An Acad Bras Cienc (2024) 96(3): e20230629 DOI 10.1590/0001-3765202420230629
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ANIMAL SCIENCE

Molecular diversity and polyparasitism of avian trypanosomes in the Brazilian Atlantic Rainforest

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Abstract: The current study proposes to investigate the diversity and phylogeny of trypanosomes parasitizing wild birds from the Brazilian Atlantic Forest. Cytological examination was carried out by light microscopy of blood smears and positive birds were selected for amplification of the 18S rDNA sequence through PCR. The resulting amplicons were subjected to purification, cloning, and sequencing analysis. Phylogenetic reconstruction was conducted, including all avian trypanosomes representative's lineages. A total of ten bird samples from species of *Turdus flavipes* (N=1/12), *T. albicollis* (N=1/8), *Tachyphonus coronatus* (N=6/121), *Thamnophilus caerulescens* (N=1/22) and *Synallaxis spixi* (N=1/8) were positive for *Trypanosoma* spp. In the six specimens of *T. coronatus*, five distinct lineages of *Trypanosoma* spp. 18S-rRNA were observed in ninety sequences obtained, and using the strategy of cloning independent PCR, it was possible to observe that two of them were related to *T. avium* (JB01/JB02), and three were closed related to *T. bennetti* (JB03/ JB04/JB05). Addionaly, all fifteen sequences obtained from *T. caerulescens*/ *S. spixi*/*T. flavipes*/*T. albicollis* were identical. The present research is the first study to access molecular diversity and polyparasitism by avian trypanosomes in Brazil. The current research exhibits the wide genetic variability in avian trypanosomes and its non-specific relationship with its avian hosts.

Key words: *Trypanosoma* spp., blood parasites, wild birds, molecular phylogeny, biodiversity, 18S-rDNA.

INTRODUCTION

Birds were firstly found parasitized by *Trypanosoma* (Kinetoplastida, Trypanosomatidae) in 1885 by Vasily Danilewsky. This original description relates the species of *Trypanosoma avium* in European owls' blood samples. Since then, avian trypanosomes have been recorded in a wide variety of bird species around the world (Bennett 1961, Votýpka et al. 2002, Sehgal et al. 2006, Reeves et al. 2007, Sebaio et al. 2010, Schoener et al. 2018, Taşçi et al. 2018, Pornpanom et al. 2019, Elikwo et al. 2020), with the largest concentration of investigations on the diversity and occurrence of avian trypanosomes related to the Northern Hemisphere (Valkiunas et al. 2011). In Brazil, data on the event of nominal species of avian trypanosomes are old and rare, and reports on this parasite are limited to studies with hemoparasites in general, where trypanosomes were described secondary (Cerqueira 1906, Lutz & Meyer 1908, Splendore 1910, Carini & Botelho 1914, Plimmer 1914, Pessoa 1935, Lucena 1938, Lainson et al. 1970, Bennett & Lopes 1980, Dias et al. 1984, Woodworth-Lynas et al. 1989, Ribeiro et al. 2005, Sebaio et al. 2010, 2012).

Historically, avian trypanosomes have been identified through morphological and morphometric analysis of the trypomastigote forms presented in blood smears (Demaree & Marquardt 1971). However, in cases where the host presents low parasitemia, the high variation in the trypomastigotes forms makes identification at species' level problematic (Valkiunas et al. 2011). Furthermore, the struggle in species identification is intensified by the presence of more than one trypanosome species parasitizing the host. Muss in taxonomy is also one of the causes of scarce information on the diversity of species of avian trypanosomes in Brazil. The confusion on taxonomy of trypanosome species is also attributed to the descriptions supported only on host specificity, without morphological and molecular data or *in-vitro* culture (Votýpka & Svobodová 2004, Valkiunas et al. 2011).

The most common method for molecular characterization of avian trypanosomes is direct Sanger sequencing of PCR amplicons. Nonetheless, it can hinder the detection of multiple genetic lineages, meaning that trypanosome co-infections can remain undetected, mainly when one trypanosome lineage is present at a lower abundance than the other (Carr et al. 2009, Fantin et al. 2013). Thus, studies should use alternative techniques such as culturing, cloning, or species-specific PCR assays to resolve co-infections related to avian trypanosomes and their genetic diversity (Paparini et al. 2011).

