (Fig. 1) markedly influence the shape of host cells from earliest stages of their development. Growing parasites were of oval or ellipsoid shapes; they closely adhered to the host cell nuclei, which were markedly enlarged, deformed and assumed crescent shapes (Fig. 1A–C). The host cell cytoplasm was present around growing gametocytes, and it was very evident. Advanced young gametocytes often possessed invaginations on their sides, which were opposite to the host cell nuclei, and that gave the growing gametocytes the shapes of giant beans with approximately equally rounded ends (Fig. 1B). Host cells assumed ellipsoid shapes from early stages of gametocyte development (Fig. 1A).

Macrogametocytes developed in fusiform host cells (Fig. 1). In *Leucocytozoon* species, as the gametocytes develop, they cause considerable distortion of the host cells, producing two distinct host cell-parasite complex forms: roundish and fusiform. In this *Leucocytozoon* species, the gametocytes only in fusiform host cells were observed. Gametocytes induced marked hypertrophy and deformation of the host cells and displacement of their nuclei, which lay on the periphery of gametocytes. The host cell nuclei acquired slender waning moon shapes; usually extending up to ½ of the circumference of gametocytes, and they could reach the fusiform processes, but they never extended into the processes (Fig. 1C–E). In both types of host cells, the nuclei looked homogenous.

The host-cell cytoplasm forms two short cytoplasmic processes located close to ends of gametocytes. Fusiform processes were variable in form (Fig. 1D, F–I) nevertheless, those processes, which length was similar to their largest width were common (Table 2), and that was a characteristic feature of the development of this species. It is worth mentioning that the cytoplasmic processes were occasionally observed as being unequally long at both sides of the same parasite (Fig. 1H and I), probably as a consequence of the host cell deformation during blood film preparation. Some remnants of host cell cytoplasm were seen covering around half of the perimeter of the gametocyte-host cell complex as a thin rim (Fig. 1G–I), which is uncommon in avian leucocytozoids. Additionally, host cells with fully grown gametocytes could possess irregular shapes and blunt processes, while they were often pointed in host cells with immature growing gametocytes (Fig. 1G–I).

The gametocyte cytoplasm was granular in appearance; it often possessed small vacuoles and tiny volutin granules (Fig. 1E and F). Vacuoles were of different sizes, but not greater than 3.0 µm in their maximum diameter; they were observed in 97 gametocytes. Vacuoles were seen in growing (immature) gametocytes (Fig. 1B). Parasite nuclei were compact; they were seen mainly in the central position (67% of reported cases) in gametocytes and were of roundish (Fig. 1D–F) or various oval (Fig. 1E) shapes. Nucleoli were visible in 50% of the gametocytes.

The general configuration of the **Microgametocytes** and other features (Fig. 1G–I) were as for macrogametocytes with the usual haemosporidian sexual dimorphic characters that were the pale stained

Table 2

Morphometric parameters of gametocytes and host cells of *Leucocytozoon grallariae* sp. nov.

Feature	<i>Leucocytozoon grallariae</i> sp. nov	
	Macrogametocyte n = 15	Microgametocyte n = 10
<i>Parasite</i>		
Length	14.7–23.6 (20.2 ± 2.5)	15.8–21.3 (18.5 ± 2.1)
Width	4.5–5.5 (5.4 ± 0.5)	4.1–6.1 (5.1 ± 0.6)
Area	79.7–109.0 (97.4 ± 9.6)	71.3–93.4 (81.7 ± 6.0)
Perimeter	39.9–54.4 (47.1 ± 4.3)	39.6–49.3 (43.5 ± 3.4)
<i>Parasite nucleus</i>		
Length	2.7–4.9 (3.6 ± 0.6)	7.2–12.9 (10.0 ± 1.9)
Width	3.1–5.4 (4.4 ± 0.6)	3.0–4.5 (3.7 ± 0.4)
Area	9.9–18.0 (13.1 ± 2.2)	23.4–49.4 (32.8 ± 9.0)
Host-cell parasite complex		
Length	24.8–32.1 (29.6 ± 2.5)	24.7–33.9 (29.4 ± 2.7)
Width	7.3–10.3 (8.5 ± 1.0)	7.0–10.3 (8.4 ± 0.9)
Area	162.6–196.4 (185.1 ± 12.8)	152.2–188.1 (168.8 ± 11.0)
Host-cell nucleus		
Length	15.8–22.9 (21.1 ± 1.8)	17.5–21.6 (18.9 ± 1.2)
Width	1.5–3.6 (2.2 ± 0.5)	2.0–2.6 (2.3 ± 0.2)
Area	27.0–49.1 (32.6 ± 5.5)	25.7–34.8 (31.3 ± 2.3)
Perimeter of parasite covered	15.3–22.1 (21.1 ± 1.8)	17.2–20.5 (18.2 ± 1.1)
Cytoplasmic processes ^a		
Length	3.3–7.6 (5.0 ± 1.0)	3.8–7.8 (5.5 ± 1.3)
Width	3.7–8.0 (5.7 ± 1.0)	4.4–8.0 (5.8 ± 1.1)
Area	15.4–35.3 (24.1 ± 3.7)	20.0–35.3 (26.4 ± 4.5)

^a Measurements are given in µm or µm² (for area). Minimum and maximum values as well as mean ± SD are provided.

^a Only one of 2 cytoplasmic processes was measured for each parasite.

cytoplasm and large diffuse nuclei (Table 2). The proportion of microgametocytes and macrogametocytes in the type material was approximately 3:5. A prominent azurophilic granule was seen only in the cytoplasm of 90% of microgametocytes (Fig. 1H and I) located close to the parasite nuclei.

3.2.2. Taxonomic summary

Type host: Undulated Antpitta *Grallaria squamigera* (Grallaridae, Passeriformes).

Additional hosts: unknown.

Type locality: Palacio Forest at the transition zone of Chingaza NNP (4° 41' N, 73° 50' W, 2950 masl), Cundinamarca, Colombia.

Distribution: This parasite has been recorded only at the type locality.

Type specimens: Hapantotype (accession No. UNAL:GERPH:PA340 -XI, intensity of parasitemia 0.25%, collected by Nubia E. Matta, 18 January 2015) and deposited in the biological collection GERPH (Grupo de Estudio Relación Parásito Hospedero) at the Universidad Nacional de Colombia, Bogotá, Colombia. Parahapantotypes (accession Nos., UNAL:GERPH:PA340 – I to UNAL:GERPH:PA340 –X and UNAL:GERPH:PA340 –XII to UNAL:GERPH:PA340 –XIX) are deposited in the same collection. Digital images of blood stages of the parasite in the type preparations are available on request from GERPH.

DNA sequences: The partial mitochondrial DNA genome (5891 bp) that includes *cox1*, *cox3* and *cytb* genes (GenBank accession number MK103895) was obtained from the type host *Grallaria squamigera*.

Site of infection: Blood cells, of which the origin is unclear due to marked deformation caused by gametocytes.

Prevalence: Only one individual of the host species was collected and found to be infected, so the sample size does not allow to estimate of prevalence. The parasite was morphologically detected in 1 of out of 684 examined birds (0.12%). This or similar parasites were not found in 840 sampled birds of different species at Palacio Forest and Los Nevados NNP (Table 1).

Etymology: The species name refers to the host genus name *Grallaria*,

which is the type host of the parasite belongs.

