A New Mycobacterium Species Causing Diffuse Lepromatous Leprosy

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Abstract

Mycobacterium leprae causes leprosy. M leprae strains collected worldwide have been genetically clonal, which poorly explains the varying severity and clinical features of the disease. We discovered a new Mycobacterium species from 2 patients who died of diffuse lepromatous leprosy (DLL). The Mycobacterium was purified from heavily infected, freshly frozen autopsy liver tissue followed by DNA extraction in 1 case. Paraffin-embedded skin tissue was used for DNA extraction in another case. Six genes of the organism were amplified by polymerase chain reaction, sequenced on cloning or from amplicons, and analyzed. Significant genetic differences with M leprae were found, including a 2.1% divergence of the 16S ribosomal RNA (rRNA) gene, a highly conserved marker of bacterial evolution, and 6% to 14% mismatches among 5 less conserved genes. Phylogenetic analyses of the genes of 16S rRNA, rpoB, and hsp65 indicated that the 2 most related organisms evolved from a common ancestor that had branched from other mycobacteria. These results and the unique clinicopathologic features of DLL led us to propose Mycobacterium lepromatosis sp nov. This species may account for some of the clinical and geographic variability of leprosy. This finding may have implications for the research and diagnosis of leprosy.

Leprosy, caused by Mycobacterium leprae, has plagued humans for millennia and remains a significant public health problem in many developing countries. The disease manifests a wide spectrum of clinicopathologic forms, ranging from tuberculoid leprosy (TT), to borderline forms, to lepromatous leprosy (LL). The lesions mainly involve skin and peripheral nerves and can be paucibacillary or multibacillary. A remarkable geographic variation also exists. For example, in India and Africa, 90% of cases are TT, whereas in Mexico, 90% of cases are LL; in Southeast Asia, the 2 forms are equally distributed.1

LL, the most severe form, may involve most internal organs of the body in addition to the skin, such as the liver, spleen, and bone marrow. The bacterial burden in these patients is massive, causing substantial mortality when not treated. A diffuse form of LL (DLL), also known as diffuse leprosy of Lucio and Latapi2,3 or leprosy with the Lucio phenomenon, is predominantly seen in patients from western Mexico and the Caribbean countries.1 DLL, initially described by Lucio and Alvarado in 1852,4 is characterized clinically by diffuse, nonnodular cutaneous infiltration and pathologically by mycobacterial invasion into endothelium along with endothelial proliferation, vascular occlusion, and/or vasculitis in the dermis and subcutis.5-7 The recurrent crops of large, sharply demarcated skin lesions (Lucio phenomenon) are considered an unusual reaction and, at an advanced stage, may become ulcerated, particularly on the lower extremities, and even generalized, leading to fatal secondary bacterial infection and sepsis.1 These features are unique to DLL.

M leprae has not been cultured on artificial media, thus hindering microbiologic research. However, passage through armadillo and the athymic nude mouse and use of molecular
tigene analysis on a Mycobacterium obtained from autopsy or biopsy tissue of 2 Mexican immigrants who died of DLL. Our results demonstrate that this organism differs remarkably from Mycobacterium leprae and represents a novel Mycobacterium species. This species may explain the unique features of DLL and, thus, account for some of the clinical and geographic variability of leprosy.

Case Reports

Case 1

A 53-year-old homeless man, originally from Mexico, was admitted to a hospital in Arizona in February 2007 for treatment of leg wounds and pain management. The patient had been seen at a community clinic a year before for this cellulitis involving the lower extremities when anemia and hypergammaglobulinemia were also found. He received antibiotics but was lost to follow-up. This admission followed a 10-day span of clinic care with trimethoprim-sulfamethoxazole with no improvement of the ulcers and swelling and numbness of the extremities.