Throughout the taxonomic history, more than 100 species of avian trypanosomes have been described globally based on morphological characteristics of trypomastigote forms in the peripheral blood of birds, geographic location, and, mainly, the host in which the parasite occurred, assuming the host-parasite specificity (Baker 1956, Molyneux 1973, Zidková et al. 2012). However, some studies suggest that the number of species may be less than previously estimated (Votýpka & Svobodová 2004, Valkiunas et al. 2011). The insertion of molecular biology analysis and phylogenetic reconstructions has helped to access accurately the trypanosome diversity in birds. In this context, the use of molecular markers, such as 18S-rDNA (Votýpka et al. 2002, Votýpka & Svobodová 2004) and GAPDH (Hamilton et al. 2004) is interesting for the characterization of *Trypanosoma* genotypes and the interpretations of the relationships between host, parasite, and the environment (Sehgal et al. 2001). In addition, these genetic regions have been used as targets for DNA Barcoding assays (Hutchinson & Stevens 2018, Lemos et al. 2015), revealing aspects of the natural history of trypanosomes. Due to the limitations of direct PCR sequencing used to identify avian trypanosomes, this study aimed to use cloning and genetic sequencing assays to investigate polyparasitism and molecular diversity of the 18S-rDNA of *Trypanosoma* spp. in wild birds of the Brazilian Atlantic Forest positives by direct microscopy.

MATERIALS AND METHODS

Birds' trapping, blood collection, and microscopic analysis

The samples were collected between the years 2013 to 2019 in two Brazilian Atlantic Forest areas. The Botanical Garden of the Federal University of Juiz de Fora (JB) (21º44'04.32''S and 43º22'10.48''W) and the Itatiaia National Park (ITA) (22°22'31''S and 44°39'44''W). The collection sites are characterized by a tropical climate going through a cold and dry season between May and September and a hot and humid season between October and April (Alvares et al. 2013). The birds' trapping was carried out using mist nets (12 meters long by 3 meters high with 36 mm mesh), arranged in linear sets of five catches, totaling a

transect of 60 meters. The collections took place for five consecutive days once each month, being extended before sunrise until noon (6 hours of duration). The nets were checked every 15 minutes. After capture, all birds were marked with metal washers provided by the Research Center for Wild Bird Conservation (CEMAVE/IBAMA), identified through field guides (Sigrist 2014), photographed, weighed, and measured to confirm identification. Soon after, passerines were restrained by hand without leather gloves, blood was collected from the birds after local cutaneous asepsis with 70 ° GL alcohol-soaked cotton through extravasation of the blood from the brachial vein with the aid of a sterile needle (13 x 4.5 mm), and the birds were released.

Blood samples were collected in 1.5mL Eppendorf microtubes and stored in -20 °C until DNA extraction. Blood smears were prepared still in field and fixed in methanol for 3 minutes after drying. Subsequently, the slides were submersed in Giemsa staining solution (1:10), for 40 minutes. An Olympus BX-51 light microscope (Olympus, Tokyo, Japan), equipped with an Olympus Evolt E-330 digital camera, was used to examine the slides at a magnification × 600. After blood smears' cytological examination, each bird specimen positive for *Trypanosoma* sp. was selected for molecular analysis.

All procedures were approved by the Ethics Committee on the animal use of the Federal University of Juiz de Fora, under Protocol No. 042/2012, and by the authorization and information system in biodiversity (SISBIO), request number 29268-3 and 55195-2.

Total DNA extraction and polymerase chain reaction (PCR)

The *Trypanosoma*-positive samples in the cytological examinations were selected for genomic DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega®, Madison, Wi, USA) according to the manufacturer's recommendations. Samples were quantified by spectrophotometer Nanodrop® ND-2000 (Nanodrop Technologies, DE, USA), and standardized at a concentration of 20 ng/µL. The total DNA was stored at -80 °C until the molecular analysis by PCR. The DNA samples were submitted to a PCR targeting the *Trypanosoma* spp. 18S-rDNA sequence. The reactions were performed using the primers 18STnF2 (5'-CAACGATGACCCCCCTGAATTGGGGA-3') and 18STnR3 (5'-TGCTCGACATATATTGCATATAC-3'), which amplify approximately 780 bp (Geysen et al. 2003).

