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# Leucocytozoon pterotenuis sp. nov. (Haemosporida, Leucocytozoidae): description of the morphologically unique species from the Grallariidae birds, with remarks on the distribution of Leucocytozoon parasites in the Neotropics

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**Abstract** We describe morphologically unique *Leucocytozoon pterotenuis* sp. nov. (Haemosporida, Leucocytozoidae), the first reported leucocytozoid species developing in fusiform host cell found in a Neotropical passeriform bird. The type host of this parasite is the Chestnut-crowned Antpitta (*Grallaria ruficapilla*, Grallariidae), an elusive native passerine bird whose natural history remains, to a large degree, unexplored. This bird was captured in Palacio forest in the damping zone of Chingaza National Natural Park, Cundinamarca, Colombia, at 2900 m above sea level (asl). Gametocytes of the new species develop both in roundish and fusiform host cells. This parasite is readily morphologically distinguishable from the described *Leucocytozoon* species because its host cells possess the narrow (needle-like) spindle-shaped processes, which length markedly exceeds their width. Additionally, the host cell nucleus markedly extends into the processes. Phylogenetic relationships were constructed based on a fragment of the mitochondrial cytochrome *b* gene and the complete mitochondrial genome. Phylogenetic analysis placed the lineage of *L. pterotenuis* in different positions depending on the length of the sequence analyzed that is likely due to poor sampling of *Leucocytozoon* species, especially from rare or non-passerine hosts, as well as a paucity of complete mitochondrial sequences of these parasites. Available data indicate that *Leucocytozoon* parasites are distributed mainly in mountain regions of the Neotropics where unique morphological forms have been recently discovered. To a better knowledge of the diversity of *Leucocytozoon* spp. and their host–vector–parasite interactions in Neotropical countries, additional deep and intensive samplings are needed, particularly in orders different to Passeriformes.

**Keywords** *Leucocytozoon* · Neotropics · Andean region · Grallariidae · Antpitta

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## Introduction

Studies characterizing biological diversity have gained momentum largely due to the goods and services that ecosystems provide to human communities. There are efforts to measure, assess, and conserve biodiversity that have incorporated parasitic species as a source of information about their hosts. Parasites are a diverse ecological guild that is present in almost all ecosystems. They affect the host behavior, growth, lifespan, and fecundity; moreover, they may provide information about the natural history of the host, trophic relationships in ecosystems in which they occur, their evolution, and distribution patterns (Pérez Ponce de León and García 2001; Poulin et al. 2011).

Currently, approximately 200 species of avian malaria parasites and related hemosporidians have been described

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based on traditional taxonomy (Valkiūnas 2005). However, it is well known that molecular tools have revealed high hidden lineage diversity (Bensch et al. 2004). Regardless this apparent species richness, limited attention has been given to the characterization and description of new species, particularly in tropical countries.

The Andean mountains have been recognized for their great variety of ecosystems and biodiversity hotspots (Josse et al. 2009). Recent studies in this region have shown new species of avian hemosporidian parasites as well as new ecological host–parasite interactions (Mantilla et al. 2013a, b; Matta et al. 2014a). Considering that these mountains harbor around 2000 avian species with almost 600 of them endemic (Herzog and Kattan 2011), it is expected that such host diversity will correlate with a high parasite diversity. Unfortunately, there are many avian species with limited or no records of their parasites. Among them, we can find those species belonging to the family Grallariidae. Birds of this family are widely distributed from Central to South America, with maximum species diversity in the tropical Andes. Twenty-seven out of 51 species described in the family have been reported in Colombia, and six are categorized as extinction risk (McMullan et al. 2011; IUCN 2014).

Here, we investigate the hemoparasites associated to the Chestnut-crowned Antpitta (*Grallaria ruficapilla*); this is a ground passerine bird belonging to the family Grallariidae that inhabits the scrublands and understory of humid Neotropical forests (McMullan et al. 2011). This species is widely distributed through the Andes mountains of Colombia, Ecuador, Peru and Venezuela between 1200 to 3600 m above sea level (asl) (BirdLife 2014). Different to other Antpittas, this bird can be easily found in heavily degraded tropical forest (Krabbe and Schulenberg 2004). There is limited information on the host parasite infections where Grallaridae species and its close relatives of the Formicariidae are involved. They have been found infected with *Plasmodium*, *Haemoproteus*, microfilaria, and *Trypanosoma* parasites (Galindo and Sousa 1966; White et al. 1978). *Leucocytozoon* infections have been detected only by molecular methods in Stripe-headed Antpitta *Grallaria andicolus* and *Grallaria* sp. at the Peruvian Andes (Witt and McNew, unpublished data). However, because abortive hemosporidian infections have been reported in birds (Levin et al. 2013), it remains unclear if the reported leucocytozoids complete life cycle or produce gametocytes in *Grallaria* species. Observation of blood stages is necessary to answer this question. In this study, two different *Grallaria* species were found infected with *Leucocytozoon* hemoparasites. In one of them, the Chestnut-crowned Antpitta, a new parasite species was found with gametocytes developing in fusiform host cells, which were reported for the first time in Passeriformes birds in South America. In this study, the morphological description and molecular characterization of *Leucocytozoon pterotenuis* sp. nov, a parasite of

Chestnut-crowned Antpitta, are provided. We discuss phylogenetic relationship and possible host association of this parasite as well as patterns of distribution of *Leucocytozoon* parasites in the Neotropics.

## Materials and methods

### Sampling area

Birds were captured using mist nets at the highland at four sites in Colombia: (1) Otun Lagoon, Los Nevados National Natural Park (NNP) from April 2010 to April 2011, (2) Pupiales (Nariño department) in December 2012, (3) Palacio Forest (Chingaza NNP) from February 2012 to February 2014, and (4) Monter Redondo Station (Chingaza NNP) from December 2008 to October 2009 and from June to July of 2012. Otun Lagoon (4° 46′ N, 75° 24′ W) is located at central mountain range in Los Nevados NNP, at 3950 m asl. Landscape is dominated by Páramo ecosystem and Andean forests with an average annual temperature of 6 °C and an annual average rainfall of 1250 mm (Vásquez and Serrano 2009).

Pupiales (0° 54′ N, 77° 39′ W) is located at the south of the Colombian Andes in Nariño department. Sampling was carried out at 3014 m asl, where montane dry and wet forests, as well as Páramo ecosystem, are present; however, the land is used mainly for agricultural activities. In the latter area, the annual media rainfall is 942.85 mm and the average temperature is 12 °C (Cadena et al. 2012). The Chingaza NNP is located in Colombian Oriental mountain range. Palacio forest (4° 41′ N, 73° 50′ W) is an Andean forest situated in the damping zone of the park at 2900 m asl in the lower altitudinal boundary of Páramo ecosystem. The temperature ranged between 12 and 18 °C in this area, but it may be under 0 °C, and the annual average of rainfall is 1900 mm (Vargas-Rios et al. 2004). Monte Redondo Station is located at 3100 m asl in an area covered mainly by Páramo ecosystem, where average temperature ranges between 6 and 7 °C; however, like in Palacio forest, temperature can fall below 0 °C, and the average rainfall in this area can reach 2900 mm (Vargas-Rios et al. 2004). For all localities, except for Pupiales, sampling dates included rainy and dry periods. Birds were identified according to the South American Classification Committee (Remsen et al. 2012).

