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 grallariae* 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; Marzl et al., 2015; Lotta et al., 2016; Moens et al., 2016; Moens and Pérez-Tris, 2016; Cadena-Ortiz et al.,...
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 apicopil (Caseinolytic protease C-Clpc) (Martinson 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 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 (Zehndijiev et al., 2012; Scher 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 squamipennis) and Green and black Fruiteteer, 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. |
|---------|-------------------------------------------------------------|
| Species | Life zone | Altitudinal range | N° cap (N° infected) per chain of mountains |
|---------|------------|-------------------|------------------------------------------|
| Anseriformes | | | |
| Anatidae | | | |
| Anas flavirostris | AF | 2900–3500 | 1(0) |
| Apodiformes | | | |
| Trochilidae | | | |
| Adelomyia melanogenys | SAF | 1800–2600 | 5(0) |
| Aglinecercus kingi | SAF | 1800–2600 | 1(0) |
| Amassia francke | SAF | 1800–2600 | 4(0) |
| Boissonneaus flavescens | AF | 2900–3500 | 1(0) |
| Boissonneaus flavescens | SAF | 1800–2600 | 2(0) |
| Coeligena | SAF | 1800–2600 | 7(0) |
| Coeligena helianthea | AF | 2900–3500 | 1(0) |
| Coeligena torquata | SAF | 1800–2600 | 3(0) |
| Coeligena torquata | SAF | 2900–3500 | 1(0) |
| Colibri coruscans | SAF | 1800–2600 | 3(0) |
| Colibri thalassinus | SAF | 1800–2600 | 3(0) |
| Doryfera ludovicia | SAF | 1800–2600 | 2(0) |
| Easaia | AF | 2900–3500 | 1(0) |
| Erisnecmus cupreoventeris | AF | 2900–3500 | 2(0) |
| Erisnecmus derbyi | AF | 2900–3500 | 1(0) |
| Helianthus | AF | 2900–3500 | 1(0) |
| Helianthus amethysticollis | | | |
| Helianthus exortis | AF | 2900–3500 | 1(0) |
| Helianthus exortis | SAF | 1800–2600 | 7(0) |
| Laffrersmya lafrasersmy | AF | 2900–3500 | 6(0) |
| Metallura tyrantina | AF | 2900–3500 | 3(0) |
| Metallura tyrantina | P | 3900–4100 | 2(0) |
| Phaeothorns guy | SAF | 1800–2600 | 4(0) |
| Phaeothorns sirratus | SAF | 1800–2600 | 3(0) |
| Schistes geoffroyi | SAF | 1800–2600 | 1(0) |
| Columbiformes | | | |
| Columbidae | | | |
| Zonaida auriculata | SAF | 1800–2600 | 2(0) |
| Charadriiformes | | | |
| Charadriidae | | | |
| Vanellus chilenis | SAF | 1800–2600 | 2(0) |
| Falconiformes | | | |
| Accipitriformes | | | |
| Rapornis magnirostris | SAF | 1800–2600 | 1(0) |
| Passeriformes | | | |
| Cinclidae | | | |
| Cinus leucocephalus | AF | 2900–3500 | 3(0) |
| Corvidae | | | |
| Cyanistrocy viridicyanus | SAF | 1800–2600 | 2(0) |
| Cyanocorax yncas | SAF | 1800–2600 | 3(0) |
| Cotingidae | | | |
| Pipreola riefferi | AF | 2900–3500 | 1(1) |
| Dendrocolaptidae | | | |
| Pseudocolaptes boissonneauti | AF | 2900–3500 | 1(0) |
| Emberizidae | | | |
| Arrammon brunneinucha | SAF | 1800–2600 | 4(0) |
| Arrammon brunneinucha | AF | 2900–3500 | 2(1) |
| Arrammon torquatus | AF | 2900–3500 | 4(0) |
| Atlapetes albinucha | SAF | 1800–2600 | 6(0) |
| Atlapetes albinucha | AF | 2900–3500 | 2(0) |
| Atlapetes palidinucha | AF | 2900–3500 | 7(1) |
| Atlapetes schistacas | AF | 2900–3500 | 6(1) |
| Zonorichia capensis | SAF | 1800–2600 | 93(0) |
| Zonorichia capensis | P | 3900–4100 | 6(0) |

