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Polymorphism evidence in *Plasmodium* (*Haemamoeba*) *lutzi* Lucena, 1939 (Apicomplexa, Haemosporida) isolated from Brazilian wild birds

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ABSTRACT

Plasmodium parasites can infect great variety of bird species around the world inflicting the so called avian malaria, an illness that could be fatal in some cases and consequently, should be monitored and widely included into conservation programs. The aim of this study was to characterize two lineages of *Plasmodium (Haemamoeba) lutzi* found in some birds in the Atlantic Forest of Minas Gerais - Brazil, that were morphologically identified after blood smears analyses under light microscopy and molecularly by sequencing the mitochondrial cytochrome *b* gene (*cyt b*). Besides these two lineages could be clearly morphologically identified as *P.(H.) lutzi*, some variations in comparison with its original description were noticed: absence of meronts and gametocytes (early and fully grown) in polychromatic erythrocytes, the larger size of pigment granules in meronts and gametocytes, and the presence of small vacuoles between pigment accumulation in fully grow macrogametocytes. Moreover, a certain degree of genetic intraspecific diversity was also observed across the lineages of *P. (H.) lutzi*, indicating the existence of polymorphisms within this taxon, which is uncommon in Haemosporida. These results allow discussion about species boundaries within avian hemosporidians and highlight the importance of multidisciplinary approaches for a more efficient species identification and characterization.

1. Introduction

Parasites of the genus *Plasmodium* (Apicomplexa: Haemosporida) can be found in several species of birds around the world, causing avian malaria. Their vectors are hematophagous dipteran, mainly of the genus *Culex* Linnaeus, 1758 and *Aedes* Meigen, 1818 [1]. Currently, about 4000 species of birds have been identified as possible hosts of blood parasites, including > 75 formally described *Plasmodium* species [1–3] distributed into five subgenera: *Haemamoeba* Grassi and Feletti, 1980; *Bennettinia* Valkiūnas, 1997; *Giovannolaia* Corradetti et al., 1963; *Novyella* Corradetti et al., 1963 and *Huffia* Corradetti et al., 1963 [1].

Plasmodium infections in wild birds can be devastating, especially when a susceptible bird population first comes in contact with the parasite. Accidental introduction of *P. relictum* and its vector into Hawaiian Islands led to population decline and extinction of native bird species [4]. Clinical signs usually appear shortly before death when birds become weak, move with difficulty, mucosae are pale, diarrhea is common, may have convulsions and paralysis. Therefore, these signs are not observed in free-living birds that in this state of debilitation are hardly sampled. These birds usually present chronic infections, in which the signs may be genuinely subclinical or asymptomatic, making it difficult to detect parasites in advance [1].

The traditional method for detection and identification of *Plasmodium* species in birds is the microscopic examination of blood smears [1,5,6]. However, several infections appear to be sub-microscopic and not all stages of the parasites can be observed on the slides. In addition, many species may be cryptic, making the use of morphological evidence difficult or even impossible in many cases [7]. Thus, many works use information contained in sequences of molecular markers, mainly the cytochrome *b* to assist in the identification / characterization process [3,8–11], and in which have led to the discovery of a much greater diversity than previous supposed.

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Polymorphism and phenotypic plasticity are common in many protist groups [12–17]. In Haemosporida these phenomena have already been observed in non-human primates, lizards and avian hosts [7,18,19], this may provide important information when there is no agreement of differentiation between morphology and genetic data [20]. In addition, these data may help to understand the parasite-host relationship as well as the delimitation of haemosporidian species.

In this study, we identified the presence of two lineages of *Plasmodium* (*Haemamoeba*) *lutzi* Lucena, 1939 in *Arremon semitorquatus* Swainson, 1838 and *Turdus rufiventris* Viellot, 1818 in fragments of Atlantic Forest in Minas Gerais, Brazil, and found evidences of the existence of intraspecific polymorphism within this *Plasmodium* species, in which data may have direct implications to species delimitation within this group of organisms.

2. Material and methods

2.1. Study sites and blood samples

We collected blood from 14 *Arremon semitorquatus* and 70 *Turdus rufiventris*, which were captured from Atlantic Forest fragments located in southeastern Brazil (21° 45 ‘51 “S, 43 21’ 01” W), using mist nets, annealed, weighed, and measured. Blood samples were taken from brachial vein punctures for preparation of blood smears, which were fixed in absolute methanol in the field and stained with Giemsa (Merck, Darmstadt, Germany) in the laboratory, according to Valkiūnas et al. [21] and, approximately 50 μl were stored at −20 °C for implementation of molecular analyses. After sampling procedures birds were released immediately. The sampling method was approved by competent Brazilian agencies, the Ethic Committee of the Universidade Federal de Juiz de Fora (protocol number 027/2012) and by the Sistema de Autorização e Informação em Biodiversidade (SISBIO), solicitation number 29268.

