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Genotyping of *Mycobacterium leprae* in humans and six-banded armadillos suggests lack of inter-species transmission in Rio Grande do Norte, northeast Brazil

Jessica da Silva Ferreira [a,b,*,1], Emilyn Costa Conceição [b,c,1], João Marcelo Azevedo de Paula Antunes [d], Amanda NB Fontes [b], Sidra Ezídio Vasconcellos [b], Ricardo José de Paula Souza e Guimarães [e], Maurício Lisboa Nobre [f], Ismênia Glauce [g], Douglas McIntosh [a], Philip Noel Suffys [b,*]

[a] *Multiuser Molecular Biology Laboratory, Department of Animal Parasitology, Institute of Veterinary Medicine, Federal Rural University of Rio de Janeiro, Rod. BR 465, km 7, CEP 23890-000 – Seropédica Rio de Janeiro Brazil*

[b] *Laboratory of Molecular Biology Applied to Mycobacteria, Oswaldo Cruz Institute, Fiocruz, Avenida Brasil 4365, CEP 21040-360 – Manguinhos Rio de Janeiro, Brazil*

[c] *South African Medical Research Council Centre for Tuberculosis Research (SAMRC), Division of Molecular Biology and Human Genetics (MBHG), Centre for Epidemic Response and Innovation (CERI), School of Data Science and Computational Thinking, Faculty of Medicine and Health Sciences (FMHS), Stellenbosch University, Cape Town, South Africa*

[d] *Veterinary Hospital Paulo Fernando Cisneiros da Costa Reis, Federal Rural University of Semi-Árido, RN, Av. Francisco Mota, 572 - Bairro Costa e Silva, CEP: 59.625-900 – Mossoró Brazil*

[e] *Evandro Chagas Institute, Health Surveillance Secretariat, Brazilian Ministry of Health, Rod. BR-316, km. 4, Ananindeua, CEP 67113-970 Pará Brazil*

[f] *State Secretary of Public Health of Rio Grande do Norte, Giselda Trigueiro Hospital, Rua Còn. Monte 110, CEP 59037-170, Trigueiro, Natal – Rio Grande do Norte, Brazil*

[g] *Faculty of Health Sciences - FACS/UERN, State University of Rio Grande do Norte, Av. Prof. Antônio Campos S/N, CEP 59625-620, – Mossoró – Rio Grande do Norte, Brazil*

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ABSTRACT

The detection of *Mycobacterium leprae* in non-human hosts, particularly armadillos, has raised questions about their role in the transmission of leprosy. In the United States, *M. leprae* strains present in *Dasypus novemcinctus* armadillos are involved in maintaining the disease. In Brazil, various armadillo species are recognized as naturally infected hosts for *M. leprae*, and the handling or consumption of armadillo meat are considered as potential risk factors for leprosy. However, genetic evidence confirming or refuting this in Brazil is lacking. To address this gap, Single Nucleotide Polymorphism (SNP)-typing and Multiple locus analysis of 16 Variable Number Tandem Repeats (VNTRs) (MLVA) were applied to genotype *M. leprae* DNA of 54 leprosy patients in the city of Mossoró, Northeastern Brazil, and from liver biopsies of 20 specimens of *Euphractus sexcinctus* armadillos, captured in proximal rural municipalities. The SNP type 4 is the most prevalent (n = 23; 71.9%) and MLVA patterns from 35 patients revealed a dominant genotype in eight patients, indicative of significant local transmission. In the case of the armadillo-derived *M. leprae*, except for a single complete 16-locus-based MLVA pattern predominantly partial genotypes were obtained. The VNTR patterns were strongly suggestive for SNP type 4. Notably, the VNTR-based *M. leprae* genotype recorded in armadillos was distinct from those obtained from the human patients. Despite limitations, this investigation suggests intense *M. leprae* transmission among *E. sexcinctus* armadillos but provided no evidence in support of zoonotic transmission to humans, in a region of Brazil where consumption of armadillo meat is a common practice.

* Corresponding authors at: Laboratory of Molecular Biology Applied to Mycobacteria, Oswaldo Cruz Institute, Fiocruz, Avenida Brasil 4365, CEP 21040-360 – Manguinhos Rio de Janeiro, Brazil.
E-mail addresses: jessicasferreira88@gmail.com (J.S. Ferreira), psuffys@gmail.com (P.N. Suffys).
[1] These authors equally contributed.

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1. Introduction

Leprosy, primarily caused by *Mycobacterium leprae* and occasionally by *Mycobacterium lepromatosis* [Han et al., 2008], is a chronic, disfiguring, and disabling disease that has afflicted humanity for millennia [Donoghue et al., 2018]. As a result of effective public health interventions and the availability of multidrug therapy (MDT), a gradual decrease in prevalence in many countries is being observed. Nonetheless, leprosy remains a neglected disease in several others, and it continues to represent a significant public health issue because it affects the skin and nerves, leading to disabilities, particularly in low-income and economically active populations. In 2024, 172,717 new cases of leprosy were registered in 188 countries, Brazil being the second highest with 22,129 new cases, which represented 12.8% of the global total [WHO, 2025].