(continued on next page)
| Table 1 (continued) | Life zone | Altitudinal range | N° cap (N° infected) per chain of mountains | Table 1 (continued) | Life zone | Altitudinal range | N° cap (N° infected) per chain of mountains |
|---------------------|-----------|-------------------|--------------------------------------------|---------------------|-----------|-------------------|--------------------------------------------|
|                      |           |                   | Eastern | Central |                      |           |                   | Eastern | Central |
| Zonotrichia capensis | AF        | 2900–3500         |         |         | 17(0) | 30(0)           | Diglossa humeralis | AF       | 2900–3500 | 11(0) |
| Fringillidae         |           |                   |         |         |        |                 |                |          |         |
| Astragalus psilopterus| AF        | 2900–3500         |         |         | 2(0)   |                 |                |          |         |
| Astragalus psilopterus| SAF       | 1800–2600         |         |         | 4(0)   |                 |                |          |         |
| Euphonia lanigera    | SAF       | 1800–2600         |         |         | 3(0)   |                 |                |          |         |
| Euphonia xanuthogaster| SAF      | 1800–2600         |         |         | 4(0)   |                 |                |          |         |
| Salator arpinensis   | SAF       | 1800–2600         |         |         | 1(0)   |                 |                |          |         |
| Furnariidae          |           |                   |         |         |        |                 |                |          |         |
| Cinclodes excelsior  | P         | 3900–4100         |         |         | 6(0)   |                 |                |          |         |
| Dendrocincus pyrrocephalus| SAF | 1800–2600         |         |         |        |                 |                |          |         |
| Hellmayria gularis   | AF        | 2900–3500         | 1(0)    |         |        |                 |                |          |         |
| Leguatathera ancilia | SAF       | 3900–4100         |         |         | 6(0)   |                 |                |          |         |
| Lepidocolaptes       | SAF       | 1800–2600         |         |         | 1(0)   |                 |                |          |         |
| lacyrmyger           |           |                   |         |         |        |                 |                |          |         |
| Margaromus squamiger | AF        | 2900–3500         | 12(0)   |         |        |                 |                |          |         |
| Premnoplex bruneensens| AF      | 2900–3500         |         |         | 1(0)   |                 |                |          |         |
| Premnoplex bruneensens| SAF     | 1800–2600         |         |         | 2(0)   |                 |                |          |         |
| Synallaxis asazae    | SAF       | 1800–2600         |         |         | 10(0)  |                 |                |          |         |
| Syroducta subaralsis | SAF       | 1800–2600         |         |         | 1(0)   |                 |                |          |         |
| Xiphocolaptes        | SAF       | 1800–2600         |         |         | 3(0)   |                 |                |          |         |
| promeroporphynaxus   |           |                   |         |         |        |                 |                |          |         |
| Grallariidae         |           |                   |         |         |        |                 |                |          |         |
| Grallaria squamigera*| AF        | 2900–3500         |         |         | 1(1)   |                 |                |          |         |
| Hirundinidae         |           |                   |         |         |        |                 |                |          |         |
| Ochotrochis murina   | AF        | 2900–3500         | 2(0)    |         | 7(0)   |                 |                |          |         |
| Ochotrochis murina   | P         | 3900–4100         |         |         | 5(0)   |                 |                |          |         |
| Pygochelidon        | SAF       | 1800–2600         |         |         | 14(0)  |                 |                |          |         |
| cyanoloxea           |           |                   |         |         |        |                 |                |          |         |
| Pyroderus scutatus   | SAF       | 1800–2600         |         |         | 4(0)   |                 |                |          |         |
| Stelgidopteryx ruficollis| SAF | 1800–2600         |         |         | 2(0)   |                 |                |          |         |
| Icteridae            |           |                   |         |         |        |                 |                |          |         |
| Molothrus bonanenias | SAF       | 1800–2600         |         |         | 2(0)   |                 |                |          |         |
| Momotidae            |           |                   |         |         |        |                 |                |          |         |
| Momotus momota       | SAF       | 1800–2600         |         |         | 1(0)   |                 |                |          |         |
| Parulidae            |           |                   |         |         |        |                 |                |          |         |
| Basilaterus strigatus| SAF       | 1800–2600         |         |         | 2(0)   |                 |                |          |         |
| Cardellina canadensis| SAF       | 1800–2600         |         |         | 8(1)   |                 |                |          |         |
| Myioborus sp.        | AF        | 2900–3500         | 1(0)    |         |        |                 |                |          |         |
| Myioborus minuatus   | SAF       | 1800–2600         |         |         | 6(0)   |                 |                |          |         |
| Myioborus oratus     | AF        | 2900–3500         | 4(0)    |         | 6(0)   |                 |                |          |         |
| Myioborus coronata   | SAF       | 1800–2600         |         |         | 1(0)   |                 |                |          |         |
| Myioborus corona     | SAF       | 1800–2600         |         |         | 5(0)   |                 |                |          |         |
| Myioborus             |           |                   |         |         |        |                 |                |          |         |
| nigricorpusus        | SAF       | 1800–2600         |         |         | 1(0)   |                 |                |          |         |
| Setophaga fusca      | SAF       | 1800–2600         |         |         | 1(0)   |                 |                |          |         |
| Rhinocryptidae       |           |                   |         |         |        |                 |                |          |         |
| Scytalopus infasciatus| AF       | 2900–3500         |         |         | 1(0)   |                 |                |          |         |
| Scytalopus micropusris| AF      | 2900–3500         |         |         | 2(0)   |                 |                |          |         |
| Thraupidae           |           |                   |         |         |        |                 |                |          |         |
| Amisognathus igniventris| AF     | 2900–3500         |         |         | 3(0)   |                 |                |          |         |
| Amisognathus         | AF        | 2900–3500         |         |         | 5(0)   |                 |                |          |         |
| lacrymosus           |           |                   |         |         |        |                 |                |          |         |
| Bulborhynchus montana| AF        | 2900–3500         |         |         | 3(0)   |                 |                |          |         |
| Catinamblyrhynchus   | AF        | 2900–3500         |         |         | 1(1)   |                 |                |          |         |
| diadema              |           |                   |         |         |        |                 |                |          |         |
| Catamenia homochroa  | P         | 3900–4100         |         |         | 12(0)  |                 |                |          |         |
| Catamenia inornata   | P         | 3900–4100         |         |         | 18(0)  |                 |                |          |         |
| Conusurus rufinucha  | AF        | 2900–3500         | 1(0)    |         |        |                 |                |          |         |
| Diglossa albaturea   | AF        | 2900–3500         | 2(0)    |         | 3(0)   |                 |                |          |         |
| Diglossa albateria   | SAF       | 1800–2600         |         |         | 1(0)   |                 |                |          |         |
| Diglossa cyanea      | SAF       | 1800–2600         |         |         | 3(0)   |                 |                |          |         |
| Diglossa humeralis   | P         | 3900–4100         |         |         | 9(0)   |                 |                |          |         |
| continued             |           |                   |         |         |        |                 |                |          |         |