Three independent PCR were performed for each sample positive for *Trypanosoma* sp. in blood smears (Figure 1). The PCR reactions were performed at a final volume of 25 µL, containing: 1x of Taq DNA polymerase buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 0.8 µM of each primer, 1U of Platinum SuperFi II DNA Polymerase – High-Fidelity enzyme (Invitrogen®), and 100 ng of genomic DNA. Thermocycling conditions were: 94 °C per 4 min followed by 40 cycles of 94 °C per 1 min, 58 °C per 1 min 30s and 72 °C per 2 min, and a final extension at 72 °C per 4 min. *Trypanosoma vivax* was used as the positive control and ultrapure water was used as a negative amplification control. The *T. vivax* DNA was obtained from bovine animals with a high parasitic load in blood smears, confirmed by PCR and DNA sequencing. PCR products were analyzed by agarose gel electrophoresis (2%) in Tris-acetate-EDTA buffer (TAE) at 5 V/cm, stained with ethidium bromide and photographed under UV light. The DNA extraction process, the PCR reactions, the inclusion of the DNA in the PCR mixture, and the electrophoresis were performed in separate rooms, following a unidirectional workflow to avoid any risk of contamination.

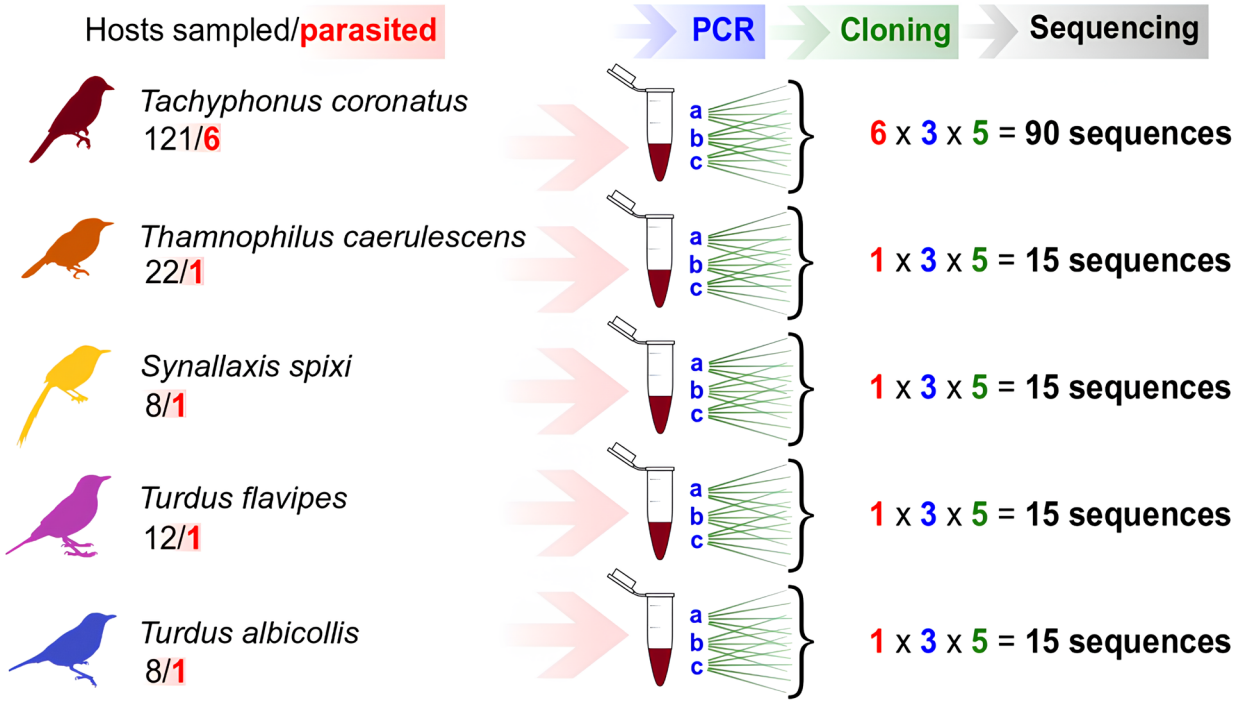

Figure 1. Graphical representation of the amplification and cloning strategy performed in positive samples for avian trypanosomes.

Cloning and Sequencing analysis

Three independent PCR were performed for each sample positive for *Trypanosoma* sp., following the conditions described above. The resulting amplicons were subjected to the purification process with the Wizard SV Gel and PCR Clean-Up System kit (Promega®, Madison, WI, USA) and cloned in a pGEM-T® Easy Vector System vector (Promega, Madison, WI, USA), following the recommendations of their manufacturers. Plasmid DNA from five positive clones from each PCR was isolated by the PureYield™ Plasmid Miniprep System kit (Invitrogen, Carlsbad, CA, USA.) totalizing 15 sequences for each specimen of bird evaluated (Figure 1). The cloned PCR amplicons were sequenced with primers 18STnF2 and 18STnR3 by Sanger method using ABI 3730 DNA Analyzer equipment (Applied Biosystems/ Perkin Elmer, CA, USA) at the Human Genome Studies Center, University of São Paulo (USP).