### Sample and blood film examination

Blood samples were obtained by bird brachial vein puncture. From each bird, three thin smears were prepared, air-dried, fixed in absolute methanol for 5 min, and stained with Giemsa (pH 7.2) for 45 min. In addition, three drops of blood

(approximately 50 μl) were stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for molecular tests.

Blood films were examined by double-blind microscopic examination, first at ×100 for 10 min and then at ×1000 for 20 min, using a Leica DM750 microscope. Digital images were taken with a Leica EC3 camera; they were processed using the LAS EZ software (Leica Microsystems, Wetzlar, Germany). Morphometric features of parasites were those described by Valkiūnas et al. (2010). At least 100 images of the new parasite species were obtained and studied; measurements were made digitally upon 31 best quality images of gametocytes using ImageJ (Schneider et al. 2012).

Intensity of infection was determined according to Muñoz et al. (1999) by counting the number of parasites in 100 microscopic fields at high magnification (×1000), where red blood cells were forming a single monolayer. Differential counts of white blood cells were done on smears by visualization of 100 white blood cells and counting eosinophils, heterophils, and lymphocytes (Hauptmanova et al. 2002).

### DNA extraction, cytochrome b gene, and complete mitochondrial genome amplification and sequencing

The parasite molecular characterization was carried out by using the mitochondrial cytochrome b gene (Escalante et al. 1998). This gene has been widely used in malarial parasites, and there is a substantial database that allows comparing our findings with those reported by others. DNA was extracted only from microscopy-positive blood samples using a standard phenol–chloroform protocol (Sambrook et al. 1989). Due to the limited availability of stored blood samples of the Grallaridae birds, only two samples out of three sampled individuals, one from Chestnut-crowned Antpitta (G. ruficapilla) and one from Tawny Antpitta (Grallaria quitensis) were processed. Molecular detection of Leucocytozoon was made amplifying cytochrome b gene according to the nested PCR protocol recommended by Hellgren et al. (2004). Amplified products were visualized on a 1.5 % agarose gel and purified using differential precipitation with ammonium acetate protocol (Bensch et al. 2000) and sequenced in both senses using a 3730 xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) through Macrogen (Macrogen Inc.).

In addition to the cytochrome b, the complete mitochondrial genome (mitochondrial DNA (mtDNA)) was also amplified, cloned, and sequenced from both individuals; Chestnut-crowned Antpitta and Tawny Antpitta were infected with different morphospecies of Leucocytozoon. We amplified 5895 base pairs (bp) of the parasite mtDNA using primers forward 5′ GA GGA TTC TCT CCA CAC TTC AAT TCG TAC TTC/reverse 5′ CAG GAA AAT WAT AGA CCG AAC CTT GGA CTC with TaKaRa LA Taq™ Polymerase (TaKaRa Mirus Bio Inc, Shiga, Japan). Details about PCR

protocol and cloning are described by Pacheco et al. (2011a, b). Both strands for at least three clones were sequenced using an Applied Biosystems 3730 capillary sequencer. The partial cytochrome b gene and the mtDNA genome sequences were deposited in GenBank accession nos. KM610045, KM610046, KM272250, and KM272251.

### Phylogenetic analysis

In order to estimate phylogenetic relationships between the new species and other hemosporidian parasites, two independent alignments were made as follows: one for partial sequences of cytochrome b gene (476 bp) and another for almost complete mtDNA genome (5456 bp excluding gaps). First, an alignment was done using 19 cytochrome b sequences with 476 nucleotides edited and aligned with MEGA5 software (Tamura et al. 2011). Sequences included in the analyses were obtained from GenBank (five sequences) and MalAvi database (six sequences) (Bensch et al. 2009) and generated in this study (eight sequences).

Bayesian methods implemented on MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) were used to do the phylogenetic reconstructions under the general time-reversible model (GTR+I+Γ), the best of 88 models according to the corrected Akaike information criterion implemented on jModelTest 2.1.1 (Darriba et al. 2012). Phylogeny with nodal support was inferred in MrBayes using two independent runs of $5 \times 10^6$ generations, sampled every 100 generations. A majority rule consensus phylogeny was obtained from 25, 000 trees after discarding the 25 % of the trees as burn-in period. In addition, a maximum likelihood analysis was conducted using RAxML Black Box through CIPRES portal (Miller et al. 2010). This analysis was carried out applying the same model as for Bayesian inference, using 1000 bootstrap replications. After, the phylogenies were visualized and edited using FigTree v1.3.1 (Rambaut 2006).

Second, the mtDNA genome alignment was made using ClustalX v2.0.12 and Muscle as implemented in SeaView v4.3.5. This alignment included the 18 mitochondrial genomes available in the GenBank for hemosporidians isolated from lizards and birds. The mtDNA alignment was further divided into four categories where each gene (cytochrome oxidase I, cytochrome oxidase III, and cytochrome b) was used as a separate partition plus the non-coding regions (Pacheco et al. 2011a). The phylogenetic relationships were estimated on mtDNA genome alignment by using both the maximum likelihood (ML) method implemented in PhyML v3.0 (Guindon et al. 2010) and Bayesian methods using MrBayes v3.1.2 with the default priors (Ronquist and Huelsenbeck 2003). The reliability of the nodes in the ML tree was assessed by the bootstrap method with 200 pseudo-replications. Bayesian support for the nodes was inferred in MrBayes using $6 \times 10^6$ Markov Chain Monte Carlo (MCMC)

steps, and after convergence was reached, we discarded 25 % of the sample as "burn-in" periods. In both cases, cytochrome *b* and mtDNA analyses, convergence is reached when the value of the potential scale reduction factor (PSRF) is between 1.00 and 1.02 and the average standard deviation of the posterior probability is below 0.01 (Ronquist and Huelsenbeck 2003). General time-reversible model (GTR+I+Γ) was also the best fit to these data, as estimated by MEGA v5.0 (Tamura et al. 2011) .

In addition, divergences among specific pairs of *Leucocytozoon* species were estimated on partial cytochrome *b* gene and mtDNA genomes using the Kimura two-parameter model of substitution as implemented in MEGA v5.0 (Tamura et al. 2011).

