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Unveiling *Trypanosoma* spp. diversity in cattle from the state of Rio de Janeiro: A genetic perspective[1]

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ABSTRACT.- Abreu A.P.M., Santos H.A., Paulino P.G., Jardim T.H.A., Costa R.V., Fernandes T.A., Fonseca J.S., Silva C.B., Peixoto M.P. & Massard C.L. 2024. **Unveiling *Trypanosoma* spp. diversity in cattle from the state of Rio de Janeiro: A genetic perspective.** *Pesquisa Veterinária Brasileira 44:e07467, 2024*. Departamento de Epidemiologia e Saúde Pública, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Rodovia BR-465 Km 7, Zona Rural, Seropédica, RJ 23890-000, Brazil. E-mail: huarrisson@yahoo.com.br

Cattle trypanosomiasis imposes significant economic burdens on the global livestock industry. The causative agents of this disease belong to the protozoan *Trypanosoma* genus. This study aims to perform detection (parasitological and molecular) and genetic characterization to analyze *Trypanosoma* spp. in cattle from 15 municipalities in the state of Rio de Janeiro, focusing on the *18S* rDNA and *Cathepsin-L* (*CatL*) gene of *Trypanosoma vivax* and *Trypanosoma theileri*. A total of 389 blood samples from 15 dairy cattle farms in the state of Rio de Janeiro were collected, and DNA was extracted for subsequent PCR amplification of *Trypanosoma* spp. *18S* rDNA and *CatL* genes. The resulting amplicons underwent sequencing and alignment for phylogenetic analysis, with comparisons made to GenBank isolates. Concerning parasitological analysis, blood smears presented 4.4% of positive cattle (n=17/389) for *T. vivax* and did not show any trypomastigote forms of *T. theileri*. The absolute frequency of *Trypanosoma* spp. through molecular detection targeting *18S* rDNA was 11.6% (45/389). However, when performing species-specific PCRs, the *T. vivax* frequency, determined through *CatL* gene PCR, was 12.8%, and the *T. theileri* frequency was 3.6%. Phylogenetic analysis based on *18S* rDNA revealed low diversity among *T. vivax* sequences, suggesting potential host segregation. This study emphasizes the high frequency of positive samples by PCR when compared to direct parasitological exams. Additionally, *T. vivax* phylogeny targeting *18S* rDNA hints at sequence clustering related to host species. Importantly, this investigation unveils, for the first time in Rio de Janeiro's cattle, the circulation of *T. theileri* lineage ThI, encompassing genotypes IIB and IF. This discovery expands our understanding of this parasite's geographical distribution and genetic diversity.

INDEX TERMS: Cattle, trypanosomiasis, *Trypanosoma* spp., genetic diversity, phylogenetic analysis, protozoan pathogens.

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RESUMO.- [**Revelando a diversidade de *Trypanosoma* spp. em bovinos do estado do Rio de Janeiro: uma perspectiva genética.**] A tripanossomíase bovina impõe significativos ônus econômicos à indústria pecuária global. Os agentes causadores dessa doença pertencem a protozoários do gênero *Trypanosoma*. Objetivou-se, com este estudo, realizar detecção (parasitológica e molecular) e caracterização genética de *Trypanosoma* spp. em bovinos de 15 municipalidades do estado do Rio de Janeiro, com foco na sequência *18S* rDNA e no gene *Cathepsin-L* (*CatL*) de *Trypanosoma vivax* e

Trypanosoma theileri. Um total de 389 amostras de sangue de 15 fazendas leiteiras no estado do Rio de Janeiro foram coletadas, e o DNA foi extraído para subsequente amplificação por PCR dos genes *18S* rDNA e *CatL* de *Trypanosoma* spp. Os amplicons resultantes foram submetidos a sequenciamento e alinhamento para análise filogenética, com comparações realizadas com isolados do GenBank. No que se refere à análise parasitológica, os esfregaços de sangue apresentaram 4,4% de bovinos positivos (n=17/389) para *T. vivax* e não mostraram nenhuma forma tripomastigota de *T. theileri*. A frequência absoluta de *Trypanosoma* spp. através da detecção molecular visando *18S* rDNA foi de 11,6% (45/389). No entanto, ao realizar PCRs específicos de espécies, a frequência de *T. vivax*, determinada por PCR do gene *CatL* foi de 12,8%, e a frequência de *T. theileri* foi de 3,6%. A análise filogenética com base no *18S* rDNA revelou baixa diversidade entre as sequências de *T. vivax*, sugerindo uma possível segregação de hospedeiros. Este estudo enfatiza a alta frequência de amostra positiva pela PCR quando comparada com a parasitológica direta. Além disso, a filogenia de *T. vivax* direcionada ao *18S* rDNA sugere agrupamento de sequências relacionado à espécie hospedeira. Importante destacar que esta investigação revela, pela primeira vez no gado do Rio de Janeiro, a circulação da linhagem ThI de *T. theileri*, abrangendo os genótipos IIB e IF. Esta descoberta amplia nosso entendimento sobre a distribuição geográfica e diversidade genética desse parasito.