Diversity and Phylogenetic analysis

Phylogenetic reconstruction was performed using a dataset containing seven sequences of the 18S-rDNA fragment from *Trypanosoma* sp. obtained in the present work (JB01, JB02, JB03, JB04, JB05, ITA01, and ITA02 codes that will be deposited soon) and a set of identified sequences obtained from the GenBank database. Sequences of crocodilian trypanosomes (*T. ralphi* and *T. grayi*) were used as external group. The sequences were aligned in the MAFFT software (Katoh et al. 2019) and then visually inspected. After removing poorly aligned positions with GBlocks (Talavera & Castresana 2007) but including the gaps, a matrix with 2083 base pairs was obtained. The inference of the *Trypanosoma*'s phylogeny was conducted under a Maximum Likelihood (ML) analysis implemented in the RaxML program (Stamatakis 2014), using the K80 + GAMMA + I model (Kimura 1980, Yang 1998), which was chosen as the best model in

JModelTest implemented in MEGA X (Kumar et al. 2016). The support values of the clades were evaluated using the RaxML bootstrap auto convergence criterion (Stamatakis 2014) with 1000 pseudo-replicates. The Bayesian inference (BI) analysis was done using the MrBayes in XSEDE v.3.2.6 program (Ronquist et al. 2012), after a million generations into two simultaneous and independent simulations of Markov Chain Monte Carlo, excluding 25% of the generated trees ("Burn-in"). The MEGA X software (Kumar et al. 2016) was employed to calculate the genetic divergence between the lineages obtained in this study, following the program's standard configuration.

RESULTS

A total of 10 samples were positive for *Trypanosoma* spp. in cytological analysis, as follows: 8.33% (1/12) from *Turdus flavipes* Vieillot, 1818 and 12.5% (1/8) from *Turdus albicollis* Vieillot, 1818 captured at ITA; and 4.95% (6/121) from *Tachyphonus coronatus* (Vieillot, 1818), 4.54% (1/22) from *Thamnophilus caerulescens* Vieillot, 1818, and 12.5% (1/8) from *Synallaxis spixi* Vieillot, 1818 captured at JB (Figure 1). In this study, it was not possible to perform morphometric analysis of *Trypanosoma* spp. due to the low parasitemia and high variation in the trypomastigote forms observed in blood smears (Figure 2). Trypomastigote morphotypes have been described on the basis of distinct morphological characteristics. Morphotype 1 has a fusiform appearance, with less stained areas, an intense purple colour, an oval/ameboid nucleus in the medial/anterior part, a kinetoplast at the anterior end and a prominent thin wavy membrane. In morphotype 2, the trypomastigotes are large, fusiform, with a more dilated medial/posterior part, a pale purple colour and different nuclear and kinetoplast