2.2. Morphological analyses

An Olympus BX-51 light microscope (Olympus, Tokyo, Japan), equipped with an Olympus Evolt E-330 digital camera and imaging software Image-Pro Plus 6.0 (Media Cybernetics, São Paulo, Brazil), was used to examine slides, prepare illustrations and schematic drawings, and take measurements. The studied morphometric features are those defined by Valkiūnas [1] and Bennett et al. [22]. Identification of the species was based on the descriptions of Valkiūnas [1] and Mantilla et al. [23]. Freehand drawings of different lineages of *P.* (*H.*) *lutzi* were created for observation of the specimens under a microscope and in papers with the morphological characterization of this species.

2.3. DNA extraction, PCR amplification, and sequencing

Total DNA from the blood samples was extracted using a Wizard® Genomic DNA Purification Kit (Promega®, São Paulo, Brazil), according to the manufacturer's protocol for blood extraction. The mitochondrial cytochrome *b* gene (*cyt b*) of the parasites, were amplified using a nested-PCR protocol [9] where in the first round, we used general primers for detection of parasites of the genus *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*: HaemFNI [5′-CATATATTAAGAGAAITATGGAG-3′] and HaemNR3 [5′-ATAGAAAGATAAGAAATACCATTC-3′]. While in the second round, the primers were HaemF [5′-ATGGTGCTT TCGATATATGCATG-3′] and HaemR2 [5′-GCATTATCTGGATGTGATA ATGGT-3′], which are specific to the genera *Plasmodium* and *Haemoproteus* [24]. The PCR were performed in a final volume of 25 μl consisting of 12.5 μl of Go Taq® Green Master Mix (Promega®), 2.5 μl of nuclease-free water, 2.5 μl of each primer (10 μM), and 5 μl of DNA template for first round reactions; and 12.5 μl of Go Taq® Green Master Mix (Promega®), 7.5 μl of nuclease-free water, 1 μl of each primer (10 μM), and 3 μl of the first round product in the second round of amplification. The PCR cycle was the proposed by Hellgren et al. [9] denaturation at 94 °C for 3 min, followed by 30 cycles of amplification at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. Amplification products were assessed by gel electrophoresis running 5 μl of the second PCR round product along with 2 μl of Blue Green Loading Dye I (LGC Biotecnologia®, São Paulo, Brazil) on an 2% agarose gel (Invitrogen®, California, USA). As negative control we used nuclease-free water instead of adding DNA template to the amplification reactions and as positive controls we used the total DNA of *Plasmodium (Haemamoeba) gallinaceum* Brumpt, 1935.

The amplified products were purified using the QIAquick® Purification Kit (Qiagen®, São Paulo, Brazil), according to the manufacturer's instructions and sequenced in both directions with the primer set HaemF e HaemR2 [24]. The two new sequences of *P.* (*H.*) *lutzi* obtained in this study were deposited in GenBank and in the MalAvi database under the following accession numbers and lineage codes: MH325387 and ASJB433 (*Arremon semitorquatus*), and KU573819 and TRJB16 (*Turdus rufiventris*).

2.4. Sequence availability and phylogenetic analyses

The phylogenetic relationship of the *P.* (*H.*) *lutzi* lineages sequenced in this work and other haemosporidian parasites was established using information from the *cyt b* genetic marker (478 bp). The dataset was generated using sequences produced here and from previous works available in the GenBank and MalAvi database, including representatives of the order Haemosporida (Danilewsky, 1885), family Plasmodiidae Mesnil, 1903 ($n = 41$) and family Haemoproteidae Doflein, 1916 ($n = 3$) (outgroup). The alignments were performed using MAFFT v 7.0 [25]. After a manual inspection, regions of an ambiguous nature and primers sequences were removed from the alignment, which was subsequently trimmed, producing an equi-length dataset, resulting in a matrix with 44 taxa and 451 characters. Subsequently, the dataset was analyzed using JModel-Test 2.1.4 [26,27] for selection of the optimal model of sequence evolution. Bayesian inference (BI) analyses were performed using MrBayes v. 3.2 [28], using GTR + G + I. Two simultaneous and independent Markov Chain Monte Carlo simulations were performed for 1000,000 generations or until the standard deviation of split frequencies were below 0.01, indicating the convergence of the two independent runs. A maximum likelihood (ML) analysis was carried out using the PhyML v 3.0 software [29], applying a GTR + G + I nucleotide substitution model. The reliability of internal branches was assessed, using the non-parametric bootstrap method with 1000 replicates.