TERMOS DE INDEXAÇÃO: Tripanossomíase, bovinos, *Trypanosoma* spp., diversidade genética, análise filogenética, protozoários patogênicos.

INTRODUCTION

Trypanosoma (*Duttonela*) *vivax* is the etiological agent of the animal trypanosomiasis, which harms primarily small and large ruminants, causing significant damage to livestock worldwide (Paiva et al. 2000). In Brazil, *T. vivax* was first observed by Floch & Lajudie (1943) and, nowadays, this pathogen is reported in all countries of Latin America and the Caribean isles (Fetene et al. 2021). Notably, the pathogen has been implicated in economic losses in the state of Rio de Janeiro, where it adversely impacts milk production and contributes to animal mortality (Costa et al. 2020).

Trypanosoma (*Megatrypanum*) *theileri* stands out as the largest trypanosome found in mammalian blood, which can infect cattle, buffaloes, and deer. Generally considered a low-pathogenic species causing latent infections in seemingly healthy cattle, recent studies have shed light on the negative repercussions of *T. theileri* parasitemia in beef and dairy cattle (Amato et al. 2019, Suganuma et al. 2022). Evidence of *T. theileri* infections in cattle has unveiled the presence of parasite lineages in Brazil, as documented by Rodrigues et al. (2006). Moreover, reports focusing on *T. theileri* infections in Brazil, particularly in the western Amazon region, have elucidated the existence of distinctive lineages, including the TthI and TthII groups, each encompassing diverse genotypes (Pacheco et al. 2018). Even though Gonçalves et al. (1998) performed the first report in the state of Rio de Janeiro, there is scarce information concerning *T. theileri* infection in cattle in this Brazilian state.

Sequencing analysis provides information for genetic identification and allows new trypanosomes to be organized within a phylogenetic tree to be compared with other isolates, aiding in the taxonomy of these parasites. For this, the *18S* gene has been widely used for trypanosomatids, as it has an alternative and variable domain, allowing it to be amplified by primers in the conserved region (Adams et al. 2010). Furthermore, genes that express cysteine protease (*cathepsin L-like*) participate in protein metabolism and control the functions of immune system evasion, cell invasion, apoptosis, virulence, and pathogenicity (Sajid & Mckerrow 2002). Garcia et al. (2011) reported that the *CatL* gene of trypanosomatids is encoded by a multigene family organized as multiple repeated copies in tandem expanded by gene duplications; this is because these genes are apparent to a concerted evolution, which allows them to be used in the evolutionary studies of kinetoplastids supporting the phylogenies based on the SSR rDNA and *gGAPDH* genes.

The present study aims to detect (parasitological and molecular) and genetically characterize *Trypanosoma* spp. through the *18S* rDNA and *CatL* genes in naturally infected cattle in Rio de Janeiro. It also intends to phylogenetically analyze the sequences of *T. vivax* and *T. theileri* obtained in the present study and compared to other sequences from Rio de Janeiro and worldwide.

MATERIALS AND METHODS

Ethical approval. This study was approved by the Research Ethics Committee of the "Universidade Federal Rural do Rio de Janeiro" (CEUA/UFRRJ) under protocol number 5931200217.

Sampling. From March 2016 to December 2018, cattle blood samples were collected by convenience in 15 municipalities in the state of Rio de Janeiro with a history of bovine trypanosomiasis outbreaks, according to a previous study (Costa et al. 2020) that was performed in the same target area of the present study. The respective georeferencing is shown in Figure 1. Many properties had a background in importing animals from dairy cattle auctions in the states of Minas Gerais or São Paulo. These states had previously reported cases of *Trypanosoma vivax* infections, naturally leading to trypanosomiasis in cattle. A total of 389 samples were collected from the bovine coccygeal vein, and the blood was placed in sterile 4mL tubes with 10% EDTA anticoagulant. Following parasitological processing, the remaining blood was stored in 1.5mL microtubes at -80°C for molecular analysis.

Blood smear evaluation. Blood samples collected for this study were utilized to create blood smears. These slides were fixed in methanol for 10 minutes, stained using the Giemsa method (1:10), and examined under optical microscopy with an immersion objective (100x) and 10x ocular magnification to directly visualize the parasites. Approximately 100 fields were evaluated per slide. The species identification was based on the visualization of morphologic characteristics following the taxonomic key proposed by Hoare (1972).

DNA extraction. Genomic DNA extraction was performed using the Wizard® DNA Genomic Purification Kit (Promega, Madison/WI, USA), following the manufacturer's recommendations. Samples were eluted in 100µL elution buffer and quantified in a spectrophotometer Nanodrop® ND-2000 (NanoDrop Technologies, DE, USA). DNA sample concentrations varied and were standardized at 20ng/µl. The DNA was stored in microtubes at -80°C for molecular analysis.