Physical examination revealed multiple, tender, well-demarcated, purpuric skin lesions with scabs on all 4 extremities. There were loss of eyebrow hairs and loss of pain sensation except in the lower extremities. No pulse from the dorsalis pedis was felt. Hepatomegaly was palpated. Laboratory tests revealed mild anemia (hemoglobin, 11.7 g/dL [117 g/L]), high levels of creatine kinase (4,234 U/L [70.7 μkat/L]), and normal alanine transaminase (ALT) and aspartate transaminase (AST) levels. While undergoing intravenous ampicillin/sulbactam therapy and further diagnostic work the next day, high fever, hypotension, tachycardia, respiratory failure, and acrocyanosis suddenly developed in the patient. Liver enzyme levels were also elevated (ALT, 210 U/L [3.51 μkat/L]; AST, 424 U/L [7.07 μkat/L]). Despite resuscitation and intensive care for 10 days, he died of presumed septic shock, but blood and skin cultures for bacteria, including acid-fast bacilli (AFB), were unrevealing. During the course of disease, skin biopsies revealed necrosis, vasculitis, and innumerable AFB, raising the possibility of leprosy. The tests for HIV and hepatitis B and C viruses were all negative. The potential exposure to the AFB among health care workers prompted an autopsy to probe the cause of death and confirm the diagnosis of leprosy.

The autopsy revealed hepatosplenomegaly and dissemination of AFB to the skin, liver, spleen, lymph nodes, bone marrow, kidneys, adrenal glands, and tracheal mucosa. As illustrated in Image 1, histopathologic examination confirmed the diagnosis of DLL as the cause of death, including nerve invasion, vasculitis and panniculitis with AFB invasion that caused skin ulceration, and massive AFB burden (globi) in internal organs. The skin necrosis was typical of Lucio phenomenon (erythema necroticans). With regard to the shock, a stormy erythema nodosum leprosum reaction was plausible considering no fever at admission, negative cultures, and sudden liver function derangement. Additional studies included the finding of a strongly positive antibody (50% end point, 1:1,500) against leprosy-specific phenolic glycolipid I by an enzyme-linked immunosorbent assay. Repeated cultures for mycobacteria from freshly frozen autopsy liver tissue, including also incubation at 30°C on chocolate agar for Mycobacterium haemophilum, were negative. Molecular studies on the AFB were then performed.

Case 2

A 31-year-old Mexican man was transferred to the burn unit of an Arizona hospital in September 2002 for treatment of extensive skin necrosis. He had been hospitalized 3 weeks earlier at another institution for necrotic skin lesions involving the lower extremities, but the lesions progressed to the trunk and face, along with the development of high fever, azotemia, and prominent leukocytosis (WBC count, 69,000/μL [69.0 × 10^9/L]) with a positive D-dimer test. Cultures of the skin lesions had grown Staphylococcus aureus and Streptococcus pyogenes. Treated with antibiotics and steroids, the sepsis with possible disseminated intravascular coagulopathy had been controlled. His medical history was notable for a presumptive diagnosis of rheumatoid arthritis treated with methotrexate, 7.5 to 10 mg weekly, and prednisone, approximately 10 mg daily for 6 years. Physical examination revealed extensive (~80% of body surface) skin necrosis evidenced by ulceration and gray to black discoloration. Admission laboratory tests revealed severe hypoalbuminemia (albumin, 1.8 g/dL [18 g/L]), mild anemia (hemoglobin, 11 g/dL [110 g/L]), and slight elevation of ALT (67 U/L [1.12 μkat/L]) and AST (117 U/L [1.95 μkat/L]) values. Extensive surgical debridement and amputation of the ears, fingers, and
lower extremities were performed; however, the patient died within several days of admission.

Pathologic examinations of many skin sections demonstrated extensive necrosis, ulceration, and superficially located infections by gram-positive cocci and yeasts. Specifically, invasion of nerves and subcutis (deeply located) by numerous AFB-laden macrophages diagnosed LL, and vasculitis with endothelial proliferation and AFB infiltration Image II was characteristic of DLL. The skin necrosis was also typical of Lucio phenomenon. Although the internal organs were not examined histologically, the findings of severe hypoalbuminemia and elevated transaminase levels were compatible with hepatic involvement by the disease.