## Results

Out of 774 birds sampled from 81 species, 22 families and 9 orders, 36 (4.65 %) were found infected with different *Leucocytozoon* species diagnosed by microscopic examination of blood film. Three individuals from Grallaridae were captured as follows: one individual of Tawny Antpitta (*G. quitensis*) in Pupiales, one individual of the same species in Otun Lagoon, and one Chestnut-crowned Antpitta (*G. ruficapilla*) in the Palacio forest. Two Tawny Antpitta, one from Pupiales and one from Otun Lagoon, were found infected with *Leucocytozoon* sp. Gametocytes of these birds were seen only in roundish host cells (Fig. 1a–c). The cytoplasm contains small vacuoles; the host cell nucleus had cap-like shape, and it extends less than one half of the circumference of gametocyte (Fig. 1a–c). However, both birds showed low intensity of infection and scarce number of morphological stages, making impossible the species identification. Nevertheless, it was possible to obtain both a cytochrome *b* lineage and complete mitochondrial DNA from the Antpitta captured in Pupiales (GenBank Accession nos. KM272251, KM610045). On the other hand, the Chestnut-crowned Antpitta captured in the Palacio forest showed an infection with morphologically unique *L. pterotenuis* sp. nov. (prevalence 0.13 %) (Table 1); its white blood cell counts were 68 % of heterophils, 18 % of eosinophils, and 16 % of lymphocytes. This individual was also found coinfected with microfilaria and *Trypanosoma* sp.

Description

*L. (Leucocytozoon) pterotenuis* sp. nov.

*Macrogametocytes (Fig. 1d–k)* Develop in fusiform (Fig. 1f–k) and roundish (Fig. 1a–c) host cells (Table 2). Gametocytes in roundish host cells are roundish in shapes (Fig. 1d, e).

Gametocytes in fusiform host cells vary from oval to ellipsoid in shapes. Parasite nucleus varies from roundish (Fig. 1j) to elongate (Fig. 1g); its position is markedly variable in gametocytes. Nucleolus was visible in 57 % of gametocytes in fusiform host cells. The parasite cytoplasm contains large number of small vacuoles (Fig. 1i, j); volutin granules were seen in 43 % of gametocytes (Fig. 1f, h). The nucleus of fusiform host cell is displaced, is deformed, and lies peripherally like a homogeneous band with ends markedly extending beyond the circumference of gametocytes and not touching the gametocytes (Fig. 1f–k). The cytoplasm of host cells forms two long, narrow spindle-shaped processes, which are of needle-like shape, and their length is markedly greater than widths (Fig. 1f–k). Host cell nucleus markedly extends into the cytoplasmic processes, a distinctive character of this species (Fig. 1f–k). Gametocytes in roundish host cells possess roundish nucleus of variable position; nucleolus was seen in 28 % of the gametocytes. Small vacuoles (Fig. 1d, e) and volutin granules were seen in 43 and 85 % of the gametocytes developing in fusiform and roundish host cells, respectively. The nucleus of roundish host cell is pushed aside, is deformed, and looks like a cap; it extended less than one half of the circumference of gametocyte (Fig. 1d, e). Remnants of the host cell cytoplasm are usually seen around the gametocytes as an envelope of variable form (Fig. 1d, e).

*Microgametocytes (Fig. 1l)* General configuration and other features as for macrogametocytes with the usual hemosporidian sexual dimorphic characters. Proportion of microgametocytes and macrogametocytes in the type material is 1:4. Measurements were taken upon one parasitized cell due to marked fragility of the gametocytes during preparation of blood smears, resulting in distorted morphology of the majority observed parasites.

Remarks

*L. pterotenuis* develops in roundish and fusiform host cells. This parasite can be readily distinguished from other described leucocytozoids due to unique shape of nuclei of its fusiform host cells. Mainly, the nuclei of fusiform host cells assume band-like forms (without thickenings on the ends); their covering between 30 and 55 % of the circumference of the gametocytes possesses markedly narrowed ends and extends into the cytoplasmic processors of host cells. These morphological characters are unique for this parasite.

Gametocytes in fusiform host cells develop in many species of *Leucocytozoon* which parasitize different orders of birds, but they are rare in passeriform birds. Only three species of leucocytozoids, which inhabit fusiform host cells, parasitize passerines. These are *Leucocytozoon balmorali*, which parasitizes Grey-headed Bush-shrike (*Malaconotus blanchoti*), Willow Warbler (*Phylloscopus*

**Fig. 1** *Leucocytozoon* spp. from *Grallaria* sp. of Colombia. *Leucocytozoon* sp. found in Tawny Antpitta *Grallaria quitensis* captured at Otun Lagoon (**a**, **b**) and Pupiales (**c**). Macrogametocytes (**d**–**k**) and microgametocyte (**l**) of *Leucocytozoon pterotenuis* sp. nov. from the blood of its type vertebrate host, Chestnut-crowned Antpitta *Grallaria ruficapilla* Antpitta. **d**–**e** Gametocytes in roundish host cells. **f**–**l** Gametocytes in fusiform host cells. *Black arrowheads* parasite nucleus, *open arrowheads* parasite nucleolus, *black long arrows* vacuoles, *open long arrows* volutin granules, *double-triangle arrows* host cell nucleus, *asterisk* host cell cytoplasm. Giemsa-stained thin blood films. *Scale bar*=10 μm
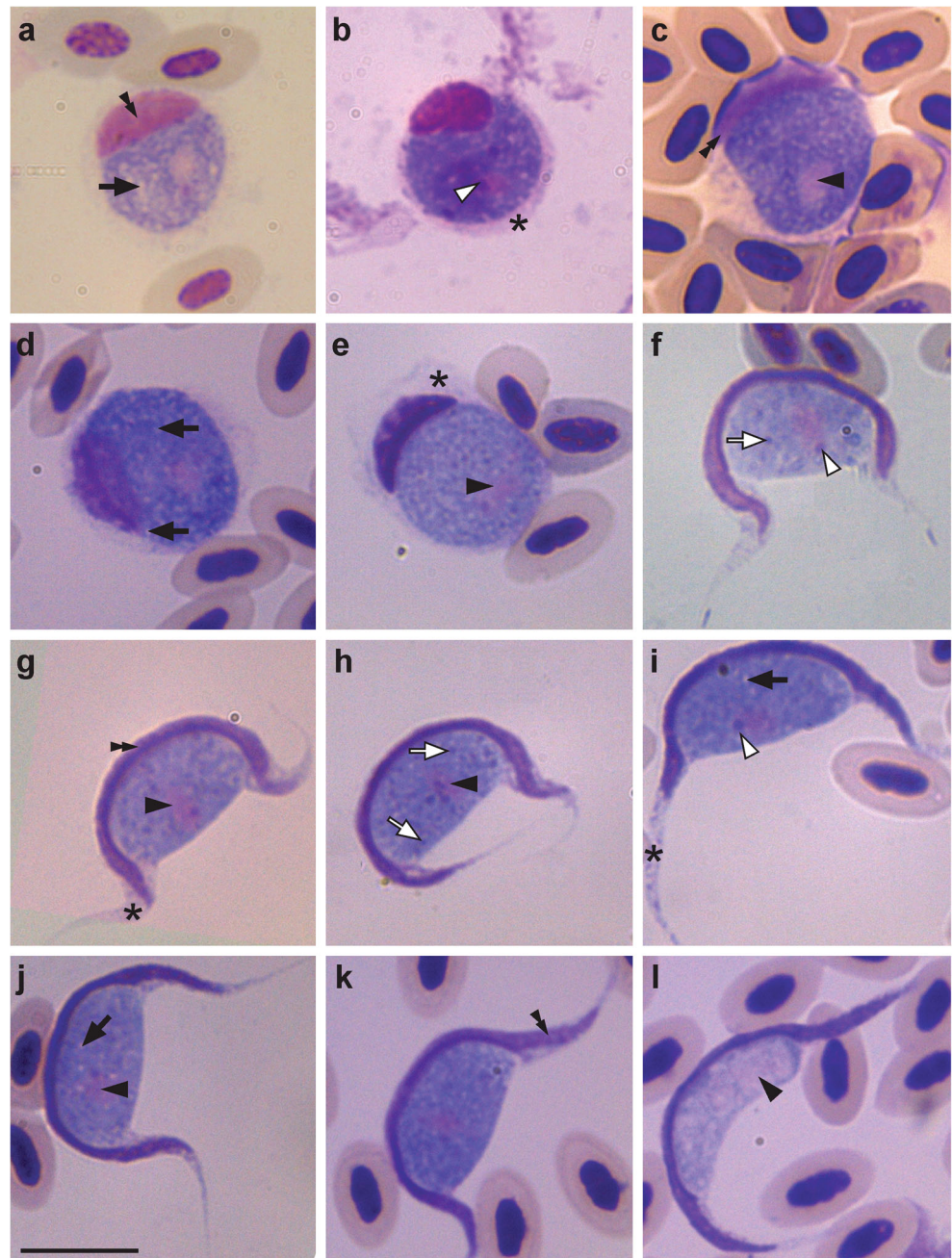