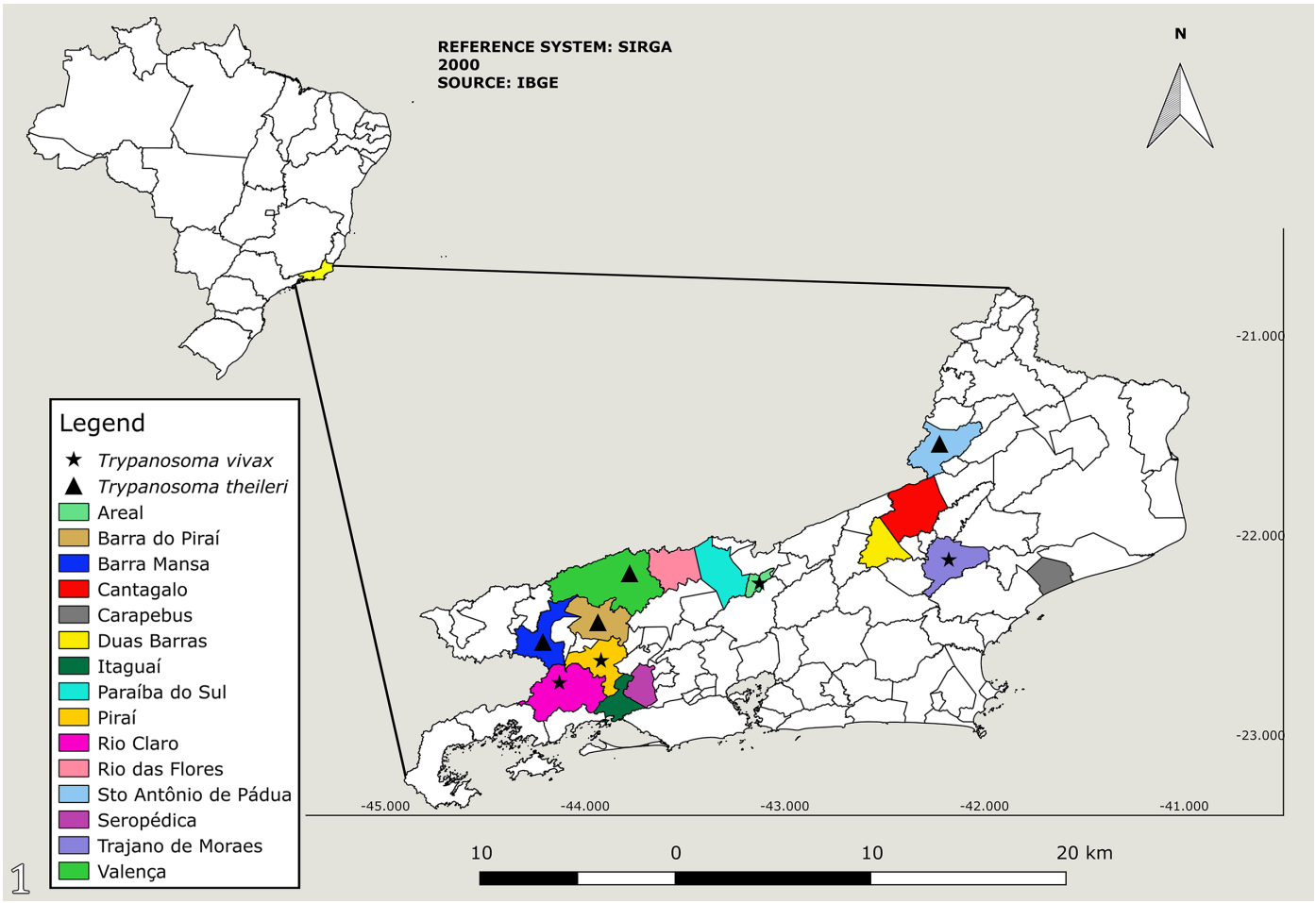

Fig.1. Map displaying the geographic distribution of municipalities in the State of Rio de Janeiro where cases of *Trypanosoma* spp. have been diagnosed in cattle.

Molecular detection. All DNA samples underwent PCR assays targeting the *Trypanosoma 18S* rDNA*, T. vivax CatL,* and *Trypanosoma theileri CatL.*

The *Trypanosoma 18S* rDNA-PCR was performed using the primers 18STnF2 (5'-CAA CGA TGA CAC CCA TGA ATT GGG GA-3') and the 18STnR3 (5'-TGC TCG ACC ATA TAT TGC ATA TAC-3'), amplifying approximately 780bp (Geysen et al. 2003). Molecular detection was executed with adaptations in the concentration of dNTPs, $MgCl_2$, and Taq, as described below. The final reaction volume was 25µL containing 13.5µL ultrapure water, 1X enzyme buffer, 2.5mM $MgCl_2$, 0.4mM each deoxyribonucleotide triphosphate, 0.8mM each primer, 1.5U of Taq DNA polymerase, and 5µL of genomic DNA. Thermocycling conditions were: 94°C for 4 min followed by 40 cycles of 94°C for 1 min, 58°C for 1 min 30 s and 72°C for 2 min, and final extension at 72°C for 4 min.