(continued on next page)
The number of birds captured per species followed of the occurrence of Leucocytozoon sp. (in parenthesis), is given. 4 hosts infected with L. neotropicais sp. nov., 4 hosts infected with L. grallariae sp. nov. Life zone as follow Sub-Andean ecosystem (SAF); Andean forest (AF); Paramo (P); and altitudinal range data is also provided.

Table 1 (continued)

| Life zone      | Altitudinal range | N’ cap (N’ infected) per chain of mountains |
|----------------|-------------------|---------------------------------------------|
|                | Eastern           | Central                                    |
| Phyllomyias    | SAF 1800–2600     | 1(0)                                       |
| nigrocapillus  |                   |                                             |
| Phyllomyias    | AF 2900–3500      | 1(0)                                       |
| nigrocapillus  |                   |                                             |
| Phylliscocetes | SAF 1800–2600     | 2(0)                                       |
| poecilotis     |                   |                                             |
| Pyrrhomyias    | SAF 1800–2600     | 4(0)                                       |
| cinamomeus     |                   |                                             |
| Sayornis nigricans | SAF 1800–2600 | 9(0)                                       |
| Serophaga cinnerea | SAF 1800–2600 | 4(0)                                       |
| Stichia flavola | SAF 1800–2600     | 3(0)                                       |
| Tyrannus melancholicus | SAF 1800–2600 | 2(0)                                       |
| Uremias agilis | AF 2900–3500      | 6(0)                                       |
| Uremias agilis | SAF 1800–2600     | 1(0)                                       |
| Zimmerius viridisflavus | SAF 1800–2600 | 12(0)                                      |