*trochilus*), Olive Bush-shrike (*Malaconotus olivaceus*), and Brown-crowned Tchagra (*Tchagra australis*) and some other bird species (Peirce 1984), *Leucocytozoon maccluri* which type host is Dark-sided Thrush (*Zoothera marginata*) (Greiner 1976) and *Leucocytozoon hamiltoni* which type host is Turkestan Tit (*Parus bokharensis*) (Valkiunas et al. 2002). None of these parasites possesses band-like host cell nuclei, which extend into the cytoplasmic processers. Due to this character, *L. pterotenuis* is similar to *Leucocytozoon simondi*, a specific parasite of birds belonging to the Anseriformes. In the latter parasite, nucleus of fusiform host cell looks like a more or less

evident dumbbell-shaped band with clear thickenings at both ends (Valkiūnas 2005). The latter character is not characteristic of the new species.

Taxonomic summary

Type host: the Chestnut-crowned Antpitta, *G. ruficapilla* (Grallaridae, Passeriformes).

Additional hosts: unknown.

Type locality: Palacio Forest at the damping zone of Chingaza NNP (4° 41′ N, 73° 50′ W, 2950 m asl), Cundinamarca, Colombia.

**Table 1** Occurrence of *Leucocytozoon* sp. and *Leucocytozoon pterotenuis* sp. nov. in Colombian birds sampled during this study

| Family and bird species | No. captured birds | Infected birds | |
|---|---|---|---|
| | | No. birds infected with *Leucocytozoon* sp. | No birds infected with *L. pterotenuis* |
| Cotingidae | | | |
| *Ampelion rubrocristatus* | 1 | 1 | 0 |
| Emberizidae | | | |
| *Arremon brunneinucha* | 5 | 1 | 0 |
| *Arremon torquatus* | 9 | 2 | 0 |
| *Atlapetes pallidinucha* | 14 | 1 | 0 |
| *Atlapetes schistaceus* | 5 | 1 | 0 |
| *Zonotrichia capensis* | 52 | 2 | 0 |
| Grallariidae | | | |
| *Grallaria quitensis* | 2 | 2 | 0 |
| *Grallaria ruficapilla* | 1 | 1 | 1 |
| Thraupidae | | | |
| *Anisognathus igniventris* | 19 | 1 | 0 |
| *Buthraupis montana* | 6 | 1 | 0 |
| *Catamenia inornata* | 80 | 2 | 0 |
| *Diglossacyanea* | 23 | 1 | 0 |
| *Dubusia taeniata* | 1 | 1 | 0 |
| *Hemispingu sverticalis* | 5 | 1 | 0 |
| *Phrygilus unicolor* | 116 | 1 | 0 |
| Trochilidae | | | |
| *Heliangelus amethysticollis*[b] | 5 | 1 | 0 |
| *Coeligena helianthea*[b] | 13 | 1 | 0 |
| *Metallura tyrianthina*[b] | 26 | 4 | 0 |
| Turdidae | | | |
| *Turdus fuscater*[a] | 21 | 6 | 0 |
| Tyrannidae | | | |
| *Myiotheretes fumigatus* | 1 | 1 | 0 |
| *Mecocerculus leucophrys* | 19 | 3 | 0 |
| *Phyllomyias nigrocapillus* | 5 | 1 | 0 |
| Total infected with *Leucocytozoon* | | 36 | 1 |
| Prevalence of *Leucocytozoon* sp | | 4.65 % | |
| Prevalence of *L. pterotenuis* sp. nov. | | | 0.13 % |
| Uninfected birds[c] | 345 | | |
| Total | 774 | | |

Type host of *L. pterotenuis* is highlighted in bold. A list of uninfected birds is provided in the end of the table

[a] Previous reports of birds infected with *Leucocytozoon* sp. were made by Lotta et al. (2013)

[b] Previous reports of birds infected with *Leucocytozoon* sp. were made by Matta et al. (2014a)