**Materials and Methods**

**Case 1**

To identify the AFB (strain FJ924) from case 1, the 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR), cloned, and sequenced. Briefly, approximately 200 mg of freshly frozen infected liver tissue was homogenized and processed with N-acetyl-L-cysteine and NaOH to enrich the AFB and decontaminate and disrupt host cells, usual for cultures. After obtaining essentially pure AFB (Image 1D), DNA extraction and PCR were performed as previously described. To be inclusive for all bacteria that might be potentially present in the tissue, highly conserved
bacterial 16S rRNA gene primers Table I, short thermocycles (30 cycles), and a low annealing temperature (55°C) were used. On verification, the PCR product was cleaned and cloned into a plasmid vector (Topo, Invitrogen, Carlsbad, CA), which was further sequenced using the standard M13 primers. Technically, this PCR cloning approach aimed to recover novel or multiple organisms on sequencing of many clones, as has been commonly used to sort out mixed or unknown or uncultivable microbial communities in gut, oral cavity, and the environment.

Three other genes were amplified and sequenced directly without cloning: the housekeeping genes hsp65 (65-kDa heat shock protein) and rpoB (for RNA polymerase β subunit) for further phylogenetic analysis and the rpoT gene (for RNA polymerase σ factor) for analysis of tandem repeats. The primers for each of these genes were designed from the conserved sequences from M leprae and other mycobacteria (Table I).

**M haemophilum Genes**

For comparative analysis, 3 M haemophilum genes that did not have GenBank accessions, including rpoT, mmaA (for methyl mycolic acid synthases 3 and 4), and ribF-rpsO (for

<table>
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<tr>
<th>Primer Pairs: Name and Sequence (5’ to 3’)</th>
<th>Gene Segment (base pairs)</th>
<th>GenBank Accession No.</th>
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</thead>
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hsp65, heat shock protein 65 gene; mmaA, methyl mycolic acid synthase gene; PCR, polymerase chain reaction; ribF-rpsO, riboflavin kinase and ribosomal protein S15 genes; rpoB, RNA polymerase β subunit gene; rpoT, RNA polymerase σ factor gene.

* These PCRs were seminested on the first PCR with RPTFO and RPTRE primers.
riboflavin kinase and ribosomal protein S15) were also amplified and sequenced directly (from strain ATCC 29548).

Case 2
The paraffin-embedded tissue blocks, archived for 5 years, were retrieved and sectioned at the Arizona hospital 7 months after autopsy of case 1. The tissue was sent to laboratories in Houston, TX, for DNA extraction, PCR, and nucleotide sequencing where the molecular analysis on case 1 had been completed for 2 months. To prevent contamination and ensure data authenticity, each of the aforementioned steps was performed at a separate laboratory (none leprosy-related) from buildings 2 miles away at a different time without overlap. Considering DNA fragmentation (to ~200-500 base pairs [bp]) during formalin fixation and paraffin embedding of tissue, the PCR primers were redesigned (Table 1) for smaller amplicons and specificity for strain FJ924 (after availability of sequences from case 1) and M leprae to avoid cross-amplification of the secondary infecting staphylococci and streptococci. The annealing temperatures were also set for high stringency. The amplicons were sequenced directly.

GenBank Accessions and Phylogenetic Analysis
The gene sequences obtained in this study were deposited in the GenBank (Table 1). Additional GenBank accessions for 16S rRNA genes included X53999 for M leprae, U06638 for M haemophilum, X52930 for Mycobacterium malmoense, AJ536037 for Mycobacterium avium, AJ536031 for Mycobacterium tuberculosis, AY457071 for Mycobacterium abscessus, X84248 for Corynebacterium diphtheriae, DQ997837 for S aureus (V. Nema and D.V. Kamboj, 2006, direct submission), and J01859 for Escherichia coli. Sequence analysis was performed through queries to the GenBank using the Basic Local Alignment Search Tool (BLAST). Phylogenetic analysis was performed using CLUSTAL W for multialignment (www.ebi.ac.uk/clustalw) and tree construction in the Newick format by Grawtree of PHYLIP (www.molgen.mpg.de). For optimal tree construction to avoid bias from varying lengths of the aforementioned GenBank 16S rRNA genes, the sequences were truncated to obtain the same aligning length (1,486 bp, including gaps).