3.2.3. Remarks

Seventeen species of *Leucocytozoon*, which gametocytes develop in fusiform host cells are reported to date (Supplementary Table S2), but only four of them, *Leucocytozoon maccluri* (Greiner, 1976), *Leucocytozoon balmorali* (Peirce, 1984), *Leucocytozoon hamiltoni* (Valkiūnas et al., 2002), and *Leucocytozoon pterotenuis* (Lotta et al., 2015) are described in birds of the.

Passeriformes. The new species described here, *Leucocytozoon grallariae*, found in a passerine bird, can be readily distinguished from the four *Leucocytozoon* species mentioned above due to two distinctive morphological characters. First, the fusiform processes are short (Table 2) in the host cells with fully grown gametocytes of *L. grallariae* (Fig. 1D–I). The length of these processes often does not exceed their largest width (Table 2, Fig. 1 G, H). This is not the case in *L. maccluri*, *L. balmorali*, *L. hamiltoni* and *L. pterotenuis*; their gametocytes develop fusiform host cells, which fusiform processes are greater in length than in largest width. Second, the host cell nuclei assume the slender waning moon shapes, and the nuclei could reach the cytoplasmic processes, but never extended into them. None of these two characters are features of *L. maccluri*, *L. balmorali*, *L. hamiltoni*, *L. pterotenuis* (see Valkiūnas, 2005; Lotta et al., 2015).

Gametocytes in many *Leucocytozoon* species described in non-passerine birds develop in fusiform host cells (Table S2). During *L. grallariae* infection, the host cell nuclei extend up to ½ of the circumference of the gametocytes, and the host cell nuclei might reach the beginning of cytoplasmic processes. This feature is not characteristic of other leucocytozoids where gametocytes developed fusiform host cells, and this feature can be used during identification of *L. grallariae*.

During microscopic examination of the type material, a co-infection with *Leucocytozoon* species with gametocytes developing in roundish host cells were detected (Fig. 3, Table S3). Gametocytes developing in fusiform host cells were ten times more often observed (parasitemia intensity is 0.25%) than gametocytes developing in roundish host cells (0.01%). In addition to the lineage associated with the GenBank No. MK103895 for the partial mtDNA genome reported above, one *cytb* lineage of 476 bp (L_GRSQU 02 GenBank No. MH909275) was amplified from the same blood sample of this type host. Genetic distance between both lineages was 0.23 (Table 5), which suggest that they belong to a different species. However, from these two lineages obtained in the same sample, we propose that lineage MK103895 obtained by cloning corresponds to the parasite, which gametocytes develop in fusiform host cells in the sample (see discussion below).

3.2.4. *Leucocytozoon (Leucocytozoon) neotropicalis* sp. nov

Macrogametocytes (Fig. 2A–E) develop in fusiform host cells; the shape of gametocytes is oval-elongate (their length is greater than width, Table 3). However, the morphology of host-parasite complexes with fusiform processes is readily distinguishable from *L. grallariae* (compare Fig. 1 F, H and Fig. 2 A, F, I). The host cell nuclei are pushed aside, deformed like a homogeneous band of variable width that extends close to half of the circumference of gametocyte (Fig. 2 A, I), but never extends into the cytoplasmic processes. This is similar in both *L. grallariae* and *L. neotropicalis* cells, so these parasites cannot be distinguished by this character (see the description of *L. grallariae* and compare Fig. 2 D, F, H and Fig. 2 A, G, I.). These species can be readily distinguished due to the length of the cytoplasmic processes. Mainly, the latter is significantly longer (Student's t-test, $\alpha = 0.05$ $P < 0.0001$) and narrower in their maximum width ($P < 0.0001$) than in *L. grallariae* (see Table 2, compare Fig. 2. F, H and Fig. 2 A, F, I).

Two long thin fusiform spindle-shaped processes of the host cells' cytoplasm reach up to 14 μm (Table 3), and that never is observed in *L. grallariae*. The length of the cytoplasmic processes can be different in the same host-parasite complex (Fig. 2J and K), and that probably is a result of deformation during the preparation of blood films.

Cytoplasmic processes are thin and often flattened appearing like a ribbon (Fig. 3F–H). It is important to note that mature gametocytes often have a flattened form on the side, located on the opposite side of the host cell nuclei (Fig. 2 H, J–K). This character is not observed in *L. grallariae* (Fig. 1I–K).

In the type material, we observed tiny volutin granules and small vacuoles (up to 0.47 μm in diameter) in 47.9% of fully grown gametocytes (Fig. 2A–E). The parasite nucleus was roundish in 49.3% of 103 observed gametocytes (Fig. 2A–C) or elongated (Fig. 2 E); its position was mainly more or less central, but sometimes was off-centre. The nucleolus was variable both in shape and position, being visible in 53.2% of 103 observed parasites (Fig. 2 B, D).

Microgametocytes (Fig. 2F–I): General configuration and other features were similar to the macrogametocytes with the usual haemosporidian sexual dimorphic characters. The proportion of microgametocytes and macrogametocytes in the type material was approximately 1:2.

Taxonomic summary.

Type host: Green-and-black Fruiteater *Pipreola riefferii* (Cotingidae, Passeriformes).

Additional hosts: unknown.

Type locality: El Bosque, Los Nevados National Natural Park (NNP) (4° 43' N; 75° 27' W, 3150 masl), Risaralda, Colombia.

Type specimens: Hapantotype (accession Nos. UNAL:GERPH:OT1354-II). The intensity of the infection of the lineage MK103894 is 0.33%, it was collected by Melisa Galarza (27 December 2015) and deposited in the biological collection GERPH (Grupo de Estudio Relación Parásito Hospedero) at the Universidad Nacional de Colombia, Bogotá, Colombia. Parahapantotypes (accession Nos. UNAL:GERPH:OT1354-I, UNAL:GERPH:OT1354-III, other data as for the hapantotype) are deposited in the same collection. Digital images of the blood stages of the parasite in the type preparations are available on request from GERPH.

Partial mitochondrial DNA genome (5811 bp) that includes *cox1*, *cox3* and *cytb* genes (GenBank accession number MK103895) was obtained from the type host *Pipreola riefferii*.

Site of infection: Blood cells, the specific cell is unknown due to the marked deformation by developing gametocytes.

Prevalence: Only one individual of the host species was collected and found infected, so the sample size does not allow to estimate the prevalence. Parasite was detected by microscopy in 1 of out of 684 examined birds (0.12%). In the type locality, 1 of 686 birds captured at Los Nevados NNP (0.14%) was infected, as determined by microscopic examination.

Etymology: The species name (*neotropicalis*) was derived from the name of the zoogeographical region where this parasite was found.

3.2.5. Remarks

Leucocytozoon neotropicalis is one of the six *Leucocytozoon* species that parasitize passerine birds and possess gametocytes developing in fusiform host cells. The main differences between *L. neotropicalis* and *L. grallariae* are specified in the description of the former parasite. Both of these new leucocytozoids have gametocytes developing fusiform host cells, which can be distinguished from other leucocytozoids due to the unique shape of their host cell nuclei (see Remarks on *L. grallariae*).

Due to the presence of long and narrow cytoplasmic processes in host cells, *L. neotropicalis* is similar to *L. lovati* (Valkiūnas, 2005) and *L. eurytomi* (Bennett et al., 1993; Valkiūnas, 2005) (Table 3). However, the nuclei of host cells never reach the cytoplasmic processes in the last two parasites. Because of this character, these species can be readily distinguished.