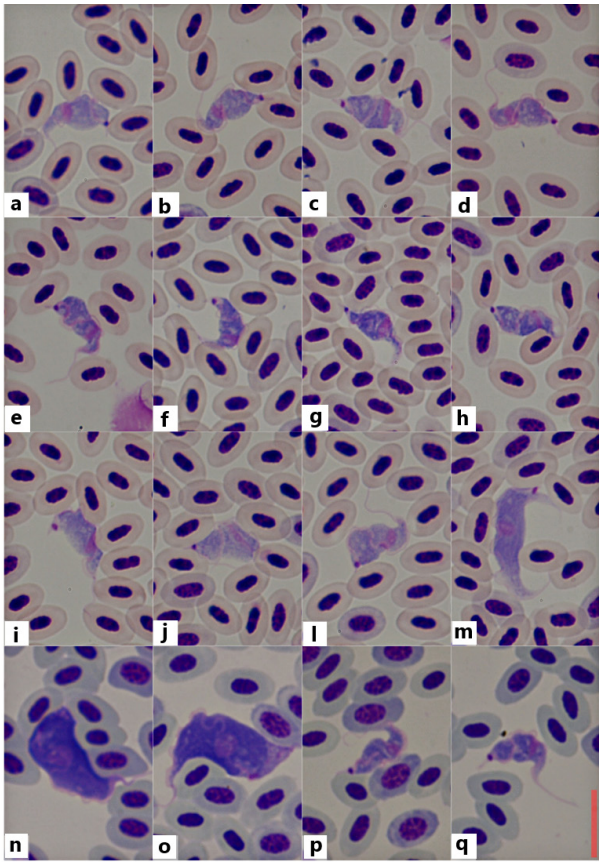

Figure 2. Morphotypes of *Trypanosoma* sp. were delineated in Giemsa-stained blood smears from wild birds, observed at a magnification of ×600 in Olympus DP73 camera on BX-51 Olympus microscope. Notably, trypomastigote forms identified as morphotype 1 were discerned in *Thamophilus caerulescens* (a–d), *Tachyphonus coronatus* (e–h), *Tachyphonus coronatus* (i–l) and *Synallxis spixi* (q–p). Morphotype 2 was exclusively observed in *Tachyphonus coronatus* (m), while Morphotype 3 was observed in *Synallxis spixi* (n–o).

characteristics to morphotype 1. Morphotype 3 presents large trypomastigotes with a prominent body, fusiform shape, intense purple colouring, a large central nucleus, a weakly prominent kinetoplast in various positions and a slightly prominent undulating membrane, similar in size to the parasite body. Trypomastigote forms identified as morphotype 1 were discerned in *T. caerulescens* (Figure 2a-d), *T. coronatus* (Figure 2e-h), *T. coronatus* (Figure 2i-l) and *S. spixi* (Figure 2q-p). Morphotype 2 was exclusively observed

in *T. coronatus* (Figure 2m), while Morphotype 3 was observed in *S. spixi* (Figure 2n-o).

All positive samples in cytological analysis were selected for the polyparasitism and molecular diversity study of *Trypanosoma*. The number of sequences obtained from each bird species is shown in Figure 1. Of the six specimens of *T. coronatus* that were naturally infected with *Trypanosoma* sp., five lineages were identified. (Figure 3). All *Trypanosoma* sp. lineages obtained from *T. caerulescens*, *S. spixi*, *T. flavipes*, and *T. albicollis* were identical. In *Trypanosoma* spp. recovered from *T. coronatus,* adopting the strategy of independent cloning PCR, two *Trypanosoma* spp. 18S-rRNA lineages (JB01 and JB02) were considered a sister group of *T. avium* and *T. thomasbancrofti*, and three was closed related to *T. bennetti* (JB03, JB04, and JB05) (Figure 3). The lineages JB01, JB02, JB03, JB04 and JB05 occurred in *T. coronatus*. The evolutionary distance between JB03 and JB04, JB03 and JB05 and between JB04 and JB05 was 0.8% and 0.7%, respectively (Table I). The lineage JB04 also occurred in the species *T. caerulescens* and *S. spixi*, and the lineage ITA01 occurred in the species *T. albicollis* and *T. flavipes*. The average evolutionary distance between all *Trypanosoma* spp. 18S-rRNA sequences obtained in this study were 0.02, according to the evolutionary distance data (Table I). It was possible to observe that the same lineage of *Trypanosoma* sp. can infect different bird species. Likewise, a bird species can be infected by different *Trypanosoma* spp. lineages.

The new lineages were grouped in different positions in the tree topology recovered in this work (Figure 3). Lineage JB02, recorded in *T. coronatus*, is a sister group of the lineages of the species *T. avium* and *T. thomasbancrofti*, while lineage JB01, also recorded in *T. coronatus*, is a sister group of the previously cited lineages. Although there is a little genetic divergence

Table I. Evolutionary distance between the new lineages of *Trypanosoma* sp. described in this study in each bird species studied. The gray gradient positively indicates an increase in similarity values. The lineage names represent the location of records. JB: Botanical Garden of the Federal University of Juiz de Fora. Brazil; ITA: Itatiaia National Park. Brazil.

New *Trypanosoma* sp. lineages – This study			**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Trypanosoma	**Host**	**Lineage**										
1. *Trypanosoma* sp.	*Turdus albicollis*	**ITA01**										
2. *Trypanosoma* sp.	*Turdus flavipes*	**ITA01**	0									
3. *Trypanosoma* sp.	*Tachyphonus coronatus*	**JB04**	0.013	0.013								
4. *Trypanosoma* sp.	*Tachyphonus coronatus*	**JB04**	0.013	0.013	0							
5. *Trypanosoma* sp.	*Tachyphonus coronatus*	**JB03**	0.017	0.017	0.007	0.007						
6. *Trypanosoma* sp.	*Tachyphonus coronatus*	**JB03**	0.017	0.017	0.007	0.007	0					
7. *Trypanosoma* sp.	*Tachyphonus coronatus**	**JB01**	0.034	0.034	0.039	0.039	0.043	0.043				
8. *Trypanosoma* sp.	*Tachyphonus coronatus**	**JB05**	0.01	0.01	0.008	0.008	0.01	0.01	0.039			
9. Trypanosoma sp.	*Tachyphonus coronatus*	**JB02**	0.041	0.041	0.038	0.038	0.041	0.041	0.008	0.04		
10. *Trypanosoma* sp.	*Thamnophilus caerulescens*	**JB03**	0.017	0.017	0.007	0.007	0	0	0.043	0.01	0.041	
11. *Trypanosoma* sp.	*Synallaxis spixi*	**JB03**	0.017	0.017	0.007	0.007	0	0	0.043	0.01	0.041	0