3. Results

Among the captured birds, one *Arremon semitorquatus* and three *Turdus rufiventris* were infected by *Plasmodium* (*H.*) *lutzi,* as revealed by both microscopic and molecular analyses representing the first documentation of this parasite in these bird species.

3.1. Morphological characterization

The evolutionary forms of two lineages of *Plasmodium* (*H.*) *lutzi* isolated in Brazil are present in Fig. 1. The morphometric characterization presented in Table 1 refers to the lineage found in the species *Arremon semitorquatus*.

Trophozoites were observed developing in polychromatic and in mature erythrocytes (Fig. 1A, B). Young trophozoites are roundish (Fig. 1A), and the “ring” stage were not observed (Fig. 1A). We observed parasites under development, which presents an irregular shape (Fig. 1B). The parasite nucleus is large (Fig. 1A–B), and we observed pigment granules small, dark-brown, and grouped in a solid mass in the margin of the parasite (Fig. 1A–B). Infected erythrocytes are deformed, and their nuclei are displaced, that are more evident in the infection of

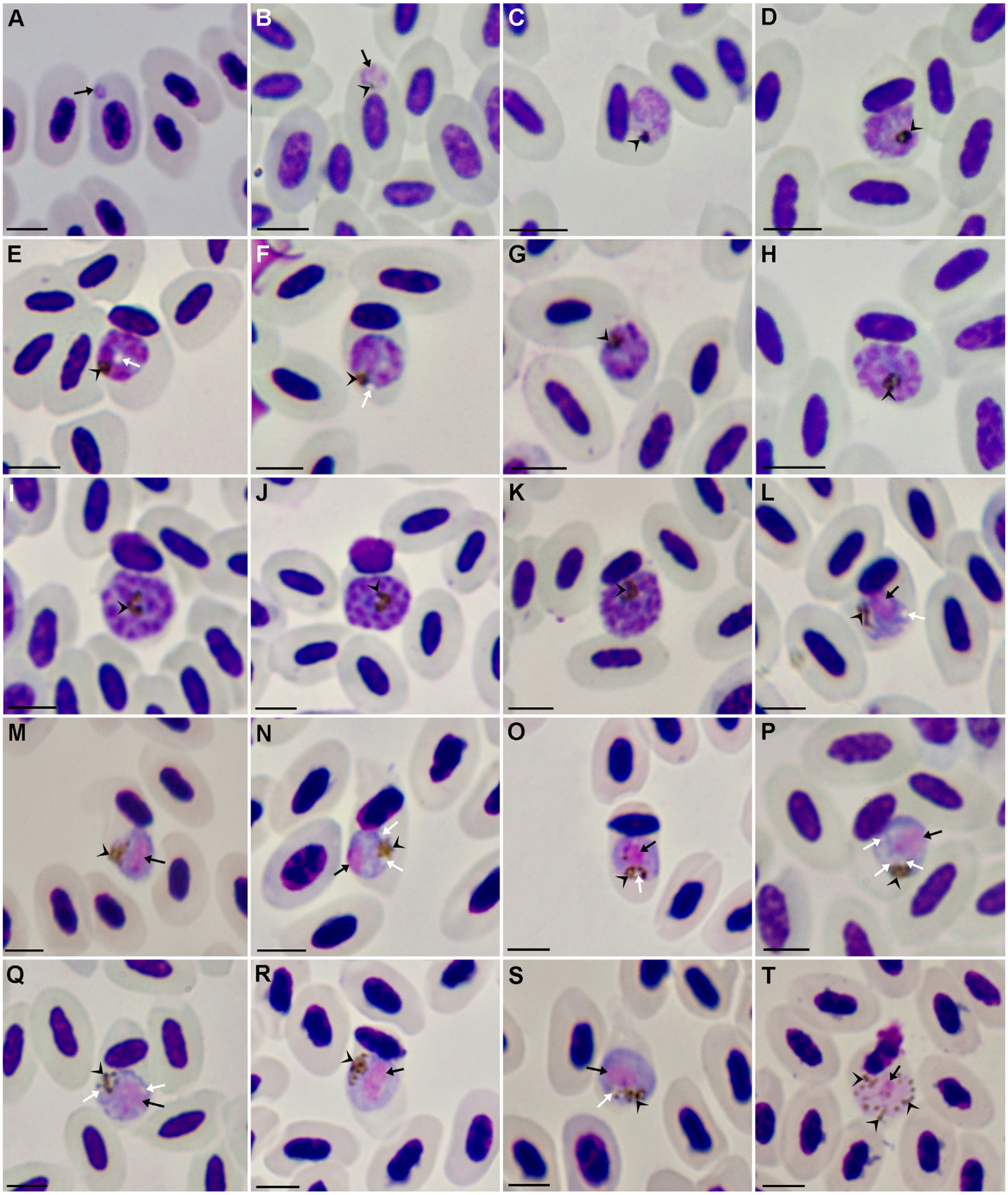

Fig. 1. Blood stages of *Plasmodium* (*Haemamoeba*) *lutzi* (lineages ASJB433 and TRJB16) from *Arremon semitorquatus* and *Turdus rufiventris* of Brazilian Atlantic Forest. (A–B) trophozoites; (C–K) meronts; (L–S) macrogametocytes; (T) microgametocyte. Arrow heads indicate pigment granules; black arrows indicate nuclei of parasites and white arrows indicate vacuoles. Giemsa stained blood films. Scale bars: 10 μm.