M. leprae was identified as the etiological agent of leprosy in 1873, but despite over 150 years of research, many aspects of the disease remain poorly understood. One area of ongoing research is the possibility that animals such as armadillos, rodents (including squirrels), and non-human primates may serve as reservoirs involved in leprosy transmission to humans. Several studies have focused on this issue [Truman et al., 2011; Balamayooran et al., 2015; Sharma et al., 2015; Avanzi et al., 2016; Honap et al., 2018; Hockings et al., 2021; Rensing et al., 2021], including a number in Brazil [Lima et al., 2022; de Souza Valois et al., 2024; Nogueira et al., 2025]. To date, the nine-banded armadillo (*Dasypus novemcinctus*) is the only animal species definitively shown to contribute to the maintenance and transmission of leprosy in the Southern states of the USA, where the disease is considered zoonotic [Truman et al., 2011; Sharma et al., 2015]. In North America this species represents the only armadillo present, and its current distribution reflects a relatively recent range expansion from Mexico into the United States. In contrast, Brazil supports a much greater diversity of armadillo species, including the six-banded armadillo (*Euphractus sexcinctus*), and the epidemiological significance of *M. leprae* infections detected in these species remains less clearly defined. The detection of *M. leprae* in various armadillo species (including *D. novemcinctus*) in Brazil, along with data indicating that contact with both six and nine banded armadillos is a risk factor for leprosy in Brazil [Aliaga-Samanez et al., 2025], suggests that leprosy might also be considered a zoonotic disease in Brazil [da Silva et al., 2018]. The ecology of *M. leprae* infection in armadillos in Brazil remains poorly understood. Although several studies have reported the detection of *M. leprae* in wild armadillos, many involve relatively small numbers of animals and employ differing diagnostic approaches, making comparisons between studies difficult. Recent meta-analyses of Brazilian studies have also highlighted the substantial heterogeneity between investigations and the difficulty of predicting infection prevalence across different regions and species. Consequently, while infection in Brazilian armadillos is well documented, robust genetic evidence demonstrating zoonotic transmission comparable to that described in the United States remains limited.

The significant levels of ongoing transmission in several regions of the world observed at the start of the 21st century stimulated the development of novel strategies to comprehend and minimize the spread of *M. leprae*. The discovery and characterization of polymorphic regions in the *M. leprae* genome, including Single Nucleotide Polymorphisms (SNPs), InDels, and Variable Numbers of Tandem Repeats (VNTRs), laid the foundation for the development of highly discriminatory genotyping methods, such as SNP-typing and Multi-Locus VNTR Analysis (MLVA). These techniques have been pivotal in leprosy genotyping research over the past 25 years [Ansari et al., 2025]. More recently, whole genome sequencing (WGS) and targeted Next Generation Sequencing (tNGS)-based assays such as Deeplex-MycLep®, have shown significant potential as robust and highly sensitive tools for extensive multi-locus genetic characterization of *M. leprae* strains [Ansari et al., 2025; Jouet et al., 2023]. Genotyping has been crucial for enhancing our understanding of *M. leprae* phylogeography and was central to deciphering the dynamics of leprosy transmission in several countries, including Brazil, China, India, the Philippines, Thailand, Mexico, Colombia, and the United States [reviewed in [Avanzi et al., 2020; Chokkakula et al., 2020; Finardi et al., 2023; Zhou et al., 2024]].

Although the state of Rio Grande do Norte (RN) has one of the lowest detection rates in the northeast region of Brazil, a notable exception is the municipality of Mossoró that is considered hyperendemic for leprosy [Amorim et al., 2016; Nobre et al., 2015]. The region is home to five species of armadillos, and intriguingly, this region maintains a substantial population of the six-banded armadillo (Euphractus sexcinctus), and the practices of hunting and consumption of these animals are commonplace in both rural and urban populations. Considering this frequent human–animal interaction and previous evidence of infection in this species, E. sexcinctus was selected as the principal focus of the present investigation. Both species are hunted and often held in captivity for fattening in rural communities before being consumed *in situ* or sold at farmers' markets in urban areas [da Silva et al., 2018; Frota et al., 2012]. Infection of specimens of*E. sexcinctus* with *M. leprae*, captured in five rural municipalities proximal to Mossoró, was demonstrated using serological and molecular methods [Ferreira et al., 2020], confirming findings from the northeastern state of Ceará [Deps et al., 2020] and the central-western state of Mato Grosso [Nogueira et al., 2025].

In line with the One Health concept [Deps and Rosa, 2021], comparative genotyping was performed using *M. leprae* DNA samples obtained from human leprosy patients in Mossoró and from the 20 *M. leprae*-infected *E. sexcinctus* specimens collected from rural municipalities nearby. The principal objectives of this study were to characterize the genetic variability of *M. leprae* in six-banded armadillos and in humans in the region and to assess the potential for interspecies transmission, thereby to evaluate the hypothesis of zoonotic leprosy transmission in Northeast Brazil.

2. Materials and methods

2.1. Study design

This descriptive, retro- and prospective study was conducted in RN, Brazil, on *M. leprae* obtained from human and animal samples.

2.2. Ethical considerations

In the case of human patients, all procedures performed were in accordance with the ethical standards of the Institutional Review Board of the State University of RN (CAAE 47,180,115.1.0000.5294) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards [World Medical Association, 2025]. Human participants provided informed consent prior to sample collection in accordance with institutional and national ethical guidelines. For the animal part of the study, the project was approved by the Ethics Committee of The Federal Rural University of Rio de Janeiro (Protocol number 8068, 280,716) and was licensed by The Brazilian Institute for the Environment and Natural Resources (IBAMA) for the capture and collection of wildlife material (Protocol number 50,564–2).