Results
The PCR cloning approach in case 1 yielded 12 clones with inserts from a single Mycobacterium (strain FJ924): 5 of the clones contained the 16S rRNA gene with identical sequences; the remaining clones, 6 containing mmaA and 1 containing ribF-rpsO, came from cross-amplification of these genes from this organism. Thus, in this open-ended, low-stringency PCR targeting bacteria broadly, the recovery of the lone Mycobacterium that infected this HIV-negative patient massively (Images 1C and 1D) proves disease-cause specificity. The heterogeneity of the PCR amplicons also explains our initial suboptimal results from direct sequencing. These cloned genes were further analyzed as follows.

The 16S rRNA Gene
Along bacterial evolution, the 16S rRNA gene is highly conserved. It has become clear recently that each species has a signatory 16S rRNA gene and that an approximately 3% sequence divergence between closest species forms the main criterion for species definition. BLAST analysis of the 1,504-bp 16S rRNA gene of strain FJ924 showed that the organism was most closely related to, but distinct from, M leprae (matching 1,475 of 1,506 bp [97.9%]). It matched second best with M haemophilum (1,465/1,505 [97.3%]), with decreasing homology with other mycobacteria. A phylogenetic tree showed that it descended along with M leprae from a common ancestor that had branched from other mycobacteria. Thus, this organism is likely a new species within the genus Mycobacterium that, despite the wide spectrum of pathogenicity, is known for tight clustering of the 16S rRNA gene among its approximately 110 species. For all M leprae strains studied to date, no variations of the 16S rRNA gene have been noted or reported. Remarkably, the 16S rRNA gene of FJ924 contained a unique 19-bp inserted sequence, TTAATTTAAACC-TATTAA, near the beginning (Figure 1B). This sequence matched poorly with its 16-bp, also AT-rich, M leprae counterpart that was known previously to be unique for M leprae. Although the precise role of the 19-bp or 16-bp insertion is unclear, this sequence, on transcription to RNA, would predict to elongate and stabilize a stem-loop structure according to the secondary structure model for the 16S rRNA. Through extensive BLAST queries, this 19-bp AT-rich sequence was not found in the 16S rRNA gene of all other bacteria (~3,200 species), despite its presence in the genomes of several subbacteria and archebacteria that have overall high A + T contents. The best match with all mycobacterial sequences (107 mega bp, including many genomes) was 11-bp without gap. Thus, this insertion was not of bacterial origin. Without evolutionary conservation, in a free-living Mycobacterium of a 4-mega-bp genome and approximately 64% G + C content, the 19-bp sequence of 16 A/Ts and 3 G/Cs would occur (0.18) × (0.32)3 × (4 × 109) = 1.6 × 10−7 times.

In contrast, this sequence was identified in the human genome, matching 18 of 19 bp or 17 bp in a row. Similarly, the M leprae sequence, AAAAATCTTTTTTTAG, all matched with humans (at other locations). Therefore, in view of the long history of human leprosy (at least millennia), chronic obligate intracellular infection, massive load of bacilli in LL,
and the reductive evolution of the *M. leprae* genome through extensive recombination (within human cells),
human origin of these short AT-rich sequences is most likely. Humans
are the only known natural host for *M. leprae* in view of the
finding that naturally infected armadillos in Louisiana likely
contracted the *Mycobacterium* from early explorers.\(^\text{18}\)

**Analysis of Other Genes**

The cloned genes *mmaA* and *ribF-rpsO* from strain FJ924 showed remarkable sequence differences from *M. leprae*, matching at 86.0% (824/958) and 91.5% (678/741), respectively. Notably, the *mmaA3* portion within *mmaA* has degenerated into a pseudogene for both organisms, albeit through separate pathways in view of the poor matches (82.0%). In addition, a 21-bp sequence within these pseudogenes, ATTCTGTTCTGGCAAGGT, was found only in the human genome but nowhere else, again suggesting human bias during degenerative evolution. Without such influence, in the 3.3-mega-bp *M. leprae* genome containing 58% G + C, this human sequence would have occurred

\[ (0.21)^{13} \times (0.29)^8 \times (3.3 \times 10^6) = 2.6 \times 10^{-7} \text{ times}. \]

Indeed, this sequence was not identified in other mycobacteria, matching at best 15 bp in a row.