*** Same *Tachyphonus* coronatus specimen. In this specimen, two Lineages of *Trypanosoma* spp. were detected (JB01 and JB05).**

between these sequences, we do not rule out the possibility that strains JB01 and JB02 are new taxa (Figure 3). Lineages JB03 and JB05, found in *T. coronatus* and JB04, founded in *T. coronatus, T. caerulescens*, and *S. spixi,* were grouped into *T. bennetti* clade. Lineages ITA01 and ITA02, identical and from different host species, were grouped into a clade containing *T. irwini* lineage, a parasite found in Koalas (*Phascolarctos cinereus*) (McInnes et al. 2009). Although lineages JB01, JB02, JB03, JB04, and JB05 of *T. coronatus* have been collected in the Botanical Garden of the Federal University of Juiz de Fora (JB), they were grouped in distinct positions in the phylogenetic tree, suggesting potentially high molecular diversity of trypanosomatids in birds.

DISCUSSION

We found in our analysis of blood smears one to three trypomastigotes per blood smear, with high morphological variability. Due to this fact, it was not possible to perform the morphological analysis of trypomastigotes using the current species description models (Votýpka et al. 2002, Valkiunas et al. 2011, Pornpanom et al. 2019). Also, the small number of trypomastigotes in blood smears did not allow the evaluation of co-infection by more than one *Trypanosoma* species, a well reported fact in parasitological studies with free-living host species (Votýpka et al. 2004, Valkiunas et al. 2011, Zidková et al. 2012). Many factors can influence the shape of trypomastigotes like temperature, density of hosts population, and maturity (Silva et al. 2005). The *Trypanosoma* genus can infect several vertebrate hosts and have a strong pleomorphism, that is, high variability in forms according to their development. The trypomastigote form of the *Trypanosoma* genus has historically shown to be pleomorphic in several studies with vertebrate hosts, such as