the same erythrocyte with several trophozoites.

Erythrocytic schizonts were observed in mature erythrocytes (Fig. 1C–K) and usually not possess vacuoles in the cytoplasm (Fig. 1C–K). We observed parasites under development, which presents the merozoites of small dimensions (Fig. 1C–K). The parasites possess a roundish or oval shape, and contains 13 to 14 (usually 13) merozoites (Fig. 1I–K). We observed pigment granules roundish, dark, and of medium size (< 0.5 μm), that were observed grouped in the margin of the parasite (Fig. 1C–K). Schizonts deform infected erythrocytes, displacing their nuclei (Fig. 1C–K) and in some cases, enucleate them (Fig. 1G).

Young gametocytes that we found, are morphologically identical to trophozoites.

Macrogametocytes were found developing in mature erythrocytes (Fig. 1L–S) and presents homogeneous cytoplasm, having small vacuoles sometimes (Fig. 1 L, N, P, Q, S). We observe fully grown gametocytes with rounded, oval and irregular shape, this last with less frequency (Fig. 1L–S). The parasite nuclear shape is variable and

Table 1
Morphometry of host cells, mature gametocytes and erythrocytic meronts of *Plasmodium* (*Haemamoeba*) *lutzi* (*cyt b* lineage ASJB433) isolated in *Arremon semitoquartus*. Minimum and maximum values are provided (in micrometers), followed by the mean and standard deviation in parentheses.