2.3. Study setting characteristics and sampling

Rio Grande do Norte is a small state in the northeast of Brazil with 167 municipalities, an area of 52,809,601 km^2 equivalent to 3.4% of the area of the northeast and 0.6% of the territories of Brazil. According to the demographic census published in 2024, among a population of 3446,071, 77.8% of the inhabitants live in urban areas and 22.2% in rural areas. The state recorded an incidence of 3.51 cases of leprosy per 100,000 inhabitants, a value significantly lower than the other Northeastern states [de Araújo et al., 2024]. However, the municipality of Mossoró, which is the second most populous in the state, reported an

incidence of 66 new cases among 303,792 people in 2021, representing almost half of the new cases in the State and was therefore classified as hyperendemic [do Nascimento et al., 2024]. The city of Mossoró covers an area of approximately 150 km^2, while the municipality, including rural areas, extends over approximately 2110 km^2."

Detailed information in relation to the collection strategy, capture locations, clinical and serological analyses applied to the 20 specimens of *E. sexcinctus* employed herein, were as reported in the study of da Silva Ferreira et al. [Ferreira et al., 2020]. For a better understanding of the present data, the individual and mean body mass of the animals is presented in **Supplementary Table 1**.

Evaluation of a possible relationship between genotype clustering and animal proximity was performed via a spatial distribution analysis including the calculation of the distance between collection sites of the 20 *E. sexcinctus*, using ArcGIS 10.4 software (https://www.arcgis.com/). A daily dispersion of this animal species of 2.25 km^2 was considered. The municipal, state, and federal boundaries were obtained from the Brazilian Institute of Geography and Statistics (https://www.ibge.gov.br/).

A single genotype obtained from *D. novemcinctus* was included in the VNTR analysis for comparative purposes. Given that this specimen originated outside the study area and represents an isolated sample, it was used only to provide biological context for the interpretation of armadillo-associated genotypes and was not considered in the evaluation of transmission dynamics within RN. The DNA sample derived from a specimen of *D. novemcinctus* had been extracted from the liver of an animal captured in the region of Ilha, São Paulo and was kindly provided by Dr. Eduardo Bagagli (UNESP, São Paulo).

2.4. Preparation of Mycobacterium leprae DNA from armadillo and human clinical specimens

The extraction of *M. leprae* DNA for genotyping analyses from animal tissue was conducted using a protocol combining mechanical and enzymatic lysis, followed by DNA purification using the DNeasy kit (Qiagen) according to the manufacturers protocol. Briefly, 25 mg of liver tissue stored in RNAlater (Ambion Inc, USA) was treated with a mixture of collagenase, dispase and DNase for 3 h at 37 °C, followed by centrifugation at 12,000 x *g* for 15 min. After discarding the supernatant, 400 µL of TE buffer (200 mM Tris–HCl, 5 mM EDTA, pH 8.0) was added to homogenize the pellet by repeated pipetting and vortexing prior to transfer to a fresh screw-capped tube containing 0.1 mm silica beads and 400 µL of tris- saturated phenol. Using a Fast Prep homogenizer, tubes were agitated for 45 s followed by centrifugation at 12,000 x *g* during 10 min, and the aqueous phase was transferred to a new microcentrifuge tube. The same volume of phenol-chloroform-isoamyl alcohol (25/24/1) was added and homogenized by inversions and centrifuged at 12,000 x g during 10 min, followed by a new extraction of the aqueous phase with phenol-isoamyl alcohol (24/1). The aqueous phase was again transferred to a new tube and a 1/10th volume of 3 M NaAc, and two volumes of pure ethanol were added and incubated at −20 °C for 1 h, then centrifuged for 20 min at 12,000 x g. The precipitate was resuspended in 20 µL of TE buffer and AL buffer of the DNeasy Blood & Tissue Kit from Qiagen, incubated overnight at 4 °C and submitted to further purification using the DNeasy kit following the manufacturer's guidelines. The DNA was eluted using 100 µL of AE buffer (10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0). In addition to the armadillo samples, the extraction procedures included (negative) controls in duplicate composed of 25 mg of bovine liver.

For extraction of *M. leprae* DNA from human tissue, slit skin smears (SSS) for collection of lymph, were produced as a component of leprosy diagnosis from 54 human patients diagnosed with leprosy at the Regional Laboratory in Mossoró. The SSS, generally from both ear lobes and elbows and occasionally from lesions (data not available), were fixed on microscopy slides by heat and stained using Ziehl-Nielsen for microscopic analysis of acid-fast bacilli (AFB). Slides positive for AFB, that had been stored at room temperature, were submitted to DNA extraction as described by Brum Fontes et al. [Brum Fontes et al., 2012]. Briefly, 25 µl of milliQ water was added to each of the sites on the slide SSS, scraped off with another glass slide and collected in a microcentrifuge tube. The same volume of 15% de Chelex-100 (Sigma-Aldrich) prepared in water was added, incubated for 30 min at 97 °C and centrifuged at 12.000 x g for 10 min with the supernatant transferred to another microcentrifuge tube and stored at −20 °C until further analysis.