Microscopic examination of blood smears from type series revealed the presence of co-infection of a parasite with gametocytes developing in roundish host cells. Overall, the configuration of the nuclei in roundish host cells resembles the same characters observed in the *L. fringillinarum* group (Fig. 3.). The reported gametocytes in roundish host

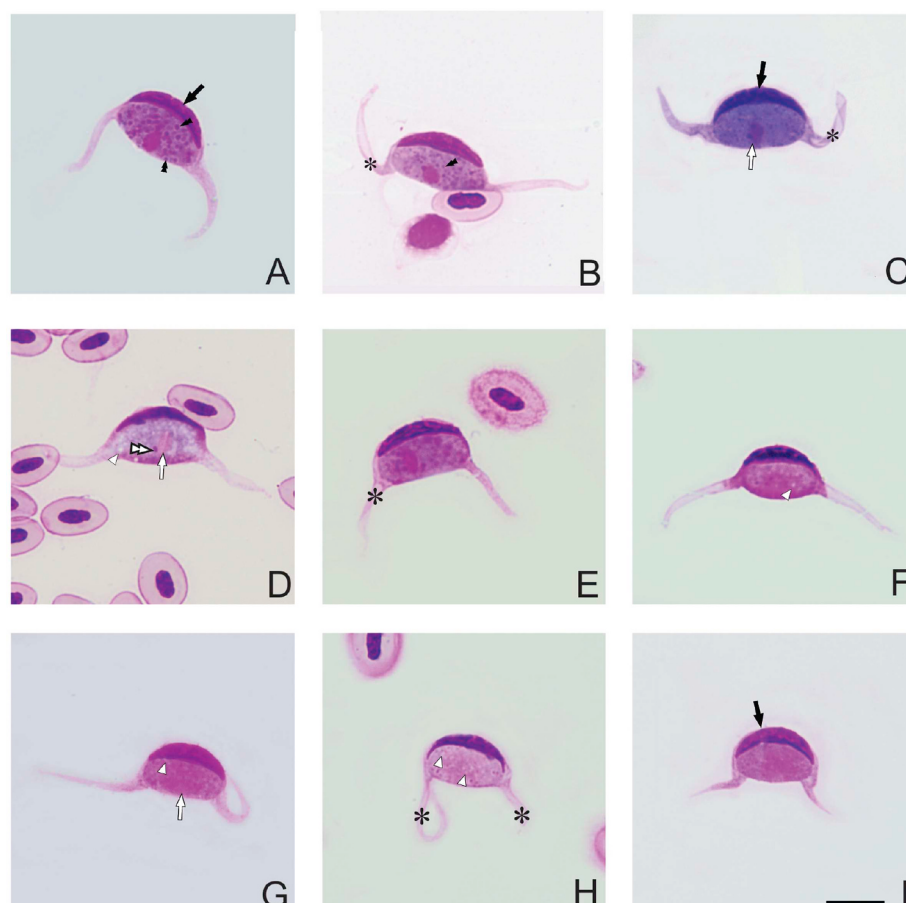


Fig. 2. *Leucocytozoon neotropicalis* sp. nov. from the peripheral blood of its type vertebrate host Green-and-black Fruiteater (*Pipreola riefferii*) captured at Los Nevados NNP, Colombia. Macrogametocytes (A–E) and microgametocytes (F–I). Black arrows (↑) indicate the deformed host cell nuclei. Parasite nuclei are indicated by white arrow (↗) and nucleoli are shown by double white arrowtips (↖). Volutin granules are indicated by double black arrowtips (↘) and vacuoles – by white arrowtips (↗). Uneven cytoplasmic processes may acquire a ribbon-like appearance (asterisk *). Giemsa-stained thin blood films. Scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cells are bigger than those observed in the sample with coinfection with *L. grallariae* (Student's t-test for parasite area: $p < 0.0001$, $\alpha = 0.05$, Table S3). In contrast to the macrogametocytes of *L. neotropicalis*, the volutin granules are not pronounced or absent in roundish gametocytes of this parasite (Fig. 3D–F). It worth mentioning that, for both new species as well as for *L. pterotenuis* (Lotta et al., 2015), the gametocytes developing in fusiform host cells were the most common, and their

parasitemia was on average ten to seventeen times higher than the species with gametocytes developing roundish host cells. Indeed, in the type material of *L. neotropicalis* parasitemia of gametocytes developing fusiform host cells was 0.21%, while it did not exceed 0.01% for gametocytes developing roundish host cells.

Two distantly related lineages with a genetic distance of 0.29 between them (L_PIRIEF_01, *cytb* gene GenBank No. MH909276 and

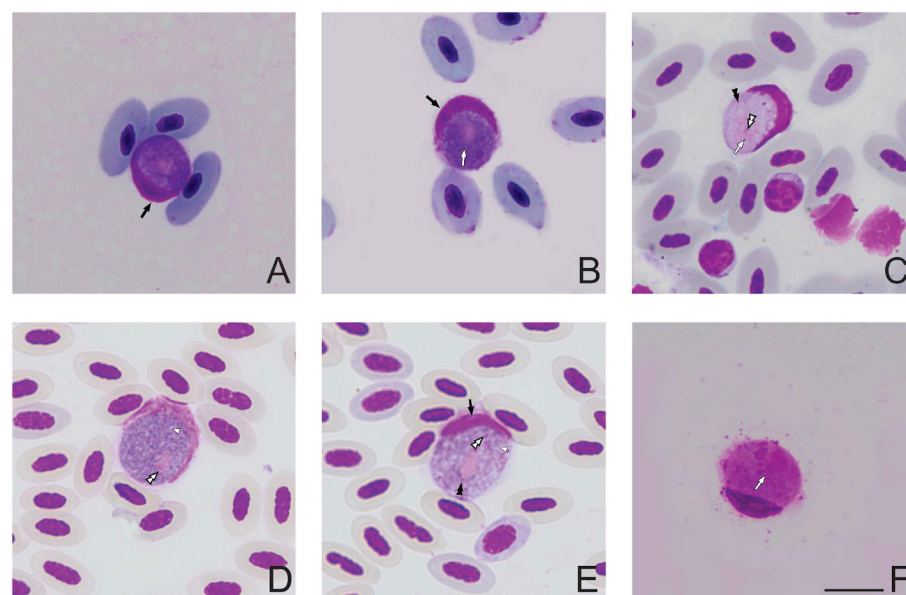


Fig. 3. Gametocytes developing roundish host cells observed in type material of *Leucocytozoon grallariae* (A–C) and *Leucocytozoon neotropicalis* (D–F). Host cell nucleus (black arrows ↑) deformed as a cap resembles the parasites of the *Leucocytozoon fringillinarum* group. The white arrow indicates the parasite nuclei (↗), double white arrow tips shows the nucleolus (↖). Vacuoles (white arrow tips ↗) are present, while volutin granules (double black arrow tips ↘) are few or absent in some gametocytes. Giemsa-stained thin blood films. Although gametocytes in roundish host cells were observed in both samples, those co-existing with *Leucocytozoon grallariae* (A–C), are significantly smaller than *Leucocytozoon* sp. gametocytes found in type host of *L. neotropicalis* (D–F) (see Table S3). Scale bar = 10 μm.