[c] Uninfected birds (number of examined individuals is given in parenthesis): Anatidae: *Anas flavirostris* (1), Caprimulgidae: *Systellura longirostris* (2), Corvidae: *Cyanolyca viricyana* (1), Emberizidae: *Atlapetes rufinucha* (1), Furnariidae: *Asthenes flammulata* (2), *Cinclodes excelsior* (21), *Leptasthenura andicola* (15), *Margarornis squamiger* (11), *Pseudocolaptes boissonneautii* (1), *Schizoeaca fuliginosa* (1), *Synallaxis albescens* (2), Hirundinidae: *Orochelidon murina* (52), Icteridae: *Amblycercus holosericeus* (2), Parulidae: *Basileuterus luteoviridis* (1), *Basileuterus nigrocristatus* (25), *Myioborus ornatus* (10), Picidae: *Colaptes rivolii* (1), Rallidae: *Fulica ardesiaca* (3), Ramphastidae: *Andigena nigrirostris* (1), Rhinocryptidae: *Scytalopus latebricola* (1), Scolopacidae: *Gallinago nobilis* (1), Thraupidae: *Conirostrum rufum* (5), *Conirostrum sitticolor* (3), *Catamblyrhynchus diadema* (2), *Diglossa albilatera* (10), *Diglossa caerulescens* (4), *Diglossa humeralis* (16), *Diglossa lafresnayii* (8), *Hemispingus atropileus* (8), *Hemispingus superciliaris* (4), *Tangara vassorii* (2), Tinamidae: *Nothocercus julius* (1), Trochilidae: *Aglaectis cupripennis* (1), *Chalcostigma heteropogon* (3), *Coeligena bonapartei* (1), *Eriocnemis cupreoventris* (9), *Eriocnemis derbyi* (4), *Eriocnemis vestitus* (12), *Heliangelus exortis* (2), *Lafresnaya lafresnayi* (1), *Metallura williami* (5), *Oxypogon guerinii* (3), *Ramphomicron microrynchum* (1), Troglodytidae: *Cinnycerthia unirufa* (23), *Cistothorus platensis* (2), *Henicorhina leucophrys* (7), *Troglodytes aedon* (2), *Trogodytes solstitialis* (2), Trogonidae: *Trogon personatus* (1), Tyrannidae: *Anairetes agilis* (6), *Anairetes parulus* (1), *Elaenia frantzii* (3), *Mecocerculus stictopterus* (7), *Mecocerculus minor* (1), *Ochthoeca cinnamomeiventris* (2), *Ochthoeca diadema* (1), *Ochthoeca fumicolor* (23), *Ochthoeca rufipectoralis* (3), *Phyllomyas uropygialis* (1)

**Table 2** Morphometry of gametocytes and host cells of *Leucocytozoon pterotenuis* sp. nov. Microgametocytes in roundish host cells were not seen in the sample

| Feature | *Leucocytozoon pterotenuis* sp. nov Lineage KM272250 L_GRRUF01[a] | | |
|---|---|---|---|
| | Gametocytes in roundish host cells | Gametocytes in fusiform host cells | |
| | Macrogametocyte $n=7$ | Macrogametocyte $n=31$ | Microgametocyte $n=1$ |
| **Parasite** | | | |
| Length | 10.3–14.8 (12.7±1.9) | 12.7–17.3 (14.6±1.2) | 17.9 |
| Width | 10.0–13.9 (11.8±1.3) | 5.4–10.0 (8±0.9) | 4.7 |
| Area | 83.5–145.6 (120.0±25.1) | 76.5–116.0 (94.2±9.7) | 72.8 |
| Perimeter | 32.4–44.4 (39.3±4.5) | 34.2–43 (38.2±2.4) | 39.8 |
| **Parasite nucleus** | | | |
| Length | 2.9–5.1 (3.5±0.7) | 2.7–5.2 (3.7±0.6) | 13.85 |
| Width | 2.2–3.4 (2.7±0.4) | 1.8–3.3 (2.5±0.4) | 4.46 |
| Area | 6.5–13.6 (8.4±2.4) | 4.2–9.9 (7.3±1.4) | 31.11 |
| **Host cell–parasite complex** | | | |
| Length | 13.3–20.1 (17.0±2.0) | 36.3–54 (45.6±4.8) | 47.1 |
| Width | 12.2–17.2 (14.8±2.0) | 6.6–11.3 (9.3±1) | 5.4 |
| Area | 130.0–240.4 (200.3±38.1) | 135.0–196.6 (165.6±13.1) | 131.8 |
| **Host cell nucleus** | | | |
| Length | 8.6–16.2 (12.0±2.8) | 29.8–46.3 (37.2±3.7) | 40.9 |
| Width | 2.2–3.3 (2.8±0.4) | 1.0–2.7 (1.6±0.4) | 1.37 |
| Area | 14.3–29.2 (24.8±5.2) | 40.6–59.1 (50.3±5.1) | 54.8 |
| Perimeter of parasite covered | 9.9–15.3 (12.0±2.1) | 20.0–27.5 (23.4±1.8) | 22.64 |
| Length of host cell nucleus, which locates inside the cytoplasmic processes | | 3.5–11.2 (6.8±1.9) | 9.12 |
| **Cytoplasmic processes[b]** | | | |
| Length | | 10.3–21.3 (14.9±3.0) | 16.0 |
| Width | | 1.4–3.4 (2.6±0.6) | 2.2 |
| Area | | 9.8–35.3 (21.4±6.9) | 21.1 |

[a] Measurements are given in micrometer or squared micrometer. Minimum and maximum values and mean ± SD are provided

[b] In each parasite, only one of two cytoplasmic processes was measured

Type specimens: Hapantotype (accession no. GERPH-07966, intensity of infection is 0.05 %, lineage KM272250 L_GRRUF01, collected by Rocio Hernández, 10 February 2014) was deposited in the biological collection Grupo de Estudio Relación Parásito Hospedero (GERPH) at Universidad Nacional de Colombia, Bogotá, Colombia. Parahapantotypes (accession nos. GERPH-07962, GERPH-07963, GERPH-07964, GERPH-07965, GERPH-07967, GERPH-07968, GERPH-07969, GERPH-07970, and GERPH-07971, other data as for the hapantotype) were 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: mitochondrial cytochrome *b* lineages L_GRRUF01 (GenBank no. KM272250) were detected from the same individual bird, from which hapantotype was originated. The *L. pterotenuis* complete mtDNA genome sequence was deposited in the GeneBank (KM610046).

Site of infection: blood cells, which origin is unclear.

Prevalence: The prevalence in the type host was 1 of 1 (100 %). In the type locality, 1 of 278 birds captured at Palacio Forest (0.36 %) was infected, as determined by microscopic examination. The overall prevalence (1 of 774 examined birds) was 0.13 % (Table 1).

Etymology: The species name (*pterotenuis*) was derived from the Latin words tenuis (thin) and *ptero* (wing); it refers to the slender elongated cytoplasmic processes of host cells of the parasite.