Feature	Measurements (μm)
Uninfected erythrocyte (*n* = 21)	
Length	9.9–11.6 (10.8 ± 0.4)
Width	5.0–7.8 (6.4 ± 0.6)
Area	48.5–66.3 (56.8 ± 5.5)
Uninfected erythrocyte nucleus	
Length	4.9–5.9 (5.3 ± 0.2)
Width	2.6–3.4 (2.9 ± 0.2)
Area	9.8–15.9 (12.3 ± 1.4)
Macrogametocyte (*n* = 22)	
Infected erythrocyte	
Length	9.5–13.0 (11.3 ± 1.0)
Width	52.0–76.4 (62.9 ± 6.4)
Area	5.8–9.68 (7.3 ± 0.9)
Infected erythrocyte nucleus	
Length	4.8–6.0 (5.3 ± 0.3)
Width	2.5–4.1 (3.0 ± 0.4)
Area	11.0–16.7 (13.3 ± 1.7)
Gametocyte	
Length	4.4–8.3 (6.4 ± 1.0)
Width	2.8–6.57 (5.2 ± 0.9)
Area	11.0–38.7 (26.9 ± 7.6)
Gametocyte nucleus	
Length	1.7–5.9 (3.4 ± 1.01)
Width	1.0–3.9 (2.1 ± 0.6)
Area	1.9–11.2 (5.9 ± 2.0)
Pigment granules	4–21 (11.5 ± 4.5)
NDR[a]	0.09–8 (0.9 ± 1.9)
Microgametocyte (*n* = 6)	
Infected erythrocyte	
Length	10.4–13.6 (11.7 ± 1.4)
Width	6.6–8.7 (7.6 ± 1.1)
Area	57.8–73.9 (67.2 ± 6.7)
Infected erythrocyte nucleus	
Length	4.9–6.5 (5.5 ± 0.8)
Width	3.4–3.8 (3.6 ± 0.1)
Area	14.6–18.2 (16.5 ± 1.8)
Gametocyte	
Length	7.6–10.5 (8.2 ± 1.1)
Width	5.8–8.3 (6.7 ± 0.9)
Area	38.7–55.7 (44.6 ± 7.4)
Gametocyte nucleus	
Length	2.4–4.3 (3.2 ± 0.6)
Width	1.5–4.2 (2.1 ± 1.0)
Area	3.3–13.6 (6.1 ± 3.7)
Pigment granules	11–21(15 ± 3.7)
NDR[a]	0.1–0.2 (0.1 ± 0.07)
Meront (n = 6)	
Length	2.1–7.3 (4.0 ± 2.1)
Width	1.7–7.2 (3.5 ± 2.3)
Area	2.4–38.1 (13.6 ± 14.4)
Area of globule	0.5–1.4 (1.0 ± 0.4)
N° of pigment granules	2–9 (4.2 ± 3.2)
N° of merozoites	13–14 (13.5 ± 0.7)

[a] NDR, nucleus displacement ratio according to Bennett and Campbell (1972).

compact (Fig. 1L–S). The pigment granules were found generally grouped in a solid mass in the margin of the parasite, round and medium in size (0.5 to 1.0 μm) (Fig. 1L–S). We recorded small vacuoles between the pigment granules (Fig. 1N–Q). We also observed macrogametocytes with pigment granules randomly scattered throughout the cytoplasm although this occurrence is not common; macrogametocytes deforming infected erythrocytes, displacing their nuclei (Fig. 1L–S) and even enucleating the host cells (Fig. 1S).

We observed that *microgametocytes* morphology resembled macrogametocytes, except for the sexual dimorphism characters, such as lighter cytoplasm staining and pigment granules dispersed throughout the cytoplasm of the parasite (Fig. 1T).

Remarks. It is important to highlight the fact that the lineages isolated in the present study show all the diagnostic characters of the species *P.* (*H.*) *lutzi* [1,23,30,31]. However, they differ in some others, especially the absence of meronts and gametocytes in polychromatic erythrocytes, the larger size of pigment granules in meronts and gametocytes (> 0.5 μm) (Fig. 1C–T), and the presence of small vacuoles between pigment accumulation in fully grow macrogametocytes (Fig. 1L–S).

3.2. Phylogenetic analyses and genetic divergence

The *cyt b* sequences from the two lineages of *P. (H.) lutzi* obtained in this work emerged in a monophyletic clade (71/0.98), together with the previously characterized *P. (H.) lutzi* isolated from *Diglossa cyanea* (Lafresnaye, 1840) (DIGCYA08), *Diglossa lafresnayii* (Boissonneau, 1840) (DIGLAF01) and *Turdus fuscater* D'Obirgny and Lafresnaye, 1837 (TFUS05) in Colombia [23,31]. Moreover, in this clade could be observed other lineages from different hosts such as SPMAG06, isolated from *Spheniscus magellanicus* Forster, 1981 in Brazil [32]; TUAMA01; TULEU03 from *Turdus amaurochalinus* Cabanis, 1850 e *Turdus leucomelas* Viellot, 1818 both in Brazil [33]; and RAMCAR01 from *Ramphocelus carbo* (Pallas, 1764) in Peru (Marzal unpubl. data) (Fig. 2). The mean genetic divergence within this monophyletic group was 1.19% (Table S1).