The cPCR targeting the *T. vivax CatL* gene applied the primers DTO 154 (5'-ACA GAA TTC CAG GGC CAA TGC GGC TCG TGC TGG-3') and DTO155 (5'-TTA AAG CTT CCA CGA GTT CTT GAT GAT CCA GTA-3'), amplifying 500bp (Cortez et al. 2009). Adaptations in dNTPs, $MgCl_2$, and Taq concentrations were executed as described below. The final reaction volume was 30µL containing 17.45µL of ultrapure water, 1X amplification buffer, 3mM $MgCl_2$, 0.4mM of deoxyribonucleotide triphosphate, 0.5µM of each primer, of 1.5U Taq DNA polymerase, and 5µL of genomic DNA. Thermocycling conditions were: 95°C for 3 min followed by 40 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, and final extension at 72°C for 10 min.

The cPCR targeting the *T. theileri CatL* gene applied the primers TthcatL1 (5'-CGT CTC TGG CTC CGG TCA AAC-3') and DTO155 (5'-TTA AAG CTT CCA CGA GTT CTT GAT GAT CCA GTA-3'), amplifying approximately 273bp (Yokoyama et al. 2015), with adaptations in the concentration of dNTP, $MgCl_2$ and Taq, as described below. The final reaction volume was 25µL containing 14.8µL of ultrapure water, 1X enzyme buffer 2.5mM $MgCl_2$, 0.4mM of each deoxyribonucleotide triphosphate, 0.5mM each primer, 1.0U of Taq DNA polymerase, and 4µL of genomic DNA. Thermocycling conditions were: 94°C for 3 min followed by 40 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min.

Positive controls for *T. vivax* (MH184514) and *T. theileri* (MN966843) were derived from cows exhibiting high parasitic loads in blood clots, subsequently confirmed via DNA sequencing of the *18S* rDNA, ensuring the absence of co-infection. Negative controls consisted of ultra-pure water for PCR, meticulously handled inside and outside the laminar flow. Amplification products were resolved on a 1.5% agarose gel at 100 V for 40 min, stained with ethidium bromide, and photographed.

PCR product purification. Following the manufacturer's recommendations, the resulting amplicons were subjected to a

purification process with the Wizard SV Gel and PCR Clean-Up System kit (Promega®, Madison/WI, USA). The quantification of the purified products could be estimated on a 2% agarose gel, where a volume of 5µL of the purified products was homogenized in 1.5µL of sample buffer. Four microliters of Low DNA Mass™ Ladder (Invitrogen®) were used as a molecular mass scale.

Sequencing. The sequencing was performed employing the Sanger method at the "Centro de Pesquisas sobre o Genoma Humano e Células-Tronco" (Center for Research on the Human Genome and Stem Cells – CEGH-CEL) of the "Universidade de São Paulo" (USP).

The sequencing chromatogram analyses and contig construction employed the CLC Main Workbench Version 20.0.3 (Qiagen Bioinformatics). Subsequently, the similarity of each sequence was assessed through the BLAST algorithm[5].

Phylogenetic inference. The newly recovered sequences from *T. theileri* and *T. vivax* (genes *18S* rDNA and *CatL*) were merged into four distinct datasets according to parasite species and genes obtained from the GenBank database. The dataset's external phylogeny lineages (outgroups) are chosen according to the parasite species' evolutive distance and lineages available in GenBank. Hence, the sequences under the accession number "KT283911" and "KT728391" encoding *Trypanosoma thomasbancrofti 18S* rDNA; "KF414042" and "KF413898" encoding *Trypanosoma congolense CatL* gene were applied as the outgroup.

The *T. vivax CatL* dataset compromised 44 sequences with 451bp. Subsequently, the *T. vivax 18S* rDNA dataset was built, consisting of 35 sequences with 628bp. The *T. theileri CatL* dataset comprised 87 sequences with 228bp. Finally, the *T. theileri 18S* rDNA dataset included 37 sequences with 617bp.

All datasets were aligned in the MAFFT software (Katoh et al. 2017) with standard options, followed by visual inspection. A homogeneous matrix of base pairs was obtained after removing misaligned positions with GBlocks (Talavera & Castresana 2007).

The phylogenetic analysis of *T. vivax*/*T. theileri* was carried out through Bayesian inference (BI) using MrBayes in XSEDE v. 3.2.6 (Ronquist et al. 2012), available on the CIPRES server (Miller et al. 2010). The BI analysis involved two simultaneous independent Markov Monte Carlo chain simulations, running for 2.5 million generations, excluding 25% of the generated trees as the 'Burn-in' phase. Subsequently, predictive models including the methods General Time Reversible (GTR), Hasegawa-Kishino-Yano (HKY), Tamura-Nei (TN93), Tamura 3-parameter (T92), Kimura 2-parameter (K2); Jukes-Cantor (JC) was created in order to find the best substitution model for phylogenetic analysis. The models presenting the lowest Bayesian information criterion (BIC) scores are considered to describe the substitution pattern the best. Non-uniformity of evolutionary rates among sites was also included in the models using a discrete Gamma distribution (+G) with five rate categories and assuming that a certain fraction of sites are evolutionarily invariable (+I). The best replacement model was conducted in MEGAX (Kumar et al. 2018) with model T92+G for *T. vivax CatL*, model K2+G for *T. vivax 18S* rDNA, model K2+G for *T. theileri CatL*, and model K2 for *T. theileri 18S* rDNA.

[5] Available at <https://blast.ncbi.nlm.nih.gov/Blast.cgi> Accessed on Aug. 3, 2020.