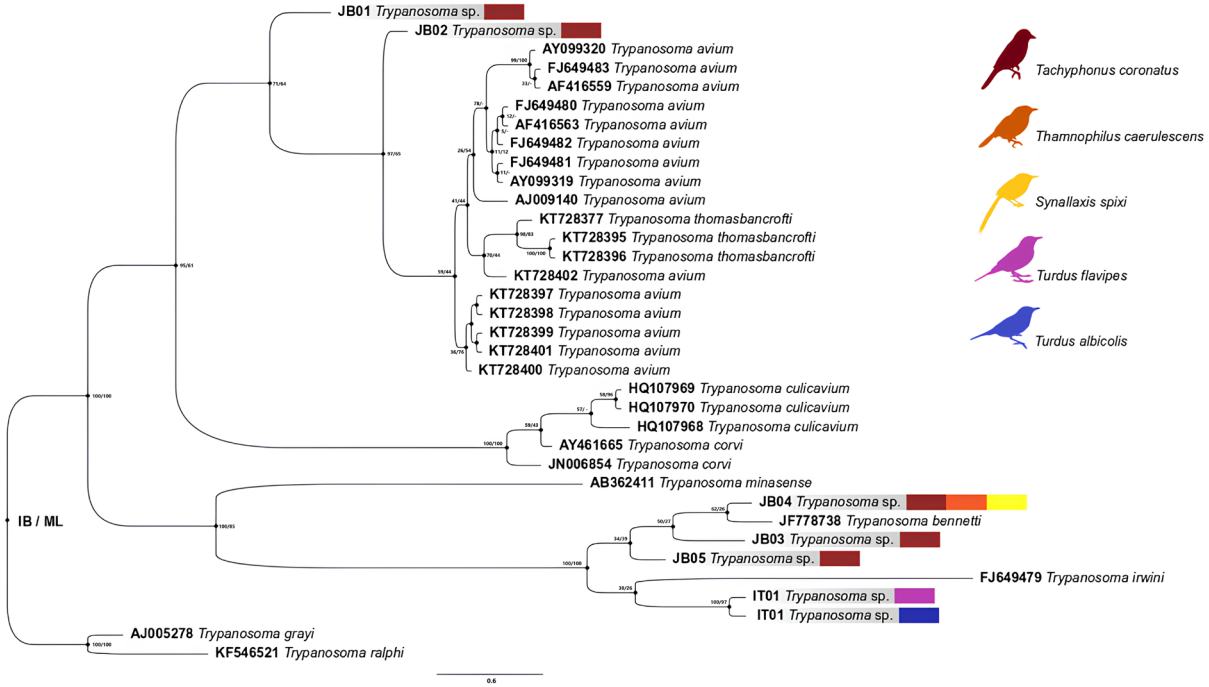

Figure 3. Phylogenetic tree of the 18S rDNA of avian trypanosomes inferred by Bayesian Inference under the evolutionary model K80 + GAMMA + I. The values close to the nodes represent the posterior probability values and bootstrap, respectively. The lineages obtained in this study are marked in colors according to hosts species. The bar represents four substitutions for positions of 100 nucleotides.

fish (Lemos et al. 2015), anurans (Ferreira et al. 2015), reptiles (Dirie et al. 1991), birds (Molyneux 1973), and mammals (Hoare 1929), suggesting that this is a characteristic shared by this genus of parasites independently of the host (Appanius 1991). Despite the experimental studies in birds realized by Molyneux & Gordon (1975), some *Trypanosoma* species can be distinguished by using morphological methods.

Although there are some studies in which birds trypanosomatids were not on focus, other studies include an in-depth morphological characterization of trypomastigotes, accompanied by information on the phylogenetic position of taxa, such as for *Trypanosoma gallinarum* Bruce, Hamerton, Bateman, Mackie and Bruce, (1911) (Sehgal et al. 2006), *Trypanosoma culicavium* Votýpka et al. (2012), *Trypanosoma anguifomis* and *Trypanosoma poligranularis* Valkiunas et al. (2011). However, these works are an exception in a context where an essential part of the described avian trypanosomatids species was poorly illustrated and often published in non-specific parasitology journals (Appanius 1991, Valkiunas et al. 2011), making most of the original descriptions of species inaccessible for morphological comparison and hindering advances in the complex diversity of the group. In the present study, the absence of morphological and morphometric data does not allow us to identify the *Trypanosoma* species found only because of their phylogenetic proximity and evolutionary distance, requiring a deeper morphological study and perhaps cultivation of these parasites. In 2012, Zídková et al. (2012) conducted a comprehensive study on avian trypanosome biodiversity, drawing upon a substantial number of isolates from both avian hosts and vectors. Employing a combination of molecular and morphological techniques, they successfully discerned approximately 11 putative avian trypanosome species. Despite the potential for a greater number of avian trypanosome species in birds, the count estimated by Zídková et al. (2012) is notably lower when compared to the 100 avian trypanosome species documented in the literature. The present study represents the pioneering use of molecular tools to explore the genetic diversity of *Trypanosoma* parasites in wild birds in Brazil, significantly enhancing public databases with the incorporation of six previously uncharacterized lineages.

In this research, an efficient molecular method was designed to detect *Trypanosoma* spp. with high sensitivity, making possible to detect co-infections. The strategy used was to clone products from different PCRs and sequence several clones to increase the probability of identifying different lineages or species of trypanosomes in the same sample. Using these methods, this study was pioneer in perform the molecular characterization of avian trypanosomes in Brazil, raising the information of high diversity of *Trypanosoma* in birds, and making possible to investigate the diversity of avian trypanosomes in other regions and biomes. Seven new 18S rDNA sequences of avian trypanosomes was made available in public databases. Also, we point out that the new lineages support the global diversity of *Trypanosoma*, which is still profoundly underestimated (Zidková et al. 2012).

The methodological approach used in this work (cloning and sequencing analysis) allowed the observation of polyparasitism between lineages and species of avian trypanosomes. Despite the limited sample size and lower bird species richness, a remarkable array of trypanosome strains was uncovered in wild birds. The Brazilian Atlantic Rainforest, designated as a vital biodiversity hotspot, offers a promising ecosystem for investigating avian trypanosomes, a group of parasites that has been overlooked in recent decades. Furthermore, the application of

large-scale sequencing techniques may prove invaluable in elucidating genetic diversity and prevalence of polyparasitism, as exemplified in the case of trypanosome species identified in marsupials (Barbosa et al. 2017). Using next-generation sequencing, Barbosa et al. (2017) and Cooper et al. (2018) demonstrated that the prevalence of polyparasitism by *Trypanosoma* spp. in marsupials was significantly higher than the prevalence of single infections. Thus, the large-scale sequencing method can reveal a greater genetic diversity and prevalence of polyparasitism in avian trypanosomes than the sequencing strategy employed in this study.