4. Discussion

Plasmodium (*H.*) *lutzi* was described by Lucena [30] in birds of the species *Aramides cajaneus* Müller, 1776 (Gruiformes) in Brazil, and it was posteriorly registered in *Turdus fuscater*, *Diglossa cyanea*, *Diglossa lafresnayii*, *Diglossa albilatera* Lafresnaye, 1843, *Hemispingius verticalis* Lafresnaye, 1840, *Anisognathus lacrymosus* (Du Bus and Gisignies, 1846) and *Anisognathus igniventris* (D'Obirgny and Lafresnaye, 1837) in Colombia [23,31]. The main taxonomic characters of this species are the trophozoites, meronts, macrogametocytes and microgametocytes rounded containing dark brown pigment granules, often clumped on the margin of the parasite [1,30]. All these diagnostic characters were observed in the two *P. (H.) lutzi* lineages (ASJB433 and TRJB16) isolated from *Arremon semitorquatus* and *Turdus rufiventris.* Moreover, this identification was corroborated by our molecular data, since their *cyt b* sequences emerged in a monophyletic clade (with significant branch support values) with other previously characterized *P.* (*H.*) *lutzi* lineages (Fig. 2) [23,31]. The *P. (H.) lutzi* lineage TRJB16 grouped with three sequences, TUAMA01, TULEU03 and SPMAG06 (Fig. 2). TUAMA01 and TULEU03 were described as *Plasmodium* sp., while SPMAG06 was originally characterized as *Plasmodium (Haemamoeba) relictum* [32]. *P.* (*H.*) *relictum* and *P.* (*H.*) *lutzi* have many characters in common, especially the roundish shape of meronts and gametocytes [1]. The morphological descriptions provided by Bueno et al. [32] to characterize the lineage SPMAG06 are not detailed enough and common to both mentioned species. Thus, we suggest this lineage was misidentified and in fact it belongs to the species *P.* (*H.*) *lutzi.*

The mean genetic distance within *P. (H.) lutzi* as estimated in this work for *cyt b* was of 1.19% (Table S1), indicating a certain degree of intraspecific variance. Moreover, the lineages ASJB433 and TRJB16 (Fig. 1; Table 1), shown morphological dissimilarities that could be noticed when compared to the original description of this species [1,30] such as absence of meronts and gametocytes in polychromatic erythrocytes, the larger size of pigment granules in meronts and gametocytes, and the presence of small vacuoles between pigment accumulation in fully grown macrogametocytes (Fig. 3 shows schematic drawings of these lineages and exposes their differences and morphological similarities). In fact, along the known *P. (H.) lutzi* genetic lineages, TFUS05 [23] is the one that mostly resembles the original description of this species [1,30] even though it was reported in association with a different host and in an unrelated geographic location.