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 cyb 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 cyb gene, two Leucocytozoon mitochondrial genomes (mtDNA) from one Undulated Antpitta (Grallaria squamigera, Grallariidae) and one Green-and-black Fruit eater (Pipreola riefferi, Cotingidae) were amplified, cloned and sequenced. PCR products were amplified with TaKaRa LA TaqTM Polymerase (TaKaRa Mirus Bio Inc., Shiga, Japan) as described by (Pacheco et al., 2011, 2018b) using primers forward 5’ GA GGA TCT TTC CCA CAC TTC AAT TCG TAC TTC and reverse 5’ CAG GAA AAT AGA AGA CCG AAC CTT GCT CCT GCA GCC. Then, a nested PCR was performed using the internal oligos forward 5’ TTT CATCCCTAAAATCTCGTAAC 3’/reverse 5’ GACCGAACCTTGGACTCTT 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 cyb 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:GER:PH:AN18). These two parasites were previously detected and described by us in other species of the Grallariidae (Grallaria ruficapilla and Grallaria quinquenis 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 cyb 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
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. dubreasi (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 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). 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

![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.](image-url)
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 Fruit-eater (Pipreola rieffelli) (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 Fruit-eaters (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 was of roundish (Fig. 1A–D) or oval or ellipsoid shapes; they closely adhered to the host cell nuclei, 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 metocytes, and it was very evident. Young gametocytes often possessed invaginations on their sides, which were opposite to the host cell nuclei, and it was very evident.

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                         | Leucocytozoon grallariae sp. nov. | Leucocytozoon grallariae sp. nov. |
|                                 | Macrogametocyte n = 15 | Microgametocyte n = 10 |
| parasite 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) |
| Parasite nucleus 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 | 152.2–188.1 |
|                              | (185.1 ± 12.8) | (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 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) |

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

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

B Only one of 2 cytoplasmic processes was measured for each parasite.
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. grrallariae (Fig. 1D–I). The length of these processes 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 and 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. grrallariae 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. grrallariae.

 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_GRSQ 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 2). However, the morphology of host-parasite complexes with fusiform processes is readily distinguishable from L. grrallariae (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. grrallariae and L. neotropicalis cells, so these parasites cannot be distinguished by this character (see the description of L. grrallariae 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, t = 0.05 P < 0.0001) and narrower in their maximum width (P < 0.0001) than in L. grrallariae (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’ cytoplast reach up to 14 μm (Table 3), and that never is observed in L. grrallariae. The length of the cytoplasmic processes can be different in the same host-parasite complex (Fig. 2 A 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. grrallariae (Fig. 11–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. 2E); 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 haemosporidan sexual dimorphic characters. The proportion of microgametocytes and macrogametocytes in the type material was approximately 1:2.

 Taxonomic summary.

 Type host: Green-and-black Fruitleteer Pipreola riefferi (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 cyt b genes (GenBank accession number MK103895) was obtained from the type host Pipreola riefferi.

 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. grrallariae 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. grrallariae).

 Due to the presence of long and narrow cytoplasmic processes in host cells, L. neotropicalis is similar to L. lovati (Valkiūnas, 2005) and L. eurystomi (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
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...
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. MK103895) corresponds to Leucocytozoon sp. 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. neotropicalis. 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. dubreuali (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. neotropicalis (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 of L. grallariae (Genbank No. MK103894) of L. grallariae samples form a clade that is the sister lineage to L.