Phylogenetic relationships of parasites

Lineages used to estimate phylogenies based on partial cytochrome *b* sequences clustered in similar clades in the trees constructed using both phylogenetic methods: Bayesian (Fig. 2a) and ML (Fig. 2b). Nevertheless, it is important to note the low support obtained in many nodes from these

**Fig. 2** Bayesian (**a**) and maximum likelihood (**b**) phylogenetic hypothesis constructed using 476 bp of cytochrome *b* sequences of *Leucocytozoon* sp. from different bird hosts. Lineage of *Leucocytozoon pterotenuis* sp. nov. is given in *bold font*. A lineage of *Haemoproteus columbae* was used as outgroup. GenBank accession numbers or alternative lineage names of sequences from MalAvi are provided before the parasite species names. Bootstrap values and posterior probabilities are shown *above the nodes*. The branch lengths are drawn proportionally to the amount of change and the *scale bars* shown substitutions per site



analyses. However, in both phylogenetic trees, the cytochrome *b* sequences of *L. pterotenuis* did not cluster with other sequences of passerine *Leucocytozoon* spp. Unlike *Leucocytozoon* sp. lineages detected in the Peruvian *Grallaria* species, the Colombian *Leucocytozoon* sp. found in birds of the same genus has clustered close to *Leucocytozoon quynzae*, a parasite recently reported in birds of the Trochilidae (Matta et al. 2014a). It is noteworthy that this parasite is located in unsolved clades in both phylogenies.

Genetic distance between *L. pterotenuis* (L_GRRUF_01 GenBank accession no. KM272250) and *Leucocytozoon* sp. from Tawny Antpitta (GRQUI_01 GenBank accession no.

KM272251) estimated on partial cytochrome *b* sequences was 4.3 %, being the smallest difference reported when compared with another leucocytozoids of passerine birds (Table 3).

The phylogeny estimated with complete mitochondrial genomes is shown in Fig. 3. In this limited dataset, two well-supported clades of *Leucocytozoon* species were found sharing a common ancestor. In one clade, *L. pterotenuis* appears as sister taxa of *Leucocytozoon sabrazesi* (AB299369) isolated from Malaysian chicken, and the other clade included *Leucocytozoon fringillinarum*, *Leucocytozoon majoris*, and *L. quynzae*. The average genetic divergences using complete

**Table 3** Genetic distance (percentage) calculated using Kimura two-parameter model of substitutions, between some cytochrome *b* lineages of *Leucocytozoon* spp. used in Fig. 2

| | | 1. | 2. | 3. | 4. | 5. | 6. | 7. | 9. | 11. | 12. | 13. | 14. | 15. | 16. | 17. | 18. | 19. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | FJ168564 | | | | | | | | | | | | | | | | | |
| 2. | FJ168563 | 9.1[a] | | | | | | | | | | | | | | | | |
| 3. | AB299369 | 18.3 | 18.5 | | | | | | | | | | | | | | | |
| 4. | FJ168562 | 22.2 | 21.9 | 21.2 | | | | | | | | | | | | | | |
| 5. | GALLUS06 | 7.5[a] | 7.3[a] | 17.8 | 22.4 | | | | | | | | | | | | | |
| 6. | ACNI1 | 20.5 | 19.9 | 18.4 | 20.9 | 18.9 | | | | | | | | | | | | |
| 7. | AEFUN02 | 9.8 | 8.4 | 19.9 | 21.3 | 7.7[a] | 23.3 | | | | | | | | | | | |
| 9. | BUBT2 | 22.7 | 21.5 | 19.9 | 23.2 | 20.0 | 9.7 | 23.0 | | | | | | | | | | |
| 8. | KF309188 (HEAME01) | 9.4[a] | 7.0[a] | 17.2 | 21.1 | 4.9 | 18.8 | 7.3 | 21.3 | | | | | | | | | |
| 9. | KM272251 (GRQUI_01) | 9.1 | 7.9 | 18.0 | 21.3 | 5.8 | 20.2 | 7.3 | 23.1 | 3.8 | | | | | | | | |
| 10. | KM272250 (GRRUF_01) | 9.8 | 8.2 | 18.3 | 22.5 | 6.5 | 18.9 | 9.6 | 19.7 | 5.2 | 4.3 | | | | | | | |
| 11. | KF717049 (L_PHNIG01) | 1.4 | 9.1 | 19.1 | 22.5 | 7.7 | 20.7 | 9.4 | 23.0 | 9.1 | 9.1 | 10.3 | | | | | | |
| 12. | KF717047 (MYFUM01) | 9.8 | 3.8 | 19.1 | 21.9 | 6.8 | 21.3 | 7.5 | 22.2 | 5.9 | 6.5 | 7.2 | 9.4 | | | | | |
| 13. | KF717051 (L_ATBRU01) | 10.1 | 4.1 | 18.8 | 22.2 | 6.0 | 21.3 | 8.2 | 21.8 | 5.7 | 7.2 | 7.7 | 9.6 | 1.0 | | | | |
| 14. | KF717066 (DICYA_01) | 8.6 | 6.8 | 17.2 | 21.5 | 6.3 | 20.5 | 9.1 | 22.8 | 6.1 | 5.4 | 6.3 | 8.6 | 6.3 | 5.6 | | | |
| 15. | KF699313. (L_TFUS11) | 5.4 | 8.4 | 18.8 | 22.2 | 6.3 | 20.4 | 8.7 | 21.6 | 7.7 | 8.6 | 8.9 | 4.7 | 8.2 | 7.9 | 8.7 | | |
| 16. | JQ988750.1 | 2.5 | 8.6 | 18.0 | 22.2 | 7.0 | 19.6 | 9.4 | 22.2 | 8.4 | 8.9 | 10.1 | 2.1 | 9.4 | 9.1 | 8.4 | 4.5 | |

GenBank accessions numbers followed by an alternative lineage names from MalAvi are provided. *Leucocytozoon pterotenuis* sp. nov. lineage is indicated in bold. *Haemoproteus columbae* lineage was used as outgroup

[a] According to Matta et al. 2014a

mtDNA and the three mitochondrial genes among *Leucocytozoon* species are given in Table 4.

## Discussion

The northern portion of the Andean mountain ranges has almost the same number of species of the Amazon basin in an area that is 14-fold smaller (Herzog and Kattan 2011). These levels of species richness and endemicity likely involve a great number of host–parasite relationships that have not been explored. Indeed, in the last 10 years, *Leucocytozoon* spp. ceased to be considered as exotic parasites in the Neotropical countries, to become common element of resident bird parasitic fauna. However, transmission of leucocytozoids seems to be restricted to mountain areas and is rare on lowlands of the Neotropic (Forrester et al. 2001; Rodríguez et al. 2009; Lotta et al. 2013; Matta et al. 2014a). Leucocytozoids are nearly absent from birds at lowlands of the Neotropics, including the Amazonian basin (White et al. 1978; Belo et al. 2011; Lacorte et al. 2013; Svensson-Coelho et al. 2013). Such markedly spotty occurrence in birds is a unique character of *Leucocytozoon* spp. distribution of the Neotropics in comparison to other zoographical regions, in which these parasites are present and often prevalent both in lowlands and highlands (Valkiūnas 2005). Experimental research on vectors of leucocytozoids is lacking in the Neotropics but is essential for understanding distribution, epidemiology, and evolution of these parasites, some species of which cause diseases, sometimes even lethal, both in domestic and wild birds (Forrester and Greiner 2008; Santiago-Alarcon et al. 2012).