The estimates of evolutionary divergence between sequences were calculated for *18S* rDNA using the K2 model, and the rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The number of base substitutions per site from between sequences is shown. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

RESULTS

The frequency of *Trypanosoma vivax* observed in the blood smear technique was 4.4% of positive cattle (n=17/389) of the analyzed samples. Seventy-six percent (n=12/17) of these animals came from the municipality of Areal, 11% (n=2/17) came from Piraí, 5% (n=2/117) came from Rio Claro, and 11% came from Santo Antônio de Pádua (Fig.1). Unfortunately, trypomastigote forms of *Trypanosoma theileri* were not detected in the blood smears.

The frequency of *Trypanosoma* spp. identified through *18S* rDNA-PCR was 11.6% (45/389). Conversely, the frequency detected via *T. vivax CatL*-PCR was 12.8% (50/242), and *T. theileri CatL*-PCR was 3.6% (14/389) described in Table 1. It is noteworthy that a considerable proportion of positive samples were obtained by PCR compared to the results of the direct parasitological exam.

In terms of *T. vivax* positivity, Trajano de Moraes (50%), Rio Claro (28.6%), Santo Antônio de Pádua (23.7%), and Areal (15.7%) exhibited the highest rates compared to other municipalities. However, it's noted that Trajano de Moraes had a relatively small sample size, which could artificially inflate the prevalence. The three municipalities with the highest positive frequency for *T. theileri* were Trajano de Moraes (50%), Valença (10%), and Barra do Piraí (22.2%). However, Trajano de Moraes also had a limited sample size, potentially skewing the prevalence (Table 1).

Table 1. Frequency of *Trypanosoma vivax* and *Trypanosoma theileri* in each municipality based on *cat-L* PCR detection

Municipality	Animals	*T. vivax* positives	*T. theileri* positives	Coninfections
Areal	146	23(15.7%)	2 (1.3%)	0
Barra do Piraí	9	0	2 (22.2%)	0
Barra Mansa	22	0	2 (9.1%)	0
Cantagalo	8	1(12.5%)	0	0
Carapebus	4	0	0	0
Duas Barras	14	0	0	0
Itaguaí	14	0	0	0
Paraíba do Sul	17	1 (5.9%)	0	0
Piraí	16	2(12.5%)	0	0
Rio Claro	14	4(28.6%)	0	0
Rio das Flores	15	0	0	0
Santo Antônio de Pádua	76	18(23.7%})	4(5.3%)	2 (2.63%)
Seropédica	2	0	0	0
Trajano de Moraes	2	1(50%)	1 (50%)	0
Valença	30	0	3 (10%)	0
TOTAL	389	50 (12.8%)	14 (3.6%)	2 (0.51%)

All sequences obtained in this study were deposited in GenBank, and the accession numbers are provided for *T. vivax 18S* rDNA (KX766453; MN966704; MN966705; MN966707; MN966708; MN966709 MN966847; MN966728; MH184514; MH184515; MH184516; MH184517; MH184518), *T. vivax CatL* (PP278989; PP278990; PP261311; PP278991; PP261305; PP261306; PP261307; PP261308; PP261309; PP261310; PP261311) *T. theileri 18S* rDNA (MN966843; MN966844; MN966845; MN966846) and *T. theileri CatL* (OL352187; OL352185; OL352186; OL352188).

In the phylogenetic analysis, *T. vivax 18S* rDNA sequences formed a distinct group separate from the outgroup (*Trypanosoma congolense*) supported by a high posterior probability value (100%). The newly recovered lineages (Fig.2, yellow circles) clustered with other Brazilian and international sequences. The topology also revealed a lineage obtained of wild antelope (Fig.2, purple circles) from Mozambique, an external cluster group mentioned above. Moreover, there was a paraphyletic group exclusively comprising cattle genotypes from Ethiopia.

In the evolutionary reconstruction of the *T. vivax CatL* gene, the newly identified cattle lineages recovered from Rio de Janeiro (Fig.2, yellow circles) were grouped with sequences of domestic cattle from East Africa, specifically Burkina Faso, Ghana, and Nigeria (Fig.3, colored in red). Concurrently, in the same analyses, lineages from West Africa compromised an internal clade with lineages of domestic cattle and wild antelope from Mozambique, Zambia, and Kenia (Fig.3, colored in blue).