2.5. Genotyping based on SNP-typing

For genotyping to the four major SNP-type level, we used PCR-Restriction Enzyme Analysis (REA) as described by Sakamuri et al. [Sakamuri et al., 2009]. Briefly, differentiation of genotypes, 1/2 from 3/4 was obtained by submitting amplicons to *Bst*UI mediated PCR-RFLP analysis of the locus at nucleotide position 2935,685 relative to the TN genome; digestion occurs in case of genotype 3/4 and lack of digestion for genotype 1/2. Differentiation of genotypes 3 and 4 was obtained by *Sml*I mediated PCR-RFLP at nucleotide position 14,676; digestion indicates SNP genotype 4 and lack of genotype 3.

2.6. Genotyping by multiple-locus variable number of tandem repeats analysis (MLVA)-typing

The DNA samples were submitted to genotyping by MLVA, based on the amplification and characterization of 16 VNTRs containing alleles, as described by Kimura et al. [Kimura et al., 2009]. Four multiplex PCR reactions with fluorescently labelled primers were performed with HotStarTaq DNA polymerase (QIAGEN, Hilden, Germany). The amplicons were denatured and subjected to capillary electrophoresis on an ABI3130 Genetic Analyzer platform (Thermo Fisher Scientific, Waltham, USA), including the internal molecular weight size standard LIZ 500 bp (Thermo Fisher Scientific, Waltham, USA). The number of allelic copies was determined using the Peak Scanner software v1.0 (Thermo Fisher Scientific) and compared to a previously calibrated MLVA pattern of the *M. leprae* strain NHDP63. In cases where one or more VNTR(s) were lacking in multiplex reactions, amplification was performed by single-plex PCR [Supply et al., 2006].

2.7. Data analysis

The copy numbers of the VNTR alleles were introduced into an Excel table and imported into BioNumerics v.7.6 (Applied Maths NV - bioMérieux, Belgium). After constructing a similarity matrix using the categorical similarity coefficient and unweighted pair group method with arithmetic mean (UPGMA), we obtained similarity values of the genotypes. In the present study, we initially considered "clusters" as formed by strains with identical genotypes based on 13-loci (not including the three alleles based on AT and TA repeats) [Fontes et al., 2009, Fontes et al., 2017]. When lacking VNTRs for cluster definition, we used the option "indifferent" in the BioNumerics software for comparison.

The allelic diversity (h) at each VNTR locus was calculated using the Hunter-Gaston Discrimination Index (HGDI) equation: $h = 1\text{-}\Sigma xi2\ [n/(n\text{-}1)]$, where n is the number of strains and xi is the frequency of the h allele at the locus. Each locus was classified as "highly discriminant" ($h \geq 0.6$), "moderately discriminant" ($0.3 \leq h \leq 0.6$) or "low discriminant" ($h \leq 0.3$) [Hunter and Gaston, 1988].

We also compared the genotypes obtained in the present study with those available in our "in house" database containing *M. leprae* MLVA based genotypes from 2.164 isolates from eight countries, including over 940 isolates from Brazil that included genotypes of *M. leprae* strains generated during published and unpublished studies

3. Results

3.1. Epidemiologic and demographic data of the leprosy patients

Among the 54 patients from Mossoró, clinical and epidemiologic data were available from only 15 individuals and as such, we could not establish any meaningful association between these data and genotype similarity or clustering.

3.2. Demographic characteristics of Euphractus sexcinctus from RN

The locations where the animals were captured was described in the study of da Silva Ferreira et al. [Ferreira et al., 2020]. Upon spatial analysis of the capture locations of the animals (Fig. 1), we observed clustering when using a radius of 2.25 Km in the regions of Afonso Bezerra, Pendências, Guamaré and Pedro Avelino, corresponding to the daily dispersion of armadillos of this species. Within the Macao municipality, the animals were more dispersed as was also the case for the animals captured in the different municipalities, and therefore less likely to having had contact.

The body mass of the animals was between 0.945 and 2.750 kg with a mean value of 1645 kg (**Supplementary Table 1**). For comparison, the mean mass of an adult *E. sexcinctus* is between 3.2 to 6.5 kg (https://www.cokesmithwildlife.com/six-banded-armadillo-euphractus-sexcinctus;39), indicating that the animals were all juveniles.

3.3. Genotyping of Mycobacterium leprae from human samples

The human slit skin smears provided *M. leprae* SNP types for 42 of the 54 patients, with 32 (71.9%) being SNP type 4, nine (28.9%) SNP type 3; a single patient was infected with *M. leprae* of SNP type 1 or 2.

The MLVA-based genotyping data for the 54 patients is presented in **Supplementary Table 2**. In 43 patients, at least one VNTR was amplified; 12 samples (22%) generated genotypes composed of all 16 VNTRs and 35 samples (65%) presented genotypes with at least 10 alleles. Analysis of allelic diversity demonstrated that the three AT-containing VNTRs presented the highest discriminatory power, followed by (GAA)21, (GTA)9 and (AC)8a, then by the moderately discriminately (AC)9, the poorly discriminatory 27–5, 18–8, 12–5, (AC)8b, 6–7, 23–3 and the non-discrimination alleles containing 6–3, (GGT)5 and 21–3. No difference in discriminatory power of the different alleles in the *M. leprae* genotypes in humans and armadillos was observed, although the number of VNTRs should be viewed as rather small.