Table 3

Morphometric parameters of gametocytes and host cells of *Leucocytozoon neotropicis* sp. nov. Measurements of *Leucocytozoon eurytomi* and *Leucocytozoon lovati* are provided for comparison. Measurements are given in μm or μm^2 (for area). Minimum and maximum values as well as mean $\pm$ SD are provided.

Feature	<i>Leucocytozoon neotropicis</i> sp. nov.		<i>Leucocytozoon eurytomi</i> ^{a,b}	<i>Leucocytozoon lovati</i> ^b
	Macrogametocyte n = 6	Microgametocyte n = 1		
<i>Parasite</i>				
Length	14.4–16.0 (15.3 $\pm$ 0.6)	11.4–17.9 (13.6 $\pm$ 1.5)	22.6–29.6 (25.2 $\pm$ 1.3)	14.1–22.0 (17.5 $\pm$ 1.4)
Width	7.0–8.2 (7.8 $\pm$ 1.0)	5.7–10.0 (7.8 $\pm$ 1.4)	7.0–12.2 (8.1 $\pm$ 1.0)	6.7–13.1 (11.0 $\pm$ 0.7)
Area	86.1–101.2 (95.2 $\pm$ 4.9)	64.9–114.9 (83.7 $\pm$ 17.4)	(182 $\pm$ 18.5)	
Perimeter	34.1–40.9 (38.8 $\pm$ 1.6)	31.4–48.9 (37.4 $\pm$ 4.8)	(63.0 $\pm$ 5.9)	
<i>Parasite nucleus</i>				
Length	2.0–3.1 (2.8 $\pm$ 0.5)	5.3–10.5 (8.0 $\pm$ 1.5)	2.8–6.4 (4.1 $\pm$ 0.4)	3.2–6.8 (4.2 $\pm$ 0.3)
Width	3.3–4.1 (4.0 $\pm$ 0.4)	4.4–7.0 (5.6 $\pm$ 0.8)	1.4–5.7 (3.5 $\pm$ 0.3)	1.8–4.4 (3.6 $\pm$ 0.3)
Area	7.0–9.6 (8.6 $\pm$ 1.0)	32.5–48.4 (41.4 $\pm$ 4.7)	(11.8 $\pm$ 2.6)	
<i>Host-cell parasite complex</i>				
Length	35.5–56.6 (44.7 $\pm$ 5.3)	29.8–55.8 (41.4 $\pm$ 8.6)	(39.2 $\pm$ 6.3)	
Width	9.5–10.8 (10.2 $\pm$ 0.5)	9.2–10.8 (9.9 $\pm$ 0.5)	(9.1 $\pm$ 0.7)	
Area	161.0–200.1 (181.8 $\pm$ 10.4)	127.4–181.7 (151.2 $\pm$ 13.0)	(202.2 $\pm$ 21.1)	
<i>Host-cell nucleus</i>				
Length	14.8–19.7 (17.7 $\pm$ 1.8)	14.2–18.3 (16.3 $\pm$ 1.3)	18.3–26.0 (21.7 $\pm$ 2.3)	9.8–16.3 (13.5 $\pm$ 1.5)
Width	2.4–3.7 (2.9 $\pm$ 3.0)	2.4–3.7 (3.0 $\pm$ 0.4)		
Area	28.2–39.6 (35.7 $\pm$ 4.3)	23.6–42.0 (33.6 $\pm$ 5.3)	(39.0 $\pm$ 7.2)	
Perimeter of parasite covered	14.1–18.2 (16.5 $\pm$ 1.8)	14.2–18.3 (16.3 $\pm$ 1.3)	(16.9 $\pm$ 2.3)	
<i>Cytoplasmic processes</i> ^c				
Length	11.3–22.0 (15.7 $\pm$ 2.8)	7.6–24.4 (14.8 $\pm$ 4.5)		
Width	2.6–5.4 (3.8 $\pm$ 0.8)	1.9–5.3 (3.7 $\pm$ 0.9)		
Area	18.2–29.0 (25.0 $\pm$ 5.3)	14.4–26.1 (21.2 $\pm$ 3.2)		

^a According to Bennet et al., (1993).

^b According to Valkiūnas (2005).

^c Only one of 2 cytoplasmic processes was measured for each parasite.

partial mtDNA genome that included *cytb*, *cox1*, *cox3*, GenBank No. MK103894), were amplified from the same sample, which makes it difficult to link the lineages with their morphotypes. Based on phylogenetic analysis, we suggest that the last one lineage (GenBank No. MK103894) corresponds to *L. neotropicis* n. sp. (see discussion below).

3.3. Sequencing of the cytochrome b gene and the DNA mitochondrial genome

Two lineages were isolated from each of the samples containing *L. grallariae* or *L. neotropicis*. The partial *cytb* fragments obtained using the primers suggested by Hellgren et al. (2004) were very distant from the *cytb* lineages obtained by the mtDNA genome amplification protocol (Pacheco et al., 2011, 2018). In other words, different *Leucocytozoon* parasite sequences were obtained in the same sample using different protocols and that corresponded to the microscopic observation of possible co-infections in these samples.

Even though both protocols for the amplification of *cytb* fragments and the mtDNA genome were run at least two times independently, each protocol amplified different lineages (lineages isolated from *G. squamigera*: GenBank *cytb* accession No. MH909275 vs GenBank mtDNA accession No. MK103895; lineages isolated from *P. riefferii*: GenBank *cytb* accession No. MH909276 vs GenBank mtDNA accession No. MK103894). Similar results were obtained when new molecular studies were performed with the Grallariidae bird samples reported by Lotta et al. (2015), where parasites were described as *Leucocytozoon pterotenuis*. With the new analysis, we realized that the *cytb* fragment obtained along with the partial mitochondrial genomes (mtDNA) identified with GenBank accession No. KM272250 and the short *cytb* fragment amplified with Hellgren's primers identified with GenBank accession No. KY646032 were different. Thereby, we will be referring to the description of *Leucocytozoon pterotenuis* (Lotta et al., 2015) as a partial description of the parasite (according to the (International Commission on Zoological Nomenclature, 1999) the description “in part”), because according to the molecular analyses performed, gametocytes in roundish host cells observed in the sample likely do not belong to *L. pterotenuis*, but to other *Leucocytozoon* species (see

discussion). Thus, the species name *L. pterotenuis* is valid only in part, mainly for gametocytes developing in fusiform host cells, but not to gametocytes in roundish host cells.

3.4. Analysis of primer affinities

The success of amplification is highly dependent on the primer's affinity for the target sequence and the parasitemia that determines the amount of parasite present DNA (Perez-Tris and Bensch, 2005; Pacheco et al., 2018a). After verification of affinities of the primers proposed by Hellgren et al. (2004) with the all complete *cytb* gene sequences of *Leucocytozoon* parasites available for passerine birds, it was noticed that oligo-sequences matched the *cytb* sequences of *L. fringillinarum* (Genbank accession No. KY653765) and *L. dubreuilii* (Genbank accession No. KY653795), which both have gametocytes developing roundish host cells (Fig. 4 A, B). In contrast, we noticed that the primer HaemR2L did not completely match with the mtDNA sequences obtained using the mtDNA genome amplification protocol used for *L. pterotenuis* (in part) (Genbank No. KM610046). Indeed, the base pairs at the 3' end of the primer, as well as the two last base pair of the 5', did not match with the *cytb* gene sequences obtained from the mtDNA genome of *L. grallariae* (Genbank No. MK103895) nor *L. neotropicis* (Genbank No. MK103894) (Fig. 4).