The *mmaA* and *ribF-rpsO* genes only matched in segments (73%-81%) with other mycobacteria, including *M. haemophilum*, *M. tuberculosis*, *M. avium*, and others, further supporting early evolutionary separation from these organisms. Owing to less conservation and/or degeneration, however, these genes were less ideal for construction of phylogenetic trees.

The genes *hsp65* and *rpoB* from FJ924, obtained through PCR sequencing, best matched *M. leprae* at 92.8% (566/610) and 94.3% (628/666), respectively. With *M. haemophilum*, they both matched at 92.0%. Similarly, between *M. leprae* and *M. haemophilum*, the 2 genes matched 92.3% and 93.7%, respectively. Thus, the divergence of approximately 7% for *hsp65* and *rpoB* is also consistent with separate species, as has been shown for other mycobacteria.  

Phylogenetic trees based on these genes were similar to that derived from the 16S rRNA gene (data not shown).
Among *M. leprae* strains, rpoT has been shown to contain 3 or 4 tandem repeats of the sequence GACATC. The rpoT from FJ924, while matching 88.0% (449/510) overall with *M. leprae*, also contained 4 such repeats. Within the 97 nucleotides flanking these repeats as used in previous studies, 5 nucleotides differed, which is notable by sequencing but not by size-based gel electrophoresis. In addition, FJ924 rpoT also contained 3 repeats of the sequence CGACCACAAATACGACT, which appears unique and absent in the *M. leprae* genome. The rpoT showed gapped matches (69%-79%) with other mycobacteria.

Taken together, the preceding multigene analyses demonstrate strongly a species-level genetic difference between strain FJ924 and all other mycobacteria. It represents a hitherto unknown and as yet uncultured *Mycobacterium* species that relates most closely to *M. leprae*. From the 4,991 nucleotides sequenced from the 6 genes, the G + C content was 58.3%, nearly identical to that of the *M. leprae* genome (57.8%), but lower than the usual 62% to 70% of other mycobacteria.

**Analysis of Case 2**

The corresponding genes from case 2 were compared with strain FJ924. All genes, albeit shorter (Table 1), matched fully (100%), including the 16S RNA gene (414 bp with the unique 19-bp AT-rich sequence), hsp65 (391 bp), rpoB (342 bp), rpoT (403 bp with the 4 GACATC repeats), mmaA (332 bp with the 21-bp likely human-biased sequence), and ribF–rpsO (250 bp). Together, these gene segments amount to 2,132 bp. Thus, the same *Mycobacterium* was demonstrated in case 2, on separation from the secondary infecting staphylococci and streptococci by the *Mycobacterium*-specific primers.

**Discussion**

By using molecular techniques, we discovered a new *Mycobacterium*, strain FJ924, from 2 patients who died of DLL. Although uncultivated as yet, FJ924 likely possesses some unique phenotypic and/or biologic traits that cause the distinct clinicopathologic features of DLL, as shown in our cases, as well as in the century-old and recent literature. Vargas-Ocampo recently studied the histopathologic features of 199 cases of DLL in Mexico and noted invariable endothelial changes, caused by mycobacterial invasion, that ranged from early endothelial proliferation to late stages of luminal obliteration along with varying degrees of leukocytoclastic vasculitis. Because these changes underpin the clinical manifestations of skin purpura, ulceration, and necrosis, the author concluded that DLL is essentially a vascular disease.