None of the patients with the full set of 16 VNTRs presented the same genotype but when excluding the three VNTRs with the highest HGDI (containing AT or TA) and thus maintaining 13 VNTRs, we observed a major cluster of eight isolates, including four (P1, P6, P16 and P19) with complete genotypes, two stains each lacking a single VNTR (P38 and P53) and two strains lacking four and six VNTRs (P33 and P48) (Fig. 2). No other groupings of considerable size were observed.

3.4. Genotyping of Mycobacterium leprae from armadillos

All armadillo samples were positive for the presence of at least one of the 16 VNTR markers but in most samples, only a subset of the alleles were amplified (**Supplementary Table 2**) A single animal (T04), rendered a complete MLVA pattern (16 alleles), one each had respectively 15 (T06), 14 (T01), 13 (T05) and 11 (T03) alleles amplified and from two animals we characterized 10 VNTRs (T02 and T07) (Fig. 2). The decision to include genotypes lacking one or more of the 13 VNTRs was because these data represent the first, albeit imperfect, report on genotype composition for strains of *M. leprae* from Brazilian armadillos.

Among the 20 *M. leprae* strains from *E. sexcinctus*, we identified divergent VNTR copy number in only three alleles among the 118 amplified from the possible total of 320, with the divergence recorded in three distinct specimens. Twelve of the 13 specimens of *E. sexcinctus*, with at least 10 alleles, presented nine copies of AC9, while sample A12 presented eight copies of this allele, the same was seen for the sample of *D. novemcinctus* from São Paulo. Two specimens (A10 and A11) of *E. sexcinctus* had divergent copy numbers, 19 rather than 17 and 16 rather than 25 of the VNTRs AT15 and TA18 respectively. However, besides being excluded for definition of clusters, these markers were amplified in only three and six animals respectively, with the TA18 allele recorded in the sample of *D. novemcinctus* (**Supplementary Table 2**)

Attempts to perform SNP analysis from the armadillo DNAs were unsuccessful due to lack of PCR products. However, based on previously described associations between VNTR copy numbers and SNP types (36,37), our data suggest that the *M. leprae* strains associated with the six-banded armadillos from RN are likely to belong to SNP-type 4.

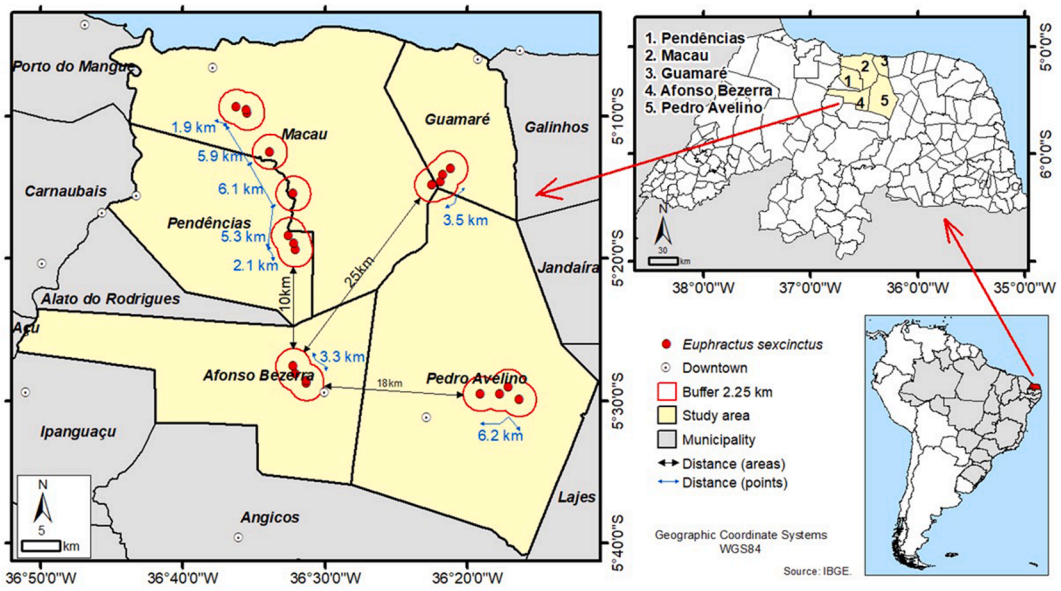

Fig. 1. Localization of the sampling sites from humans and animals. Part A presents the localization of the State of Rio Grande do Norte in Brazil and the South American continent. Part B shows the five Municipal Districts where the 20 different Euphractus sexcinctus were captured and Part C their relative distance and their clustering.