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Table 3

| Feature                        | Leucocytozoon neotropicalis sp. nov. | Leucocytozoon eurystomi | Leucocytozoon lovati |
|-------------------------------|-------------------------------------|-------------------------|---------------------|
| Macrogametocyte n = 6        |                                     |                         |                     |
| Length                        | 14.4–16.0 (13.3 ± 0.6)              | 11.4–17.9 (13.6 ± 1.5)  | 22.6–29.6 (25.2 ± 1.3) |
| Width                         | 7.0–8.2 (7.8 ± 1.0)                 | 5.7–10.0 (7.8 ± 1.4)    | 7.0–12.2 (8.1 ± 1.0) |
| Area                          | 86.1–101.2 (95.2 ± 4.9)             | 64.9–114.9 (83.7 ± 17.4)| (182 ± 18.5)        |
| Perimeter                     | 34.1–40.9 (38.8 ± 1.6)              | 31.4–48.9 (37.4 ± 4.8)  | (630 ± 5.9)         |
| Parasite nucleus              |                                     |                         |                     |
| Length                        | 2.0–3.1 (2.8 ± 0.5)                 | 5.3–10.5 (8.0 ± 1.5)    | 2.8–6.4 (4.1 ± 0.4)  |
| Width                         | 3.3–4.1 (4.0 ± 0.4)                 | 4.4–7.0 (5.6 ± 0.8)     | 1.4–5.7 (3.5 ± 0.3)  |
| Area                          | 7.0–9.6 (8.6 ± 1.0)                 | 32.5–48.4 (41.4 ± 4.7)  | (11.8 ± 2.6)        |
| Host-cell parasite complex    |                                     |                         |                     |
| Length                        | 35.5–56.6 (44.7 ± 5.3)              | 29.8–55.8 (41.4 ± 8.6)  | (39.2 ± 6.3)        |
| Width                         | 9.5–10.8 (10.2 ± 0.5)               | 9.2–10.8 (9.9 ± 0.5)    | (9.1 ± 0.7)         |
| Area                          | 161.0–200.1 (181.8 ± 10.4)          | 127.4–181.7 (151.2 ± 13.0)| (202.2 ± 21.1)     |
| Host-cell nucleus             |                                     |                         |                     |
| Length                        | 14.8–19.7 (17.7 ± 1.8)              | 14.2–18.3 (16.3 ± 1.3)  | 18.3–26.0 (21.7 ± 2.3)| 9.8–16.3 (13.5 ± 1.5) |
| Width                         | 2.4–3.7 (2.9 ± 3.0)                 | 2.4–3.7 (3.0 ± 0.4)     | (39.0 ± 7.2)        |
| Area                          | 28.2–39.6 (35.7 ± 4.3)              | 23.6–42.0 (33.6 ± 5.3)  | (16.9 ± 2.3)        |
| Perimeter                     | 14.1–18.9 (16.5 ± 1.8)              | 14.2–18.3 (16.3 ± 1.3)  |                     |
| Cytoplasmic processes         |                                     |                         |                     |
| Length                        | 11.3–22.0 (15.7 ± 2.8)              | 7.6–24.4 (14.8 ± 4.5)   |                     |
| Width                         | 2.6–5.4 (3.8 ± 0.8)                 | 1.9–5.3 (3.7 ± 0.9)     |                     |
| Area                          | 18.2–29.0 (25.0 ± 5.3)              | 14.4–26.1 (21.2 ± 3.2)  |                     |

* According to Bennet et al., (1993).
* According to Valkiūnas (2005).
* Only one of 2 cytoplasmic processes was measured for each parasite.
pterotenuis (in part) (KM272250) and Leucocytozoon sp. (KM272251). These parasites are closely related to L. sabrazesi (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. neotropicalis, 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. dubreuili (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. neotropicalis, 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. sabrazesi (synonym of Leucocytozoon macleani) (AB299369) and L. danilewskyi (KY653781) (Fig. 5B clade II). Both Leucocytozoon sabrazesi 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. sabrazesi and L. pteroteunuis (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. sabrazesi, L. pteroteunuis (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. pteroteunuis (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 Neotropic leucocytozoids (Lotta et al., 2016, 2015) and are rare in leucocytozoids parasitizing Passeriformes birds (Valkiūnas, 2005). Nevertheless, the standard PCR protocols used to diagnose and characterize the leucocytozoids (i.e. Bensch et al., 2006; 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. pteroteunuis (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; Londóno 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. dubreuili*. Also, different studies have proved their efficiency in the detection of other leucocytozoids 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. pteroteunuis*, *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. dubreuili* 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. pteroteunuis* (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 leucocytozois. 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 lineages isolated from colorectal 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. pteroteunuis* (in part, Lotta et al., 2015), the authors found that cytB lineages isolated from *Grallaria ruficapilla* and *Grallaria quinquis* (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. pteroteunuis* (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, Dessier (1967) proved that *Leucocytozoon simondi* has gametocytes that develop both roundish and fusiform host cells, respectively. In this, after the publication of *L. pteroteunuis* (in part, 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. 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. pteroteunuis* (in part, Lotta et al., 2015).
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. Fig. 5.

![A Bayesian phylogenetic hypothesis of Leucocytozoon species constructed only with partial mitochondrial genomes (5485 bp excluding gaps) and (B) partial cyt b gene sequences of leucocytozoids. 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 cyt b 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.)](image)

### Table 4

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

Lineages amplified and cloned from.