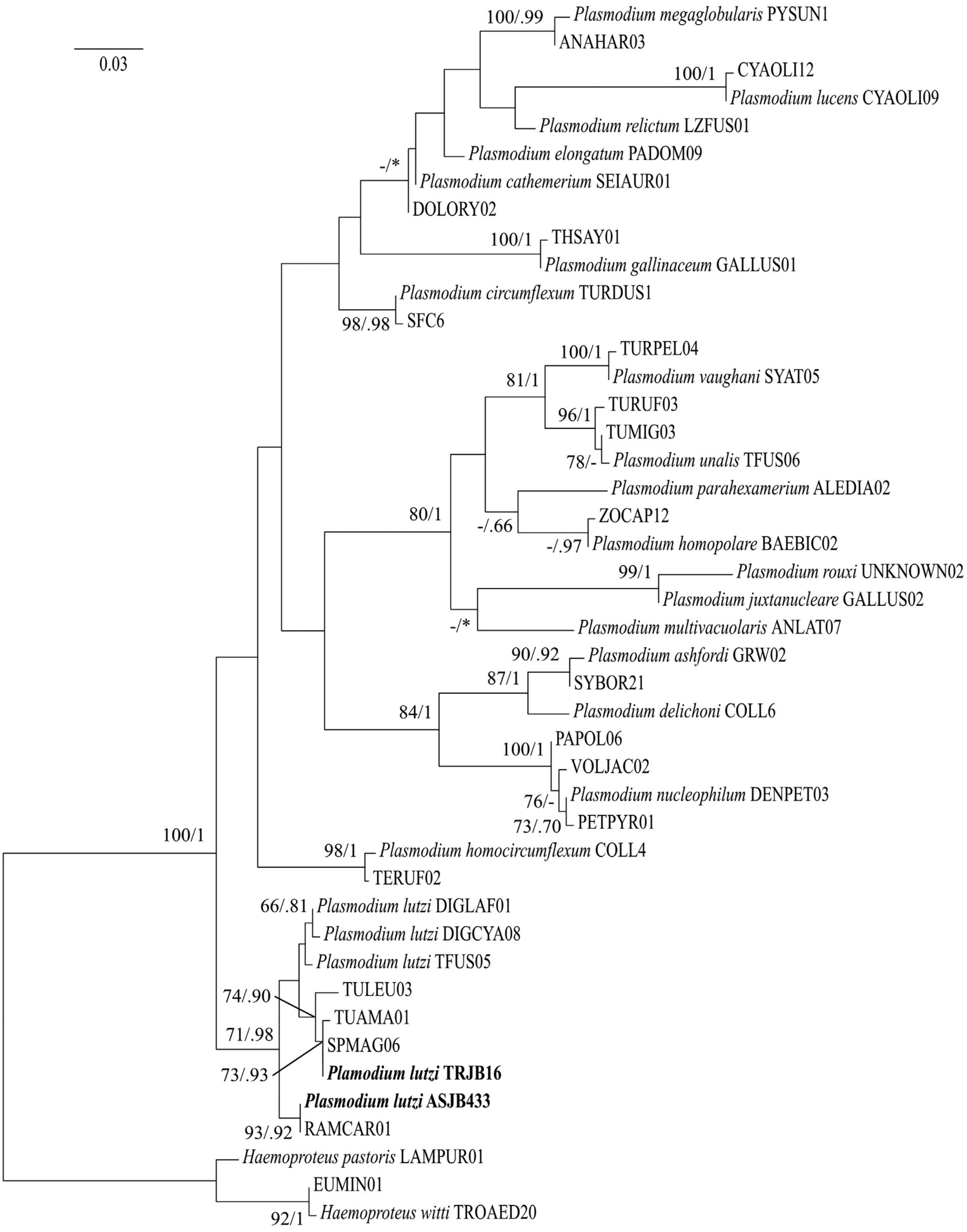

Fig. 2. Maximum likelihood tree of *Plasmodium* spp. (Apicomplexa, Haemosporida) based on mitochondrial *cyt b* sequences. *Haemoproteus* spp. were chosen as outgroup. The values on the branches represent the maximum likelihood (ML) bootstrap values and the posterior probability (BI). The symbol (−) indicates bootstrap values of and/or posterior probability < 50%/0.5. The new sequences of the species *Plasmodium* (*Haemamoeba*) *lutzi* obtained in this study are highlighted in bold. The scale bar corresponds to 3 substitutions per 100 nucleotides.

The lineages DIGCYA08 and DIGLAF01 [31] are the most morphologically similar to the lineages presented in this work, all sharing the same morphological dissimilarities as we present previously (Fig. 3). On the other hand, the lineages DIGCYA08 and DIGLAF01 [31] are genetically closer to TFUS05 [23] than to ASJB433 and TRJB16 (average genetic divergence 0.3% while 1.9% to ASJB433 and TRJB16) (Table S1). This data highlights the fact that great morphological similarity is not, necessarily, related to low genetic divergence in this group of parasites.