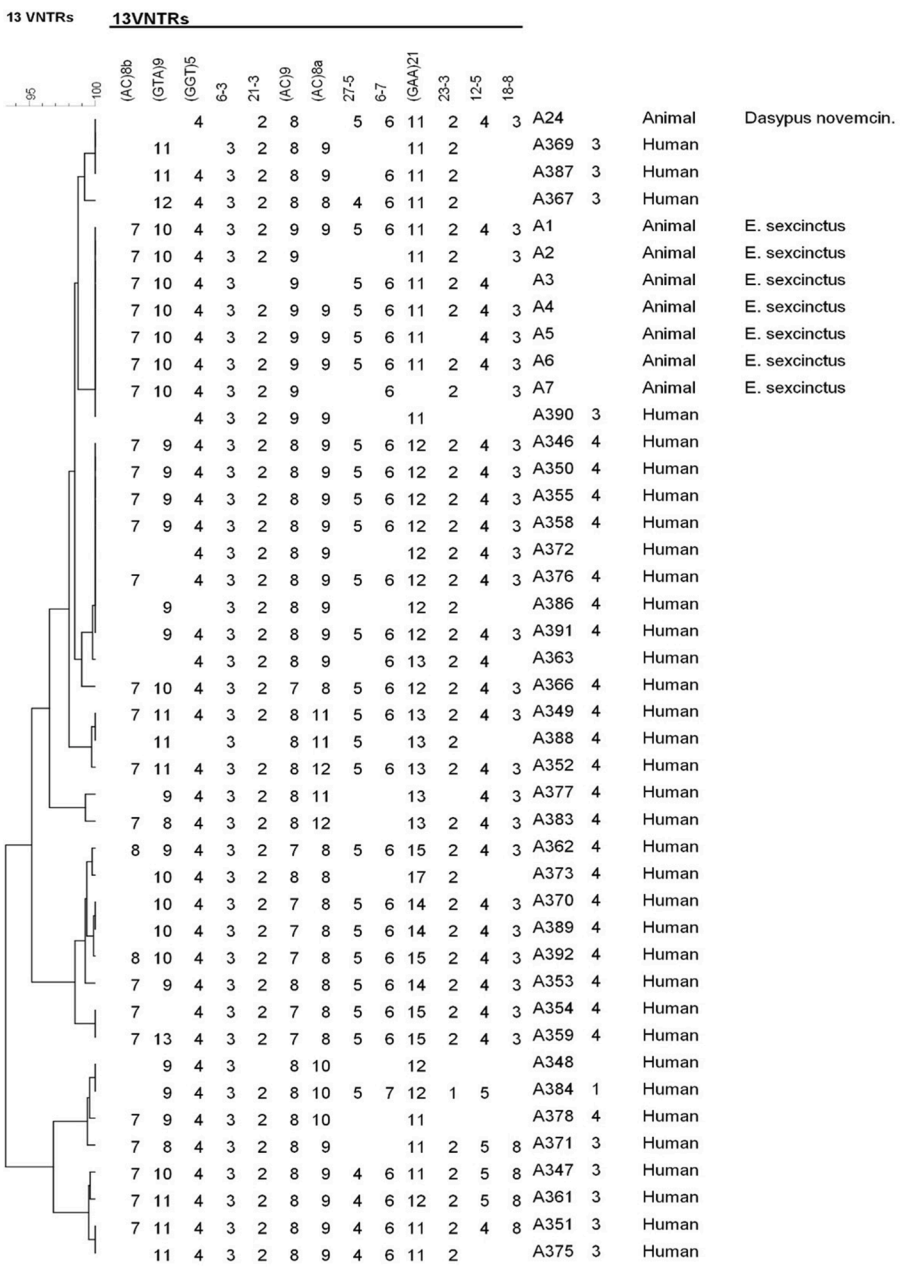

Fig. 2. UPGMA-based representation of the genetic proximity of *Mycobacterium leprae*. The Figure included the number of VNTR copies present in 13 different alleles, followed by the sample name, SNP-type and sample origin.

A comparison between the MLVA profile of the *M. leprae* strains present in Brazilian *E. sexcinctus* and those reported in *D. novemcinctus* from the southern USA is presented in **Supplementary Table 3**. Notable differences were observed for the VNTRS AC8a (9 vs 10), AC8b (7 vs 10), AC9 (9 vs 8) and 12–5 (5 vs 4), the latter probably associated with SNP-type difference between the two species. Unfortunately, we could only compare six alleles from the single *D. novemcinctus* from Brazil and the USA with alleles 6–7 and 12–5 being different, with respectively six and four copies in Brazil and seven and five copies in the US, To what extent these differences are regional or armadillo-species related needs to be further investigated but the 6–7 VNTR six-copy number is observed in all but one samples from RN while the strong relation between SNP-type 3 and four copies of VNTR 12–5 seems maintained.

3.5. Combined analysis of Mycobacterium leprae genotypes derived from humans and armadillos

According to UPGMA based analysis (Fig. 2), along the genotype cluster composed by eight *M. leprae* strains from human origin, we observed another genotype shared by eight *M. leprae* isolates, seven of animals' origin and one from a single human strain. As a caveat, it is pertinent to note that the human representative was defined by only six VNTRs and therefore could simply be clustered due to lack discriminatory power. Moreover, the human strain closest to the armadillo strain is the only from this species carrying nine copies of (AC)9 while the rest presented seven copies.

When comparing the *M. leprae* genotype from *E. sexcinctus* with those in our database, we observed a very similar pattern (12 identical VNTRs) in an isolate (301,019) from a patient from Fortaleza, Ceará that was also of the SNP type 4 genotype [Brum Fontes et al., 2012]. Only small

differences were observed in the VNTRs with highest variability with respectively 15, 21 and 10 copies in AT15, TA18 and GAA21.