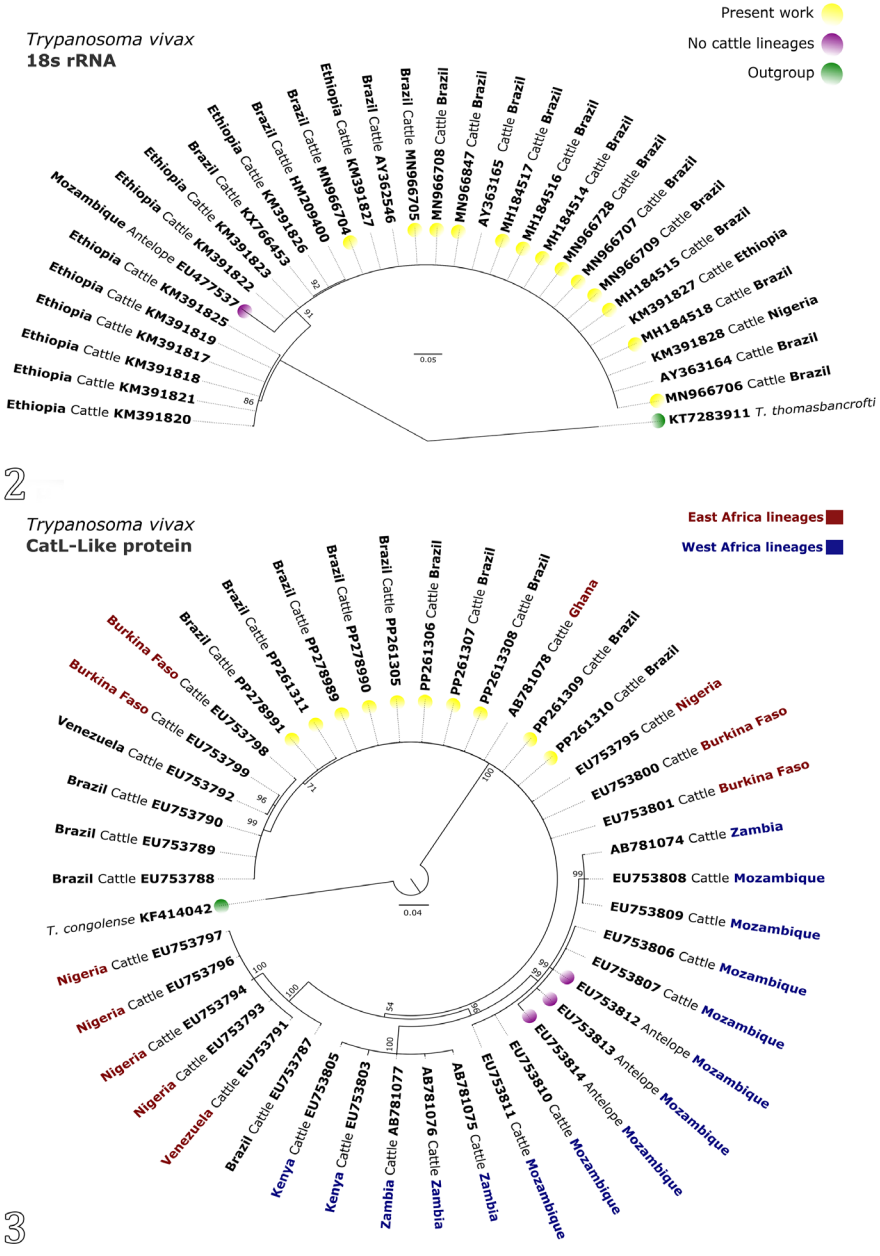

Fig.2-3. Bayesian phylogenetic trees of (**2**) *Trypanosoma vivax: Cathepsin-Like* gene and (**3**) *18S* rDNA sequences, with posterior probability values at key nodes.

Furthermore, it was noticed that there is a smaller distance between isolates from Brazil and Nigeria, with 0.002, which is directly related to their position in the same clade. In contrast, a more substantial evolutionary distance of 0.04 was noted between the Brazilian sequences (cattle) and those from Mozambique (antelope).

The phylogenetic analysis of *T. theileri* applying the conserved molecular marker *18S* rDNA reveals sequences forming a distinct cluster apart from the outgroup (*Trypanosoma congolense*) supported by a high posterior probability value (100%). The recent lineages recovered (Fig.4, yellow circles) position within the clade of cattle lineages from Italy, Japan, and the USA, alongside bison lineages from Poland (Fig.4, purple circle); and the *Glossina fuscipes* lineage from Central Africa Republic (Fig.4, purple circle). In the topology, non-cattle lineages of bison from Poland and deer from Japan (Fig.4, purple circles) group with cattle lineages from the USA and the UK.

By contrast, phylogeny targeting *T. theileri CatL* displays the grouping of both *T. theileri* lineages and genotypes. The phylogenetic reconstruction of the *T. theileri CatL* gene shows the placement of the newly identified Brazilian lineages (Fig.5, yellow circles) from cattle of Rio de Janeiro in three different positions. The four sequences obtained in the present study were included in the TthI lineage. Lineages OL352186/OL352188 group with cattle lineages from Brazil, Philippines, Sri Lanka, USA, and Vietnam (Genotype IB), while lineages OL352185/Ol352187 form an internal clade containing cattle lineages from Brazil and Sri Lanka (Genotype IF). The topology also reveals the grouping of *T. theileri CatL* lineages from buffalo (Fig.5, purple circles) in different tree positions, indicating a lack of specific clade formation from lineages of this host.

Finally, the evolutionary distance shows a smaller genetic distance between isolates from Japan, the Central African Republic, and some sequences from the USA, ranging from 0.002 to 0.005. In contrast, a more substantial evolutionary distance is observed between Brazil and Poland, measuring from 0.07 to 0.017.