3.5. Phylogenetic analysis

In the phylogenetic reconstructions based on partial mitochondrial genomes and 476 *cytb* fragments (Fig. 5 and S1), two main clades that resemble the classification of parasites according to morphological features were observed. Thus, parasite lineages of leucocytozoids with gametocytes developing round host cells were part of a separate clade (Fig. 5 and S1 clade I). An exception is *L. danilewskyi*, in which the gametocytes develop both roundish and fusiform host cells. Meanwhile, parasites that produce gametocytes in fusiform cells are part of a separate monophyletic group (identified as clade II). Within this, lineages Genbank No. MK103894 of *L. neotropicis* and Genbank No. MK103895 of *L. grallariae* samples form a clade that is the sister lineage to *L.*

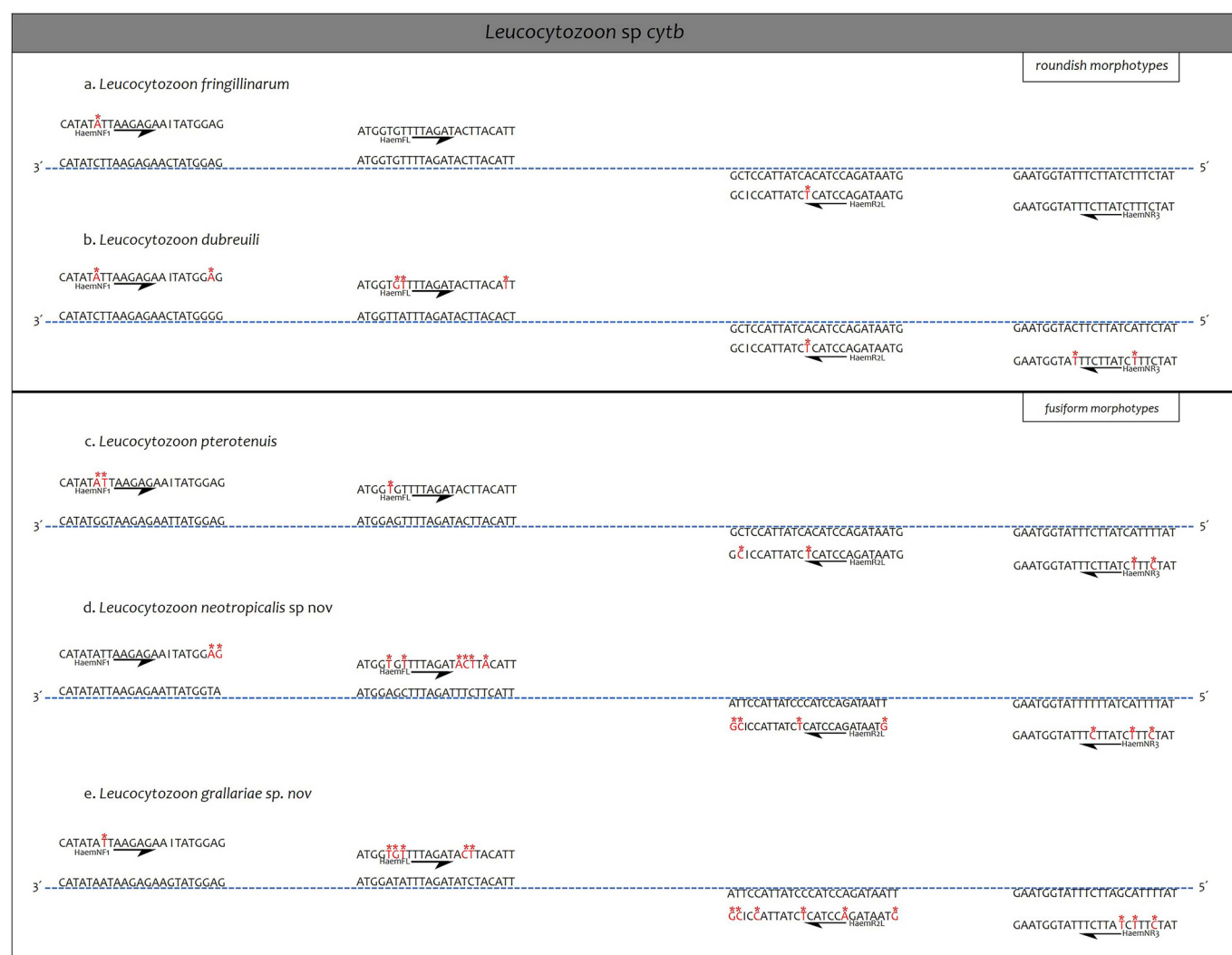


Fig. 4. Primer affinity analyses of the primers suggested by Hellgren et al. (2004). Parasites with gametocytes developing roundish host cells are: (a) *L. fringillinarum*, and (b) *L. dubreuilii*; and parasites with fusiform host cell are (c) *L. pterotenuis* (in part), (d) *L. neotropalis* sp. nov., and (e) *L. grallariae* sp. nov. An asterisk over the base pair highlights mismatches between the sequences and the primers. Note that primers HaemNR3 and HaemR2L are presented in 3'-5' sense to fit the parasite sequences.

pterotenuis (in part) (KM272250) and *Leucocytozoon* sp. (KM272251). These parasites are closely related to *L. sabrazei* (a morphological synonym of *Leucocytozoon macleani*, AB299369), a parasite infecting Galliformes birds, in whose gametocytes develop in fusiform host cells (Fig. 5A, Table 4). Thus, the phylogenetic analyses suggested a link between the parasite morphotypes and their sequences for both samples with co-infection.

It is worth noting that, since parasite mitochondrial genomes (mtDNA) corresponding to the partial *cytb* fragments of the MH909275 and MH909276 sequences could not be amplified, they were not included in the phylogenetic hypothesis constructed with mtDNA (Fig. 5A).

Interestingly, in clade I, the partial *cytb* lineages MH909275, MH909276 and KY646032 obtained from samples of *L. neotropalis*, *L. grallariae* and *L. pterotenuis* (in part) respectively using the primers proposed by Hellgren et al. (2004) share a recent common ancestor with *L. fringillinarum* and *L. quynzae* (Fig. 5B and S1). This suggests that these lineages likely correspond to the roundish host cell morphotype coexisting with the fusiform host cell morphospecies present in the samples infected with *L. neotropalis*, *L. grallariae* and *L. pterotenuis* (in part). On the other hand, *cytb* fragments obtained from mtDNA genome lineages KM272250, MK103894 and MK103895 form a monophyletic

group (Fig. 5B clade II) that is a sister clade of parasites developing roundish host cells plus *L. sabrazei* (synonym of *Leucocytozoon macleani*) (AB299369) and *L. danilewskyi* (KY653781) (Fig. 5B clade II). Both *Leucocytozoon sabrazei* and *L. danilewskyi* are parasites with gametocytes that develop both roundish and fusiform host cells.