In Haemosporida, even small divergence in mtDNA may have

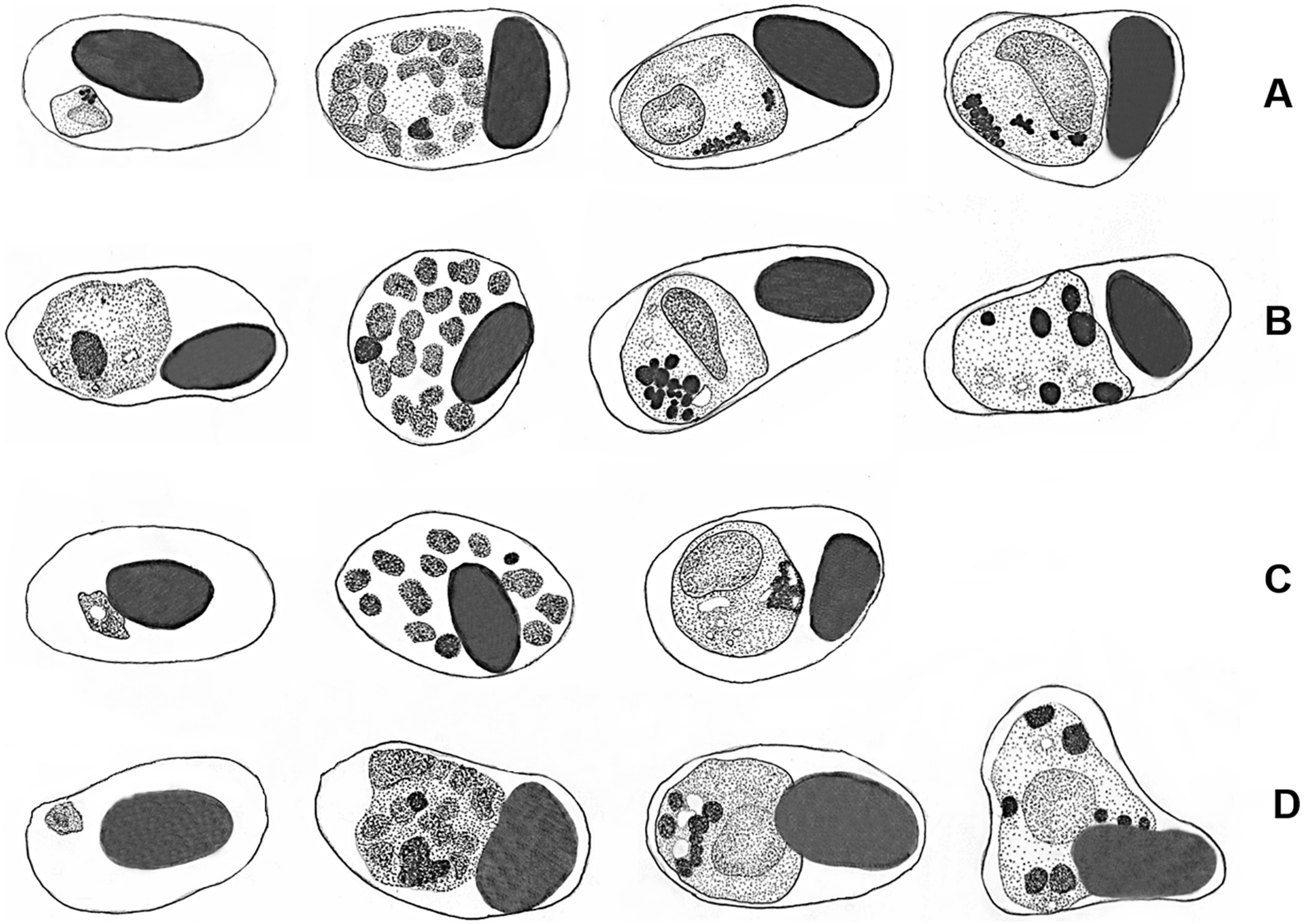

Fig. 3. Schematic drawings of different lineages of *Plasmodium* (*Haemamoeba*) *lutzi*. (A) original description (Lucena 1939; Valkiūnas 2005); (B) lineage isolated in *Turdus fuscater*, Colombia (Mantilla et al. 2013); (C) lineage isolated in *Diglossa cyanea*, Colombia (Gonzàlez et al. 2015); (D) present study.

significant implications for species definition, for example, *P. vivax* and *P. cynomolgi* of mammals showed low genetic divergence (1.2%) in the complete sequence of the mitochondrial cytochrome *b* gene, were separated into two species. The same was observed between *P. yoelii* and *P. berghei* [23]. Another example was the low genetic divergence (0.6%) recorded in the partial cytochrome *b* gene between *Haemoproteus jenniae* and *H. iwa* which showed distinct morphological characters [34]. This shows how difficult it is to define species in this group of parasites where often the values of genetic divergence do not corroborate the morphology. Nevertheless, the *cyt b* gene is widely used to identify lineages of avian haemosporidian [3,35] and the correct identification of morphologically distinct species using a fragment of this gene (478 bp) has been demonstrated by Pacheco et al. [20]. However, *cyt b* gene information should be used with caution and the use of multigene analyzes together with morphological and biological criteria may be indispensable for a better understanding of the evolutionary relationships between the haemosporidian species and specially to species delimitation in this group.

Thus, studies that demonstrate morphological polymorphism and intraspecific genetic variability are required to solve taxonomic problems, since many species of hemosporidians inhabit numerous species of birds, and the same species of parasites can present morphological differentiation and variety of lineages [36].

Our results raise discussions about the difficulty of delimiting species of avian haemosporidian parasites, due to the occurrence of polymorphisms and due to disagreement over metrics that can be used to diagnose species. According to Outlaw and Ricklefs [3], it is very important conducting multidisciplinary studies involving the use of new approaches, such as: the use of more informative markers that currently employed [37,38], the investigation of other morphological characters and aspects of the biology of parasites and hosts, targeting more consistent descriptions that allow the understanding of the processes that generate biodiversity in order Haemosporida.

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