4. Discussion

The discovery of naturally infected populations of nine-banded armadillos in the USA in the seventies was initially viewed as advantageous for leprosy research as it provided a model system for multiplication of the organism [Sharma et al., 2013]. The natural infection of armadillos with *M. leprae* is believed to have occurred relatively recent (during the last 500 years) during the colonisation of the Americas by Europeans and the forced introduction of enslaved Africans, together with their infectious agents [Truman, 2005; Balamayooran et al., 2015]. Armadillos are part of the natural habitat of several Latin American countries and nine-banded armadillos expanded their range into the United States in the mid-1800s from Mexico [Taulman and Robbins, 1996]. The recent confirmation of zoonotic leprosy involving *D. novemcinctus* in the southern USA [Truman et al., 2011] provides an elegant example of how invasive anthropogenic behaviour can have consequences for human health.

Mossoró and itś municipalities have a history of hyperendemicity and local transmission chains that call for support measures to intensify surveillance activities and act more effectively in combating the leprosy endemic [Franca et al., 2023]. Although the clustering rate among humans in our study was not very high, we observed a cluster of considerable size that could represent either a more virulent *M. leprae* strain or a group of patients with higher risk for recent transmission. Unfortunately, the lack of robust epidemiological data did not permit a more detailed investigation.

In addition to studies on human disease, more integrated approaches including non-human species uncovered the participation of armadillos in zoonotic leprosy in the Southern USA [Truman et al., 2011; Sharma et al., 2015]. There is a growing body of molecular, histopathological and epidemiological data that points to the same role for nine-banded armadillos in Brazil [Frota et al., 2012; Deps et al., 2008; da Silva et al., 2018; Deps et al., 2021; Monsalve-Lara and Romero-Alvarez, 2024; Lima et al., 2025], Colombia [Cardona-Castro et al., 2009], Mexico [Vera-Cabrera et al., 2022], Paraguay and Bolivia [Romero-Alvarez et al., 2024]. Nevertheless, other investigations have questioned the contribution of armadillos to leprosy in Brazil [Pedrini et al., 2010; Schmitt et al., 2010; Stefani and Costa, 2019]. Recently, infection by *M. leprae* of non-armadillo animal species has been described in Brazil such as small rodents and marsupials of the order *Didelphimorphia* [de Souza Valois et al., 2024] and other animals such as Artiodactyla, Carnivora, Cingulata, Pilosa, Primates, Rodentia and Didelphimorphia [Nogueira et al., 2024, Nogueira et al., 2025]. Although most studies related to *M. leprae* infected armadillos have focussed on *D. novemcinctus*, a growing number of studies in Brazil have demonstrated infection of *E. sexcinctus*, including PCR-positive tissue sample from two animals in the state of Ceará [Frota et al., 2012], one PGL-I seropositive animal from Mato Grosso [Nogueira et al., 2025] and a study on 20 free living animals in the state of Rio Grande do Norte, showing infection both by anti-PGL-I in serum and/or PCR/nested-PCR in liver [Ferreira et al., 2020].

The same 20 animals as described by da Silva Ferreira et al. [Ferreira et al., 2020] were investigated in the present study and the positivity for at least one VNTR amplicon in each animal confirmed the PCR data reported in that study. Most samples of armadillos yielded partial VNTR profiles and no amplification was observed with the SNP assay, most likely reflecting the relatively low quantity of *M. leprae* DNA present in several of the tissue samples analyzed. Variation in bacillary load between animals, particularly in younger individuals or those at early stages of infection, may limit the success of multilocus amplification approaches such as VNTR genotyping. The markers used for genotyping were single copy markers and the difficulties encountered in their amplification may reflect the lower sensitivity of the multiplex PCR assays compared to the PCR or nested PCR assays using a repetitive element (RLep) as target, as was applied in our earlier work. In the study by Truman et al. [Truman et al., 2011], 33 nine-banded armadillos generated high quality *M. leprae* genotypes, but the authors do not report on the overall yield obtained and the exact number of studied animals. In that study, liver, spleen and/or lymph-node tissue were examined by means of homogenization followed by DNA extraction with the use of a DNeasy kit, while in our study, we used the highly efficient combined procedure of tissue homogenization combining enzymatic and mechanical lysis followed by extraction with organic reagents and silica, as such we believe that the latter procedure is at least as efficient as the one used in the US. In addition, both studies used PCR-based procedures for genotyping and although with some differences, shared the VNTR-based approach. Conceivably, there may be armadillo species–related differences in bacterial load within infected tissues. However, a more plausible explanation relates to differences in ecological and demographic history. In Brazil, armadillos have occupied their natural habitats for centuries, whereas in the United States the species was introduced and expanded relatively recently, undergoing rapid population growth over a short time [Taulman and Robbins, 1996]. In addition, the current absence of natural predators in the United States likely allows armadillos to survive longer. Prolonged survival would, in turn, permit longer disease progression, potentially resulting in higher *M. leprae* bacterial loads. Consistent with this interpretation, the mean body mass of *E. sexcinctus* individuals examined in our study suggests that these animals were relatively young and therefore may not have had sufficient time to develop advanced stages of leprosy associated with high bacterial burden.