Phylogenetic relationships of parasite lineages with sequences isolated from South American birds are depicted in Supplementary Figure S1 (see also Supplementary Table S1). It is noteworthy that the *cytb* lineage L_GRSQU_02 (Genbank No. MH909275) obtained by PCR from the sample infected with *L. grallariae* was placed in a well-supported clade along with the lineage KY646032 isolated from the type material of *L. pterotenuis* (in part) (Fig. S1, clade E). Within the clade E, a partial *cytb* sequence of *L. quynzae* and the lineage KF874769 obtained from a Peruvian *Grallaria erythroleuca* specimen were included. Genetic distances between the isolate L_GRSQU_02 (Genbank No. MH909275) and the lineages of parasites previously reported in other species of Grallariidae (KY646032, KY646033, and KF874764) were 0.05, and 0.06 respectively; while it was 0.05 for *L. quynzae* (Fig. S1, Table 5). Furthermore, the lineage L_PIRIE_02 (Genbank No. MH909276) fell into a clade composed by parasites infecting other species of *Pipreola* (Fig. S1, Clade IB). Genetic distances between this lineage and the Peruvian lineages isolated from *Pipreola* (*P.*) *intermedia* (Genbank accession No.

KF874740, KF874814) and *Pipreola arcuata* (Genbank accession Nos. KF874701, KF874796) ranged between 0.01 and 0.03 (clade B).

Consistently with phylogenetic reconstructions performed with mtDNA genome, lineages MK103895 and MK103894 fell into a clade that included *L. sabrazei* and *L. pterotenuis* (in part) (Fig. 5A, clade II). The genetic distance between the mtDNA genome lineage MK103894 of *L. neotropicalis* and its sister taxa (KM272250) was 0.25, while the genetic distance of lineage MK103895 *L. sabrazei*, *L. pterotenuis* (in part) and MK103894 were 0.21, 0.17, and 0.25 respectively. The large genetic distances estimated using both the partial mtDNA and the *cytb* sequences obtained from the new species as well as *L. pterotenuis* (in part) using the methodologies above mentioned are reported in Table 5.

4. Discussion

The combined use of information obtained from morphological and molecular characterizations provided opportunities to distinguish and describe two new parasite species *L. neotropicalis* and *L. grallariae*. Gametocytes of both species develop fusiform host cells that are quite scarce in the Neotropical leucocytozooids (Lotta et al., 2016, 2015) and are rare in leucocytozooids parasitizing Passeriformes birds (Valkiūnas, 2005). Nevertheless, the standard PCR protocols used to diagnose and characterize the leucocytozooids (i.e. Bensc et al., 2000; Hellgren et al., 2004; Perkins and Schall, 2002) likely underestimate the *Leucocytozoon* diversity. The mispriming found in this study explain how such protocols failed to detect parasite lineages that, in this case, seem to be endemic from the Neotropical region. Besides other factors that cause a sub-estimate of *Leucocytozoon* parasite prevalence and diversity are the sampling bias of avian hosts (most of them belong to orders Passeriformes or Apodiformes), as well as that some studies rely solely on molecular methods and did not use microscopic examination for the parasite detection (i.e., Galen and Witt, 2014; Harrigan et al., 2014).

4.1. Prevalence of *Leucocytozoon* parasites

In the Neotropics, the overall percentage of naturally infected birds with *Leucocytozoon* parasite is generally low (e.g., Harrigan et al., 2014; Lotta et al., 2016; Fecchio et al., 2018). Indeed, the above-cited studies report 0.06%, 1.16%, 4.6% respectively. The percentage found in this study used microscopy solely so chronic non-patent infections could be overlooked by this methodology. However, the percentage obtained (1.2%) was similar to the ones reported in the two studies mentioned above, where molecular detection of all samples was performed. However, this value is lower when it was compared with other prevalence data obtained in other zoogeographical regions (e.g. 16.2% in the Holarctic region, see Valkiūnas, 2005). New molecular protocols together with microscopy may change our perspective of how frequently these parasites are found in the neotropics. In the avian species studied in Colombia, there is marked variation in the frequency of *Leucocytozoon* parasites' found across different families. For example, all Grallariidae species studied so far are infected (100%), followed by Thraupidae (29.2%), Emberizidae (19.4%) and Turdidae (7.9%) (Lotta et al., 2016).

In this study, 4 out of 4 examined grallariids were infected. Although the sample size is small, we can speculate that these birds are highly susceptible to *Leucocytozoon* infection. Indeed, different *Leucocytozoon* species were found in these birds even in sympatric transmission, as is the case of *L. pterotenuis* (in part) in *Grallaria ruficapilla* described by (Lotta et al., 2015) and *L. grallariae* (*Grallaria squamigera*) which showed high genetic diversity (Table 5) and distinct morphological differences (Figs. 1 and 2; see also Fig. 1 in Lotta et al., 2015). An ecological aspect that can drive this feature is the preference of these birds to inhabit areas and build their nest near streams and small brooks (Stiles and López, 1995; Londoño et al., 2004), being them a readily available blood source for simuliids, their parasite vectors. Simuliids also have preferences for running and clean waters (Coscarón

and Coscarón-Arias, 2007).

4.2. Analysis of primer affinities

With the affinity analysis of the primers proposed by Hellgren et al. (2004) (Fig. 4), we determine that those primers can amplify *Leucocytozoon* parasites with gametocytes that developed round host cells, like *L. fringillinarum* and *L. dubreuilii*. Also, different studies have proved their efficiency in the detection of other leucocytozooids with gametocytes in fusiform host cells, such as *L. buteonis* (Krone et al., 2008), *L. danilewskyi* (Ortego and Cordero, 2009), and *L. simondi* (Smith and Ramey, 2015). However, these primers did not match properly with the *cytb* sequences of *L. pterotenuis*, *L. grallariae*, and *L. neotropicalis*; which gametocytes develop in fusiform host cells recently described in the Neotropics. One possible explanation for these results may be due to the design of the Hellgren et al. (2004) primers, particularly the primer HaemR2L, since only the *cytb* sequences of *L. dubreuilii* and *L. simondi* were available at that time (Perkins and Schall, 2002).

It is worth mentioning that despite of microscopic report of two different morphologies of *Leucocytozoon* parasites in blood films of type samples of *L. neotropicalis*, *L. grallariae* and *L. pterotenuis* (in part, previously described by Lotta et al., 2015), the presence of co-infections were confirmed only when molecular analyses (partial *cytb* and mitochondrial DNA genome amplification) were performed in parallel.

None of the partial *cytb* lineages obtained by mtDNA amplification could be amplified using the primers and protocols suggested by Hellgren et al. (2004). That calls for the development of a new set of primer for nested PCR-based methods diagnosis of avian leucocytozooids. The new mtDNA genomes sequences obtained in this and previous studies conducted in the Neotropics (i.e., Matta et al., 2014; Lotta et al., 2016; Pacheco et al., 2018b) and now available in public databases can be helpful for the design of a new set of primer and PCR protocols.

4.3. Diversity of *Leucocytozoon* lineages and primers used

Two different parasite lineages were amplified in each sample where a new parasite species was described, and that was consistent with microscopic examination. The genetic distances between the *cytb* fragments obtained by direct sequencing of the PCR product and the cloned mitochondrial lineages ranged from 0.20 to 0.29 (Table 5), indicating that two different species were co-infecting each sample. Similar to this, after the publication of *L. pterotenuis* (in part, Lotta et al., 2015), the authors found that *cytb* lineages isolated from *Grallaria ruficapilla* and *Grallaria quitensis* (obtained using the Hellgren's protocol, Hellgren et al., 2004) and used for the reconstruction of phylogenetic relationships were different from those obtained using the protocol for the parasite mtDNA genome amplification. Nevertheless, both sets of lineages - partial mtDNA genomes (GenBank accession numbers KM610045 and KM610046) and partial *cytb* genes (GenBank accession numbers KY646032 and KY646033) are true lineages. The last one we presume belongs to a morphotype also present in co-infection with *L. pterotenuis* (in part, Lotta et al., 2015).