The discovery of the same lineages in different host species allowed to demonstrate the unspecific relationship betweenthe trypanosome's lineages and their hosts, a fact also observed in other studies (Sehgal et al. 2001, Votýpka et al. 2004, Votýpka & Svobodová 2004, Sehgal et al. 2006, Taşçi et al. 2018, Pornpanom et al. 2019). About the species *T. coronatus* captured in JB, it was possible to observe five lineages in their host species and the co-infection by two lineages of *Trypanosoma* in a single sample. One of these *Trypanosoma* lineages recorded in *T. coronatus*, JB04, was also found in the species *T. caerulescens* and *S. spixi*. These results allow us to reaffirm Votýpka et al. (2004) findings that demonstrated that *Trypanosoma avium* and *Trypanosoma corvi* could parasitize the same avian host and being transmitted by different species of Ceratopogonidae. Field studies supported by molecular biology have shown that, in nature, *Trypanosoma* species can infect a wide range of host species in avian communities (Sehgal et al. 2001, Votýpka et al. 2004, Taşçi et al. 2018, Pornpanom et al. 2019).

Borges et al. (2021) investigated Trypanosoma's phylogeny using trypanosomatids' sequence and question the avian clade's monophyly occurred in our study. Avian clade paraphilia has been pointed out in some molecular studies, highlighting the presence of mammal trypanosomatids as *T. irwini* (koala) and *T. minasense* (neotropical primates) grouped among bird trypanosomatids (Votýpka et al. 2004, Hamilton et al. 2007, Šlapeta et al. 2016). According to Kostygov et al. (2021), the paraphyletic Avian clade is divided into three monophyletic subgenera: *Avitrypanum* (*T. corvi* and *T. culicavium*) is closer to the subgenus *Trypanomorpha* (*T. avium*, *T. gallinarum*, and *T. thomasbancrofti*), and both form a sister group with the artiodactyl trypanosomatids. The subgenus *Ornithotrypanum* (*T. anguiformis*, *T. bennetti*, *T. everetti*, *T. naviformis*, and *T. polygranularis*) is less related to the other groups of bird trypanosomatids and closer to the species *T. irwini*, found in Koalas. In our study, the lineages JB01 and JB02, although evolutionarily closed to *T. avium* and *T. thomasbancrofti*, could not be identified as being species already described based on morphological data or new taxa that need a formal description. The strains JB03, JB04, and JB05 that grouped with *T. bennetti* demonstrated the low specificity of this taxon and prompt further studies of geographic distribution and parasite specificity. Lineages ITA01 and ITA02, identical and from different host species, were grouped into a clade containing a lineage of *T. irwini*, a koala parasite, and raises a co-evolutionary investigation of host exchange (bird-mammals). Although still inconclusive about a safe definition of species, phylogenetic analysis allows retrieving other equally relevant information.

In the present study, the small number of trypomastigotes found in blood smears of birds, their high pleomorphism, and the co-infections shown by molecular analysis preclude the identification of *Trypanosoma* at species level. The phylogenetic position and the evolutionary distance of the lineages found were not enough

for the species identification. Currently, studies that use various tools to assess the diversity of *Trypanosoma* species, such as optical and electron microscopy, culture, and molecular data with multiple markers, are almost non-existent in avian parasitic trypanosomes compared to human parasitic trypanosomes. This study fills a significant gap in sampling and knowledge of the diversity of avian *Trypanosoma* lineages in South America. The present study is the first to access molecular diversity and polyparasitism by avian trypanosomes in Brazil. It was possible to reveal seven new sequences of the 18S-rDNA of avian trypanosomes, contributing mainly to studying the group's global diversity. Furthermore, the wide distribution of the parasite and its non-specific relationship with its avian hosts were reaffirmed.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant number 429638/2018-3], and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) (PPM2016, grant number PPM-00734-16), Brazil.

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How to cite

DUARTE RG ET AL. 2024. Molecular diversity and polyparasitism of avian trypanosomes in the Brazilian Atlantic Rainforest. An Acad Bras Cienc 96: e20230629. DOI 10.1590/0001-3765202420230629.

Manuscript received on June 5, 2023;
accepted for publication on November 16, 2023

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