According to the genotypes of the *M. leprae* strains we obtained from armadillos, it is tempting to conclude that the animals were infected by the same *M. leprae* strain, although originally from different municipalities with some difference between clustering radii. The presence of a single *M. leprae* genotype in the armadillos is like what was observed in *D. novemcinctus* in southern USA, a study with a totally different epidemiologic background [Truman et al., 2011]. In the latter study, the animals were infected by *M. leprae* with SNP-type 3I-2-v1 and a MLVA pattern identical to the genotype recorded in a substantial proportion of their patient samples but were clearly distinct from the *M. leprae* genotype of the present study. The deduced SNP type 4 observed in our animal samples was the same as that of most patients and to that most frequently observed in other regions of Northeast Brazil [Fontes et al., 2017], including those from Fortaleza, origin of the only human derived *M. leprae* with (an almost) identical MLVA based genotype. This observation suggests that this genotype is not exclusively associated with armadillo-derived *M. leprae*.

Possibly the most striking finding was that none of the human derived *M. leprae* genotypes from patients from Mossoró, apart from a sample with only six defined alleles, was in a cluster with the armadillo derived genotypes. That human derived strain was the only one that presented nine copies of AC9, a feature apparently characteristic for our *E. sexcinctus* derived *M. leprae* genotypes. However, it should be kept in mind that AC9 is a dimer repeat and not easy to determine a one copy difference during the MLVA assay, possibly leading to erroneous interpretation or slight deviation during semi-automated reading against the reference strain. Unfortunately, neither DNA nor the electropherogram file was available to repeat the MLVA or the reading. In addition, the VNTR (GTA)9 clearly differentiated the two clusters, presenting respectively 10 and 9 copies in animal and human derived strains.

There are some limitations to the present study. In particular, the sampling strategy and the number of human genotypes obtained were not optimal for investigating transmission of *M. leprae* between armadillos and humans, to our knowledge this is the only study in Brazil describing *M. leprae* genotypes in armadillos or in any non-human animal species. The epidemiological limitations are comparable to those reported by Truman et al. [Truman et al., 2011], in which information on prior contact with armadillos was available for 15 patients, but it was

not possible to confirm that those animals were infected with *M. leprae*. A more detailed comparison between the two studies is not appropriate due to differences in the size of the study areas, the lack of data on patient migration into regions where infected animals were present, and, in our case, the absence of investigation of *M. leprae* infection in armadillos collected in the city of Mossoró, particularly animals traded for human consumption. Nevertheless, although the number of patients included in our study was like that of the USA study, the broader distribution of *M. leprae* genotypes among human cases in Brazil may reduce the likelihood of detecting clear evidence of interspecies transmission.

Performing surveys of wild animal populations for zoonotic agents is not straightforward. In the case of armadillos, difficulties begin with the capture of the animals but extend to the need to respect environmental legislation that limits the invasive and lethal sampling methods that can be employed. These constraints frequently restrict the number of animals and species that can be included in field-based molecular studies. Consequently, although the present investigation focused on *E. sexcinctus*, future studies incorporating additional armadillo species, including *D. novemcinctus,* will be important for clarifying the role of different armadillo hosts in the epidemiology of *M. leprae* in Brazil.

Although the genotypes obtained from *M. leprae* strains from *E. sexcinctus* in Rio Grande do Norte and the patients from Ceará showed some slight differences, the copy number of the more stable VNTRs are identical. Migration of humans and armadillos between those neighbouring states may have contributed to this but caution must be exercised to draw conclusions from genotype comparisons based only on VNTR markers only, since they do not always have similar HGDI in different populations. Therefore, for comparison of genotypes of *M. leprae* from larger geographic areas, SNP-based data should be included as suggested by Ansari et al. [Ansari et al., 2025].

Taken together, these findings highlight the need for additional comparative genetic studies involving multiple armadillo species and human populations in Brazil to better understand the potential role of wildlife hosts in the ecology and transmission dynamics of *M. leprae*.

5. Conclusions

In conclusion, our data confirmed the high infection rate with *M. leprae* observed in six-banded armadillos in the state of Rio Grande do Norte, by means of an additional molecular method, but they are strongly suggestive of those animals being infected by a single bacterial strain. Given that this strain seems to be distinct from those infecting humans in the area, our data support efficient transmission between *E. sexcinctus* but do not provide evidence of transmission between armadillos and humans in the sampled population. Nonetheless, we have demonstrated that armadillos are a reservoir of infective and transmissible *M. leprae* and that further studies on a larger scale, and if possible, using a more efficient genotyping methods such as the recently developed Deeplex-MycLep assay [Ansari et al., 2025; Jouet et al., 2023] are needed. Taken as a whole, the findings of this study underscore the importance of a One Health framework that integrates human, animal, and environmental data to understand the ecology of *M. leprae* and to inform leprosy control strategies in endemic settings.

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CRediT authorship contribution statement

Jessica da Silva Ferreira: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization. **Emilyn Costa Conceição:** Conceptualization, Data curation, Investigation, Methodology, Formal analysis, Visualization. **João Marcelo Azevedo de Paula Antunes:** Investigation, Resources. **Amanda NB Fontes:** Data curation, Formal analysis, Investigation, Validation. **Sidra Ezídio Vasconcellos:** Data curation, Investigation, Resources. **Ricardo José de Paula Souza e Guimarães:** Formal analysis, Investigation, Resources. **Maurício Lisboa Nobre:** Investigation, Resources. **Ismênia Glauce:** Investigation, Resources. **Douglas McIntosh:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Philip Noel Suffys:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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