The presence of parasites with gametocytes developing fusiform and roundish host cells in the smears does not always implicate a co-infection. Indeed, Desser (1967) proved that *Leucocytozoon simondi* has gametocytes that develop both roundish and fusiform host cells depending on the stage of exo-erythrocytic development. Mainly, merozoites from the hepatic meronts and megalomeronts produced gametocytes that develop in roundish and fusiform host cells, respectively. Nevertheless, in all our phylogenetic reconstructions, partial *cytb* fragments fell into a different clade to those *cytb* sequences derived from the mtDNA sequences. Furthermore, the microscopic examination revealed that fusiform host cells were more often seen than roundish ones in all the three samples where we have found new *Leucocytozoon* species. For example, in *G. ruficapilla*, parasitemia of *L. pterotenuis* gametocytes in

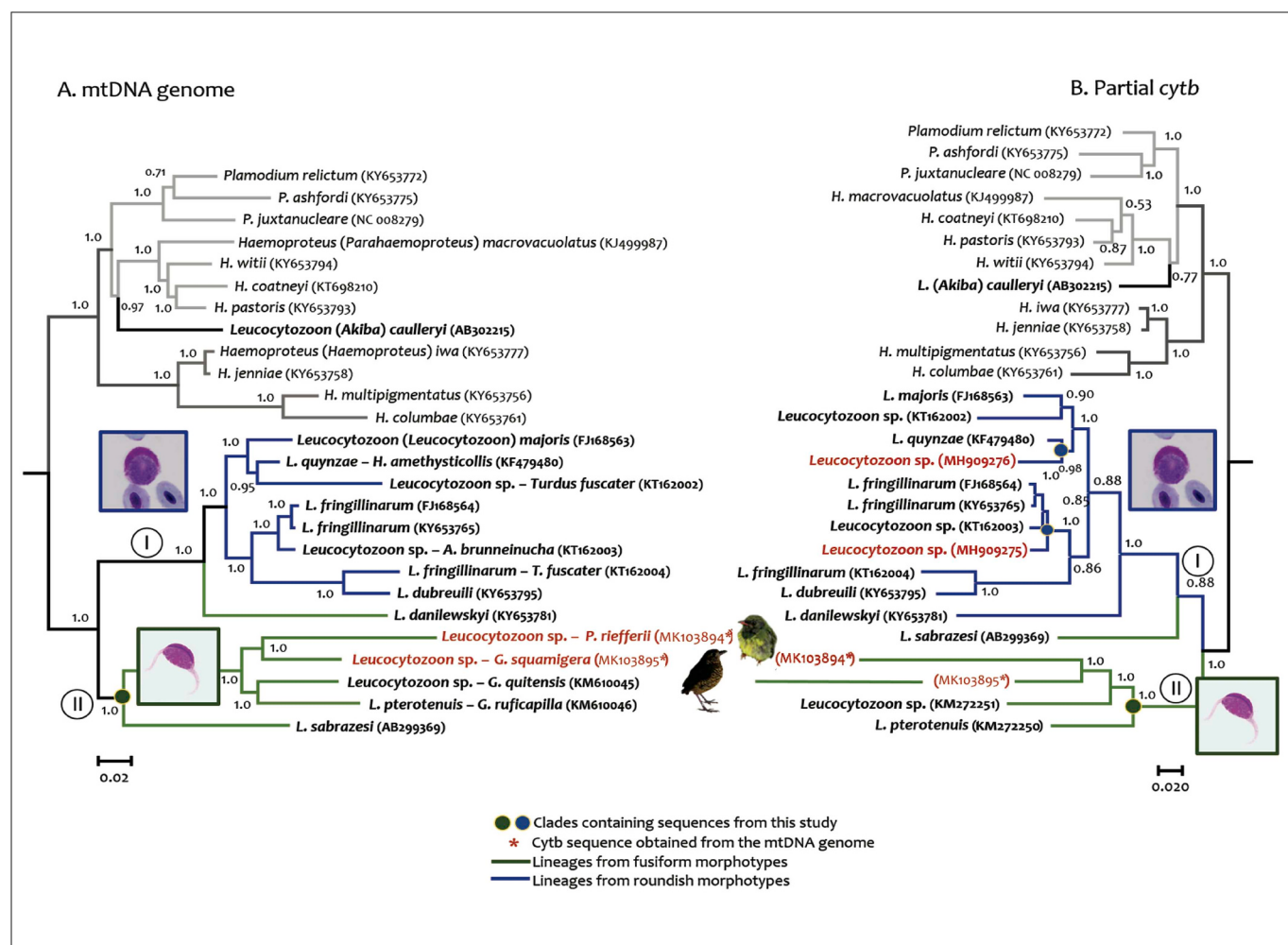


Fig. 5. (A) A Bayesian phylogenetic hypothesis of *Leucocytozoon* species constructed only with partial mitochondrial genomes (5485 bp excluding gaps) and (B) partial *cytb* gene sequences of leucocytozooids. Branch colors indicate the parasite morphology, with green branches representing parasites in fusiform host cells, and blue branches correspond to a species that develops in roundish host cells. Notice that, since parasite mitochondrial genomes (mtDNA) corresponding to the partial *cytb* fragments of the MH909275 and MH909276 sequences could not be amplified, they were not included in the phylogenetic hypothesis constructed with mtDNA (Fig. 5A). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4

Genetic distance (d) and standard errors (SE) between pairs of species using *cox1*, *cox3*, *cytb* and mitochondrial genomes (mtDNA) sequences of *Leucocytozoon* lineages (Fig. 5).

	Genetic distance (d ± SE)			
	<i>cox1</i>	<i>cox3</i>	<i>cytb</i>	mitochondrial genome
<i>L. neotropicalis</i> ^a vs. <i>L. grallariae</i> ^b	0.090 ± 0.007	0.105 ± 0.011	0.129 ± 0.007	0.095 ± 0.004
<i>L. neotropicalis</i> vs. <i>L. pterotenuis</i> ^c	0.103 ± 0.008	0.116 ± 0.011	0.141 ± 0.007	0.108 ± 0.004
<i>L. grallariae</i> vs. <i>L. pterotenuis</i>	0.095 ± 0.007	0.119 ± 0.011	0.080 ± 0.006	0.082 ± 0.004
<i>L. neotropicalis</i> vs. <i>L. sabrazei</i>	0.156 ± 0.010	0.169 ± 0.013	0.176 ± 0.006	0.149 ± 0.005
<i>L. grallariae</i> vs. <i>L. sabrazei</i>	0.095 ± 0.009	0.188 ± 0.014	0.136 ± 0.006	0.131 ± 0.005
<i>L. quynzae</i> vs. <i>L. neotropicalis</i>	0.160 ± 0.009	0.161 ± 0.012	0.141 ± 0.008	0.146 ± 0.005
<i>L. quynzae</i> vs. <i>L. grallariae</i>	0.167 ± 0.010	0.181 ± 0.014	0.080 ± 0.007	0.133 ± 0.005
<i>L. fringillinarum</i> vs. <i>L. neotropicalis</i>	0.171 ± 0.009	0.178 ± 0.013	0.175 ± 0.007	0.150 ± 0.004
<i>L. fringillinarum</i> vs. <i>L. grallariae</i>	0.176 ± 0.010	0.192 ± 0.014	0.140 ± 0.007	0.139 ± 0.005
<i>L. majoris</i> vs. <i>L. fringillinarum</i>	0.089 ± 0.007	0.099 ± 0.011	0.048 ± 0.004	0.058 ± 0.004

Lineages amplified and cloned from.