DISCUSSION

Although bovine trypanosomiasis is considered endemic, it is capable of causing sporadic outbreaks by *Trypanosoma vivax* following the introduction of infected animals into disease-free areas and by *Trypanosoma theileri* in immunocompromised hosts or when genetically different strains are present (Rodrigues et al. 2003, 2006).

The prevalence of *Trypanosoma* spp. in cattle causally depends on the sensitivity of the detection method applied (Bastos et al. 2020). The prevalence of *T. vivax* was lower (31%) of positive animals from Nigeria reported by Gier et al. (2020), which may have occurred due to inappropriate mass therapeutic interventions carried out by rural producers (Giordani et al. 2016) or the chronic nature of the disease in the region, which, according to Batista et al. (2009), renders parasitemia undetectable in laboratory tests. However, the frequency found in the present study is higher than the prevalence (8.84%) in Brazil's central region (Bastos et al. 2020). The frequency of *T. theileri* was lower than expected (10 to 90%), according to Lee et al. (2010).

The significant number of positive PCR results to the findings of the direct parasitological examination is notable. This is consistent with Ahmed et al. (2013), Fikru et al. (2014), Mossaad et al. (2020), and Gier et al. (2020), who assert that molecular biology is a fundamental tool in the diagnosis of animal trypanosomiasis, as it exhibits high sensitivity and specificity even in cases of low parasitemia, which frequently occurs in cattle infected with *T. theileri* or chronically infected with *T. vivax*.

Although the first report of *Trypanosoma* species infection in cattle in the American continent dates back to the last century (Leger & Vienne 1919), this current investigation marks the first molecular characterization study of *Trypanosoma* spp. in the region.

Molecular screening targeting conventional *Trypanosoma* molecular markers has not only confirmed the etiological cause of vector-borne disease outbreaks in cattle from the state of Rio de Janeiro. However, it has also unveiled the presence of two *Trypanosoma* species in the targeted area, namely, *T. vivax* and *T. theileri*.

Phylogenetic inferences based on the *18S rDNA* of *T. vivax* present a distinct divergence between clades supported by high posterior probability values (Fig.2-3). One clade displays sequences exclusively from Ethiopia, including isolates from *Glossina* spp. free and infested areas (Fikru et al. 2016). There is also a restrictive clade exhibiting solely the *T. vivax* isolated of nyala antelope from Mozambique. Finally, all Brazilian isolates (including sequences obtained in this study) cluster in the clade with other *T. vivax* sequences from countries such as Venezuela, Kenya, Nigeria, and other Ethiopia isolates. This evolutionary history mirrors the phylogenetic network presented by Fikru et al. (2016), indicating a group exclusively for Ethiopian isolates, a clade with a unique sequence represented by the *T. vivax* isolate from nyala antelope in Mozambique, and a third group featuring Brazilian isolates clustering with isolates from Nigeria and Ethiopia. These evolutionary inferences suggest that the host species may influence this parasite's phylogeny. *T. vivax* has already been recorded in more than 20 species of domestic and wild hosts (Fetene et al. 2021). This disparity reflects the great genetic richness of *T. vivax* and how vital studies with wild species are to understand the parasite's transmission dynamics and reservoir hosts.

By contrast, the *T. vivax* evolutionary history targeting the *CatL* gene displays the formation of four distinct clades, which corroborates that this is a polymorphic molecular marker compared to the *18S rDNA*. Notably, the *T. vivax- CatL* phylogeny shows Brazilian cattle sequences from Rio de Janeiro grouping with isolates from West Africa (Ghana, Nigeria, Burkina Faso). Moreover, clear segregation is observed between Brazilian and West African sequences and East African sequences (Kenya, Mozambique, and Zambia). Similar results were reported by Cortez et al. (2009), Pimentel et al. (2012), Garcia et al. (2011), Rodrigues et al. (2015), and Jaimes-Dueñez et al. (2018), which observed that *Trypanosoma vivax* isolates from West Africa are more congruent to South America isolates. This clustering reinforces the historical hypothesis of *T. vivax* introduction in South America through infected cattle imported into the Caribbean islands from West Africa. It shows that Rio de Janeiro *T. vivax* lineages have the same origin.

Regarding the *T. theileri* phylogeny, the *18S* rDNA grouped all sequences in a unique clade, which is expected of this traditional molecular marker due to the high conservancy