Antpittas (Grallaridae) are territorial ground passerines, which are of broad altitudinal distribution in Colombia; they have been reported between 300 and 4000 m asl. This genus reaches its highest diversity in Andean mountains above 800 m.; the four endemic species reported for this country are distributed upon 1200 m asl. Few individuals of Grallaridae have been reported as infected by avian hemosporidans (Galindo and Sousa 1966; White et al. 1978). The scarce number of infections of Antpittas might be explained by the ecological and behavioral traits of these birds (Kattan and Beltran 1999); since they are difficult to capture by mist netting. It is noteworthy that 100 % (3 of 3) of individual birds belonging to this genus sampled in Colombia were infected with *Leucocytozoon* spp. In general hemoparasite infections may be overlooked due to low parasitemias that make difficult to detect parasites using microscopic examination (Valkiūnas et al. 2008). It is noteworthy that the type host was coinfected with other two blood parasites (*Trypanosoma* and microfilaria) and the proportion of eosinophils was high when compared with previous data of white blood cell counts in birds (Hauptmanova et al. 2002;

**Fig 3** Phylogenetic hypothesis constructed using complete mitochondrial genomes (5485 bp excluding gaps) of *Leucocytozoon* spp. The values above branches are posterior probabilities together with bootstrap values (in *bold*) as a percentage obtained for the maximum likelihood tree (see "Materials and methods" section)
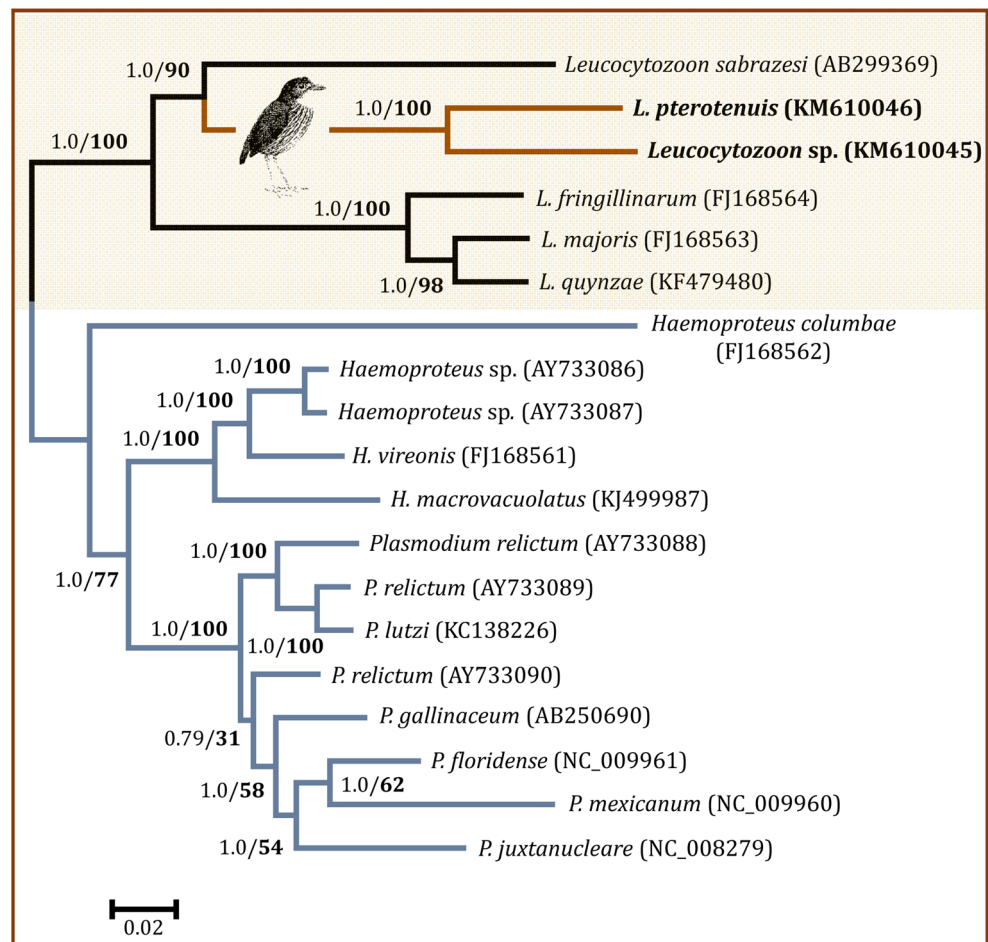


Gálvez et al. 2010). It has been reported that coinfections may have synergistic or antagonistic effect on birds (Palinauskas et al. 2011). In this way, the effective detection of this parasite by microscopy could be the result of an increase in the

**Table 4** Genetic distance and standard errors between pairs of species using mitochondrion genomes (mtDNA) sequences of *Leucocytozoon* lineages (Fig. 3)

| Species | Genetic distance (d ± SE) | | | |
|---|---|---|---|---|
| | COXI | COXIII | CYTB | Complete mtDNA |
| *L. pterotenuis* vs. *Leucocytozoon* sp. (AN18B) | 0.090±0.009 | 0.142±0.014 | 0.163±0.014 | 0.085±0.005 |
| *L. pterotenuis* vs. *L. sabrazesi* | 0.163±0.012 | 0.244±0.019 | 0.222±0.017 | 0.152±0.005 |
| *Leucocytozoon* sp. (AN18) vs. *L. sabrazesi* | 0.219±0.017 | 0.161±0.012 | 0.250±0.019 | 0.150±0.005 |
| *L. pterotenuis* vs. *L. fringillinarum* | 0.193±0.013 | 0.230±0.020 | 0.220±0.016 | 0.154±0.006 |
| *L. pterotenuis* vs. *L. majoris* | 0.196±0.013 | 0.218±0.020 | 0.237±0.019 | 0.157±0.006 |
| *L. pterotenuis* vs. *L. quynzae* | 0.186±0.013 | 0.211±0.018 | 0.218±0.017 | 0.149±0.007 |
| *L. sabrazesi* vs. *L. fringillinarum* | 0.223±0.015 | 0.230±0.019 | 0.210±0.017 | 0.148±0.005 |
| *L. sabrazesi* vs. *L. majoris* | 0.211±0.014 | 0.220±0.019 | 0.210±0.017 | 0.147±0.005 |
| *L. sabrazesi* vs. *L. quynzae* | 0.197±0.013 | 0.220±0.019 | 0.191±0.015 | 0.141±0.005 |
| *L. quynzae* vs. *L. majoris*[a] | 0.073±0.010 | 0.047±0.006 | 0.058±0.008 | 0.040±0.003 |
| *L. quynzae* vs. *L. fringillinarum*[a] | 0.109±0.013 | 0.093±0.008 | 0.069±0.008 | 0.059±0.003 |
| *L. majoris* vs. *L. fringillinarum*[a] | 0.105±0.012 | 0.095±0.008 | 0.081±0.009 | 0.058±0.003 |

*d* genetic distance, *SE* standard error

[a] According to Matta et al. 2014a

intensity of infection, caused by a synergistic effect of these parasites.