^a *P. riefferii* infected with *L. neotropicalis*, (GenBank MK103894).

^b *G. squamigera* infected with *L. grallariae* (GenBank MK103895), and.

^c *G. ruficapilla* infected with *L. pterotenuis* (Lotta et al., 2015 (partim)) (GenBank KM610046).

fusiform host cells and roundish host cells was 0.06% and 0.01%, respectively (Lotta et al., 2015). The same pattern was found in the birds infected with *L. grallariae* and *L. neotropicalis* (see above in the

description remarks). The parasitemia and aforementioned analyses of primers suggest that DNA fragments of parasites from gametocytes in roundish host cells more likely were amplified by the Hellgren et al.

Table 5
Genetic distance between cytochrome b lineages of *Leucocystozoos* spp. shown in FigS1. Calculations were made using Kimura two-parameter model of substitutions.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1 AB299369																									
2 JQ988502PERU	0,19																								
3 JQ988749PERU	0,18	0,23																							
4 JQ988751PERU	0,18	0,03	0,22																						
5 KF479480	0,17	0,09	0,19	0,08																					
6 KF874701PERU	0,18	0,05	0,23	0,03	0,09																				
7 KF874740PERU	0,18	0,10	0,21	0,09	0,07	0,09	0,10																		
8 KF874751PERU	0,18	0,04	0,22	0,04	0,10	0,02	0,03	0,09																	
9 KF874796PERU	0,16	0,05	0,21	0,04	0,08	0,02	0,02	0,07	0,02																
10 KF874797PERU	0,17	0,07	0,22	0,06	0,09	0,07	0,06	0,10	0,06	0,06															
11 KF874801PERU	0,17	0,05	0,21	0,04	0,08	0,03	0,03	0,07	0,03	0,01	0,06														
12 KF874814PERU	0,18	0,24	0,05	0,23	0,21	0,22	0,23	0,23	0,23	0,21	0,22	0,21													
13 KM272250COL	0,23	0,21	0,15	0,21	0,22	0,20	0,21	0,22	0,21	0,20	0,24	0,20	0,17												
14 KM272251COL	0,17	0,09	0,22	0,08	0,12	0,08	0,09	0,14	0,09	0,08	0,10	0,09	0,23	0,22											
15 KT247892COL	0,18	0,10	0,19	0,10	0,05	0,10	0,11	0,08	0,10	0,08	0,10	0,08	0,20	0,24	0,12										
16 KY646032COL	0,18	0,09	0,19	0,09	0,04	0,10	0,10	0,07	0,10	0,08	0,10	0,08	0,21	0,23	0,23	0,04									
17 KY646033COL	0,18	0,12	0,23	0,11	0,08	0,10	0,11	0,07	0,11	0,08	0,10	0,09	0,23	0,26	0,13	0,10	0,08								
18 MG714923BRAZIL	0,19	0,09	0,23	0,09	0,07	0,09	0,10	0,08	0,09	0,08	0,10	0,08	0,23	0,23	0,11	0,08	0,08	0,08							
19 NC.012450	0,18	0,04	0,23	0,03	0,09	0,04	0,03	0,09	0,03	0,03	0,06	0,04	0,23	0,21	0,08	0,10	0,09	0,11	0,09						
20 NC.012451	0,21	0,24	0,15	0,24	0,22	0,26	0,27	0,24	0,25	0,24	0,26	0,24	0,17	0,18	0,23	0,22	0,22	0,25	0,24	0,24					
21 MK103895	0,31	0,30	0,24	0,29	0,30	0,30	0,32	0,28	0,30	0,29	0,30	0,30	0,26	0,25	0,27	0,30	0,29	0,29	0,30	0,30	0,25				
22 MK103894	0,19	0,04	0,22	0,02	0,10	0,02	0,01	0,10	0,02	0,03	0,06	0,03	0,23	0,21	0,08	0,11	0,09	0,11	0,09	0,03	0,25	0,30			
23 MH909276	0,18	0,10	0,19	0,09	0,06	0,10	0,10	0,07	0,10	0,09	0,10	0,09	0,22	0,24	0,14	0,06	0,05	0,08	0,08	0,10	0,23	0,28	0,10		
24 MH909275	0,21	0,22	0,27	0,22	0,21	0,22	0,22	0,23	0,24	0,22	0,24	0,22	0,24	0,29	0,21	0,23	0,21	0,24	0,22	0,22	0,28	0,32	0,22	0,22	
25 FJ168562																									

Lineages obtained from the type samples of *L. gulariae* sp. nov. and *L. neotropicalis* sp. nov. are indicated in bold. Lineage of *Haemoproteus columbae* was used as outgroup, and it is indicated in italics.

primers (Hellgren et al., 2004).

4.4. Phylogenetic relationships of parasite lineages

The association of molecular lineages and morphospecies described in this study cannot be definitively proved. However, the close relationship between the 476 bp *cytb* lineage obtained in the *L. neotropicalis* (GenBank accession No. MH909276) sample with *L. fringillinarum* (Fig. 5B, genetic distance 2.8% Table 5) suggests that the *L. neotropicalis* lineage analyzed correspond to the parasite developing in roundish host cells, which resemble *L. fringillinarum* morphological species group. On the other hand, *Leucocytozoon* parasites with gametocytes in fusiform host cells are rare in passerine birds (Lotta et al., 2015; Valkiūnas, 2005). Although *L. grallariae* lineage is part of the same clade as *L. neotropicalis* and is closely related to *L. pterotenuis* (in part), patterns of host specificity of these infections deserve more in-depth studies. It is important to mention that the *cytb* lineages obtained both for *L. grallariae* and *L. neotropicalis*, grouped with other lineages previously reported in Peru and Colombia (Lotta et al., 2016) (Fig.S1. clade B and E).

In the past decade, leucocytozoids have been the subject of intense study in the Neotropical countries (Merino et al., 2008; Rodríguez et al., 2009; Matta et al., 2014; Galen and Witt, 2014; González et al., 2014; Harrigan et al., 2014; Lotta et al., 2015, 2016). New parasite species have been described in birds with distribution limited to the Andean mountains (Matta et al., 2014; Lotta et al., 2015). Due to the bias of the bird's capture method, mainly birds of Passeriformes and Apodiformes out of the 25 bird orders present in these mountain ranges (Herzog and Kattan, 2011) have been extensively sampled. Besides, due to the high endemism and richness of host species in the Neotropics, it is possible that new host-parasite relationships and coevolution have contributed to the generation of new lineages, which are significantly different to previous ones reported in other geographical regions. We encourage researchers to perform more in-depth sampling of birds belonging to other bird orders as well as to go further in genomic studies and life cycle characterization that are helpful for estimates parasite diversity and evolutionary history of avian malaria and relative haemosporidian parasites.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijppaw.2019.05.002>.

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