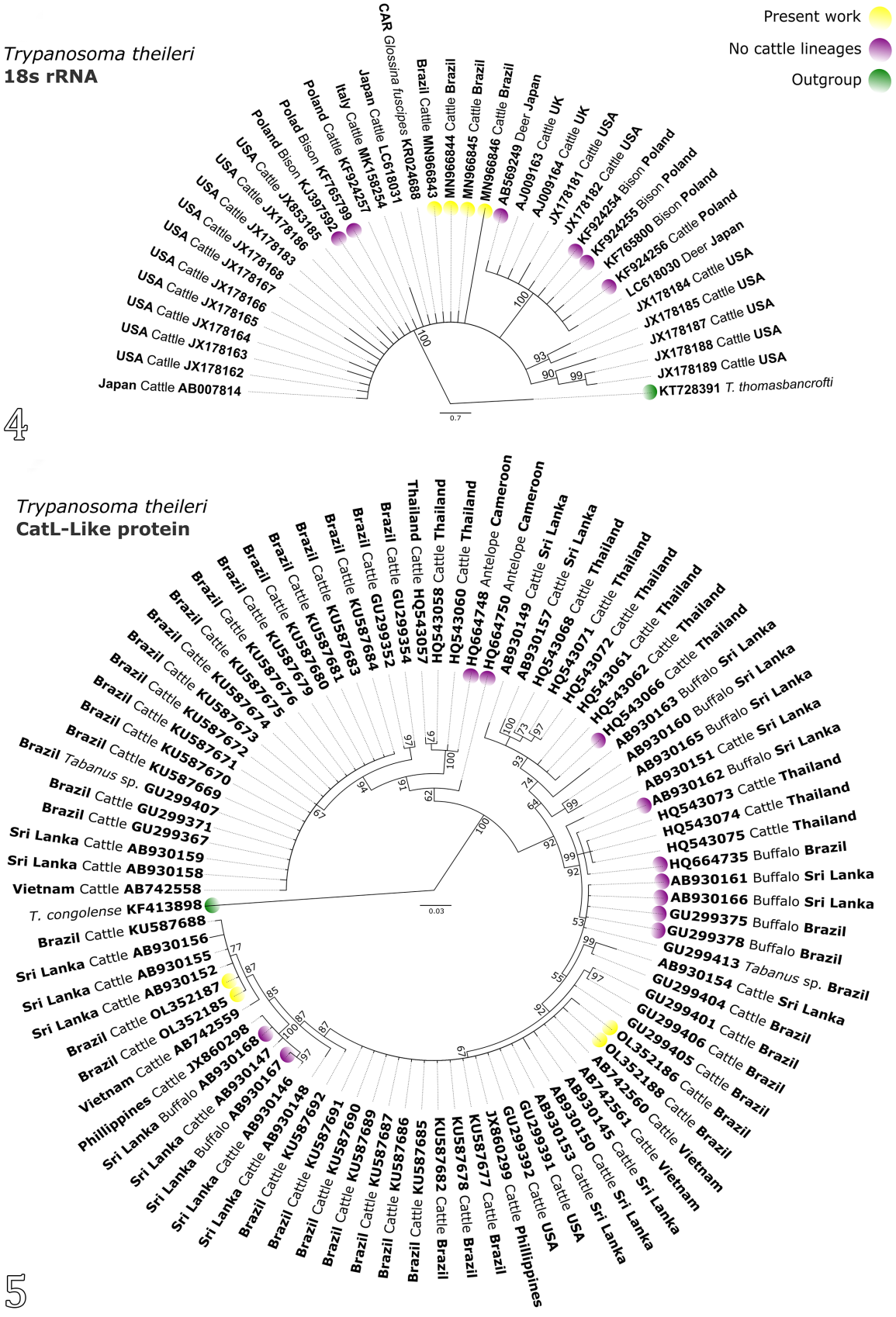

Fig.4-5. Bayesian phylogenetic trees of (**4**) *Trypanosoma theileri*: *Cathepsin-Like* gene and (**5**) *18S* rDNA sequences, with posterior probability values at key nodes.

of this sequence (Rodrigues et al. 2006, Pacheco et al. 2018). Nevertheless, the *CatL* gene effectively highlights *T. theileri* lineages and genotypes in this research (Fig.4-5). Previous phylogenetic studies identified both *T. theileri* lineages, compromising six genotypes distributed across Brazilian regions (Rodrigues et al. 2008, Pacheco et al. 2018). The current investigation aligns with Rodrigues et al. (2008), reporting the ThI lineage genotype IB circulation in the Southeastern Brazilian region. However, the genotype IA was not detected in the present study. The genotype IB has been reported in other Brazilian regions as well as the Philippines, Sri Lanka, and Iran (Rodrigues et al. 2008).

Moreover, an investigation targeting cattle from the western Amazon in the North region revealed the presence of the genotype IF from the ThI lineage in Brazil for the first time (Pacheco et al. 2018). The genotype IF was previously reported only in cattle from Sri Lanka and Vietnam in the Asian continent (Sivakumar et al. 2013, Yokoyama et al. 2015). Interestingly, the current study also unveils the circulation of genotype IF occurring in the state of Rio de Janeiro, a geographically distant area from the previous report. This result underscores the incomplete characterization of *T. theileri* diversity, particularly in Brazil, with its extensive geographical expanse. Including new sequences from less-explored regions in the previously established *T. theileri* phylogeny will contribute to a comprehensive understanding of this parasite's diversity (Pacheco et al. 2018).

CONCLUSION

The phylogenetic analysis of *Trypanosoma vivax*, particularly focusing on the *18S* rDNA, reveals a noteworthy potential for sequence clustering associated with the host species. Notably, this investigation unveils a novel occurrence of *Trypanosoma theileri* lineage ThI, encompassing genotypes IIB and IF, in cattle from the state of Rio de Janeiro. This discovery contributes to expanding our understanding of the parasite's geographical distribution and genetic diversity in this region, highlighting the need for continued surveillance and research to elucidate the dynamics of *Trypanosoma* infections in livestock comprehensively.

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