- P. riefferii infected with L. neotropicalis, (GenBank MK103894).
- G. squamigera infected with L. grallariae (GenBank MK103895), and.
- 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 *Leucocytozoon* 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 | 0.19 |
| 2 | JQ984502PERU | 0.18 | 0.23 |
| 3 | JQ984504PERU | 0.18 | 0.22 |
| 4 | KF479480 | 0.17 | 0.09 | 0.19 | 0.08 |
| 5 | KF874701PERU | 0.18 | 0.05 | 0.22 | 0.04 | 0.09 |
| 6 | KF874740PERU | 0.18 | 0.05 | 0.23 | 0.03 | 0.11 | 0.02 |
| 7 | KF874751PERU | 0.18 | 0.10 | 0.21 | 0.09 | 0.07 | 0.09 | 0.10 |
| 8 | KF874796PERU | 0.18 | 0.04 | 0.22 | 0.04 | 0.10 | 0.02 | 0.03 | 0.09 |
| 9 | KF874797PERU | 0.16 | 0.05 | 0.21 | 0.04 | 0.08 | 0.02 | 0.02 | 0.07 | 0.02 |
| 10 | KF874801PERU | 0.17 | 0.07 | 0.22 | 0.06 | 0.09 | 0.07 | 0.06 | 0.10 | 0.06 | 0.06 |
| 11 | KF874814PERU | 0.17 | 0.05 | 0.21 | 0.04 | 0.08 | 0.03 | 0.03 | 0.07 | 0.03 | 0.01 | 0.06 |
| 12 | KM272505COL | 0.18 | 0.24 | 0.05 | 0.23 | 0.21 | 0.22 | 0.23 | 0.23 | 0.21 | 0.22 | 0.21 |
| 13 | KM272510COL | 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 | KT247892COL | 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 | KY646035COL | 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 | KY646034COL | 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 | 0.12 | 0.04 |
| 17 | MG719231BRAZIL | 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 | NC_012450 | 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_012451 | 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 | MK103989S | 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 | MK103989S | 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 |
| 22 | MH909297 | 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 | MH909297 | 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 | J01685562 | 0.21 | 0.22 | 0.27 | 0.22 | 0.22 | 0.22 | 0.21 | 0.21 | 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 |

Lineages obtained from the type samples of *L. gralariae* 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 cyt b lineage obtained in the L. neotropicalis (GenBank accession No. MH909276) sample with L. fringillinarum (Fig. 5A, 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 gamotoctyes 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. neotropicals and is closely related to L. pteronotus (in part), patterns of host specificity of these infections deserve more in-depth studies. It is important to mention that the cyt b lineages obtained both for L. grallariae and L. neotropicals, 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 have been described in birds with distribution limited to the Andean mountains (Mattia et al., Lotta et al., 2015). Due to the bias of the bird’s capture method, mainly birds of Passeriformes and Apodiformes have been described in birds with distribution limited to the Andean mountains (Matta et al., Lotta et al., 2015). Due to the bias of the bird’s capture method, mainly birds of Passeriformes and Apodiformes have been described in birds with distribution limited to the Andean mountains (Matta et al., Lotta et al., 2015). Due to the bias of the bird’s capture method, mainly birds of Passeriformes and Apodiformes have been described in birds with distribution limited to the Andean mountains (Matta et al., Lotta et al., 2015). Due to the bias of the bird’s capture method, mainly birds of Passeriformes and Apodiformes have been described in birds with distribution limited to the Andean mountains (Matta et al., Lotta et al., 2015).

The evolution of parasite lineages based on the gene encoding cyt b and the nuclear 12S rRNA is ongoing and will be published in a separate article. The association of molecular lineages and morphospecies described in this study cannot be definitively proved. However, the close relationship between the 476 bp cyt b lineage obtained in the L. neotropicalis (GenBank accession No. MH909276) sample with L. fringillinarum (Fig. 5A, 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 gamotoctyes 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. neotropicals and is closely related to L. pteronotus (in part), patterns of host specificity of these infections deserve more in-depth studies. It is important to mention that the cyt b lineages obtained both for L. grallariae and L. neotropicals, grouped with other lineages previously reported in Peru and Colombia (Lotta et al., 2016) (Fig.S1. clade B and E).

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