*L. pterotenuis* is the first parasite described in birds of the Grallaridae; also, this is the first parasite with gametocytes developing in fusiform host cell that is described in Passeriformes in South America. This is an important finding regardless the intensive samplings on the Andean passerine birds as well as in other localities from Neotropical countries (Gabaldon et al. 1974; Gabaldon et al. 1975; Gabaldon and Ulloa 1976; Bennett et al. 1980; Rodríguez et al. 2009; Levin et al. 2012; Lacorte et al. 2013; Marzal et al. 2014; Galen and Witt 2014). The only one *Leucocytozoon* with fusiform gametocytes reported in South America is *Leucocytozoon toddi*, which infects Accipitridae (Falconiformes) (Forrester et al. 2001).

A single report of *L. pterotenuis* in only one avian host does not provide much information about the parasite and its ecology. This parasite is morphologically similar to *L. simondi*. Unfortunately, the sequence available for *L. simondi* has poor quality in the region that overlaps with our alignment so we had to exclude it from our phylogenetic analyses. On the other hand, it is worth noting that *L. pterotenuis* is phylogenetically close to *L. sabrazesi* (possible synonym of *Leucocytozoon macleani* according to Valkiūnas 2005), a parasite of the Galliformes birds. Interestingly, the latter parasite also develops gametocytes both in roundish and fusiform host cells, but it has not been reported in the Neotropics so far (Valkiūnas 2005). We could speculate that the presence of fusiform host cells in the type avian host might be a recent host switch from hosts in which fusiform host cells are common. Indeed, it is important to mention that there is sympatric presence of Andean Guan (*Penelope montagnii*, Cracidae) in Palacio forest; however, we have not sampled this species for parasitology research. Because parasites in new hosts often are highly virulent and even cause mortality (Olias et al. 2011), prevalence of such infections should be low in wildlife populations (Toft and Karter 1990). Determining whether this parasite relates with others found in Cracidae, for example, is simply a speculation at this point. It seems that the putative narrow distribution of *L. pterotenuis* is better explained by the poor sampling of these rare passerines and many species of non-passerines in Andes (Matta et al. 2014b).

The phylogenetic relationships reconstructed for *L. pterotenuis* showed different results depending on the length of the sequences used. Reconstructions obtained using only a fragment of the cytochrome *b* gene placed *L. pterotenuis* inside unsolved nodes closely related with a parasite from Apodiformes birds. Phylogenies constructed with different methods showed low nodal supports, mainly on the deep nodes. It is associated with the size and nature of this mitochondrial marker. The relatively high rate of evolution of cytochrome *b* gene makes it ideal to discriminate between closely related species (Escalante et al. 1998;

Perkins et al. 2011; Pacheco et al. 2011a); however, the use of a small fragment of the gene may restrict the informative sites for the analysis (Matta et al. 2014a). Additionally, analyses carried out using a barcoding approach on sequences like cytochrome oxidase subunit I or, in our case, cytochrome *b* require a good baseline traditional taxonomy that allows to discern intraspecific from interspecific genetic divergences (Moritz and Cicero 2004; Valkiūnas et al. 2014). Another factor that could affect the analysis is the use of paralogous genes. Mainly, due to the methodology used to amplify the fragment of cytochrome *b*, there is a probability to amplify copies inserted on nuclear genome that have evolved independently from target gene (Funk and Omland 2003), leading to a non-reliable result. This is not our case since we also confirmed the cytochrome *b* fragment with the one derived from the complete (cloned) mtDNA. A more critical problem in the literature is the use of direct sequencing of PCR amplicons since mix infections in avian parasites are common in nature (Pérez-Tris and Bensch 2005; Valkiūnas et al. 2014), as has been reported in mammals (Pacheco et al. 2013).

In the case of primate malarias, it is well known that partial cytochrome *b* sequences lead to spurious phylogenetic relationships or problems separating species (Pacheco et al. 2013). The interest on avian malaria and related hemosporidians has generated a great number of lineages from a variety of birds; unfortunately many sequences are short and are the result of direct sequencing. Regardless this growing interest, there is still poor knowledge about infections in non-passerine birds and rare passerines as is the case with Antpittas (Dimitrov et al. 2014). The observed poor nodal support as well as the contradictions in the position of *L. pterotenuis* in different phylogenies might be due to limited sampling of cytochrome *b* in leucocytozoids developing in fusiform host cells in passerine or non-passerine birds, as well as the length of the sequences used.

Evolutionary relationships based on complete mitochondrial genome shows that *L. pterotenuis* is more closely related to *L. sabrazesi* than to other passerine leucocytozooids, supporting the similarity of morphological traits between these two parasites, which both have gametocytes in fusiform host cells (Valkiūnas 2005). In this phylogeny, polytomies seen in the analysis obtained using only cytochrome *b* fragments were solved with a good nodal support. In this way, an increase of information, preferably from genes of different origins, can improve our interpretation of the evolutionary history of these parasites. It is important to keep in mind that there are only few complete mitochondrial genomes available for *Leucocytozoon* species. In this way, despite of the new informative sites gained by the inclusion of these genes to the analysis, results obtained with few taxa could be widely criticized by its sensitiveness to homoplasy (Soltis et al. 2004). To obtain a better estimated of the phylogenetic relationships, ideally, these analyses should be constructed using a

larger number of informative genes and taxa (Rokas and Carroll 2005).

Most of the surveys addressing avian hemosporidians use mist nets; it is well known that this capture method is biased toward small passerine birds (Valkiūnas et al. 2003). For better estimation of avian hemoparasite, biodiversity is desirable to use different catching methods, for instance, Noose carpet traps (Gosler 2004) and/or Cannon nets and Bow nets (Bennett et al. 1992).

In addition to a morphological description, this study provides the complete mitochondrial genome for the new parasite species, which, eventually coupled with other markers, will allow establishing more accurately phylogenetic relationships among *Leucocytozoon* species. Additionally, for a better understanding of evolutionary relationships and ecology of these parasites, more studies analyzing vector–host–parasite interactions should be developed.

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