Therefore, we propose *Mycobacterium lepromatosis* sp nov (le-pro-ma-to’s sis, N.L. gen. fem. n. lepromatosis of lepromatous leprosy) to distinguish it from *M. lepra*, a genetically homogeneous species. Recognizing this etiology may explain the endemic nature of DLL in western Mexico that borders Arizona where the patients sought care. Recently, we also documented *M. lepromatosis* as the cause of the 2 fatal DLL cases from Singapore that have been previously published (X.Y. Han, H.H. Tan, K.C. Sizer, et al, unpublished data, 2008). At present, we are making an effort to determine whether this species also caused the DLL cases reported in the Middle East, Brazil, and northern Africa. Our patients exhibited the typical features of end-stage DLL, owing to delayed diagnosis. In the United States, physicians seldom encounter leprosy owing to its rarity; there are 100 to 200 new cases annually, mostly in immigrants. This fact highlights the importance of skin biopsy in making the correct diagnosis, a key to early and successful therapy. In retrospect, the second patient’s rheumatoid arthritis was probably a manifestation of DLL, a common feature, but, unfortunately, the treatment with methotrexate and prednisone for years, without concurrent antileprosy therapy, might have hastened his disease progression by immune suppression. Therefore, these cases serve to reeducate us on leprosy, especially DLL, which is rare but not gone, despite the success of worldwide control.

*M. lepromatosis*, *M. lepra*, and *M. haemophilum*, being most related, all infect the skin primarily, suggesting a preference for cooler growth conditions. Indeed, *M. haemophilum* can be cultured at 30°C incubation. *M. lepra* is uncultivable so far, however, owing to the degenerative genome and consequent metabolic defects. *M. lepromatosis* also heavily infects internal organs, implying growth at 37°C, higher virulence, and, possibly, a shorter doubling time than the 14 days for *M. lepra*. Despite our current culture failure, future efforts can be made toward supplementation of nutrients, use of newer liquid mycobacterial culture media or cell cultures, and passage through armadillo and/or athymic mouse.

The genome of *M. lepra* strain TN from Tamil Nadu, southern India, contains 3 repeats of GACATC in rpoT. A recent study of 100 northern India strains also reports 3 repeats containing 3 repeats. These data correlate with the predominance (90%) of TT in India. In contrast, an analysis of 27 strains from Mexico showed 25 (93%) with 4 repeats, paralleling the vast majority (90%) of LL in Mexico. Yet, given our finding of 4 repeats in *M. lepromatosis*, indistinguishable from *M. lepra* if not sequenced, the possibility exists that some of these Mexican strains might actually be *M. lepromatosis*. While there is little doubt that *M. lepra* causes TT, future analysis of many strains along with clinical data for diverse regions may elucidate whether *M. lepromatosis* mainly causes DLL and whether one or both cause LL or the borderline forms. To address these questions, the unique gene sequences of *M. lepromatosis* (Table 1) may be useful. Conceivably, however, not recognizing *M. lepromatosis*...
earlier likely implies its overall low prevalence, geographic restriction, unique susceptibility to the organism in a human population, or misdiagnosis or a combination thereof.

The 3.3-mega-bp \( M \) \( \text{leprae} \) genome is exceptionally stable over time, more than the 4.4-mega-bp \( M \) \( \text{tuberculosis} \) genome, as indicated by the clonality of the worldwide \( M \) \( \text{leprae} \) strains.\( ^{17,18} \) Thus, the reductive evolution of the \( M \) \( \text{leprae} \) genome must have antedated the global spread of \( M \) \( \text{leprae} \) strains that occurred via human activity and migration during the last approximately 100,000 years.\( ^{17,18} \) Indeed, in a recent study comparing the genomes of \( M \) \( \text{leprae} \) and \( M \) \( \text{tuberculosis} \), as well as analyzing the \( M \) \( \text{leprae} \) pseudogenes, Gómez-Valero et al\( ^{43} \) estimated that \( M \) \( \text{leprae} \) genome decay took place in the last 20 million years. Our data on the phylogenetic distance between \( M \) \( \text{lepromatosis} \) and \( M \) \( \text{leprae} \) (Figure 1) also suggest that the 2 species bifurcated well before global dissemination. Furthermore, several commonalities for both species support the idea that the process of genome reduction and degeneration started from the common ancestor: (1) culture difficulty; (2) degeneration of \( \text{mmaA3} \); (3) presence of the unique, likely human-derived or biased sequences within 16S rRNA gene and \( \text{mmaA3} \); (4) identical 6-base tandem repeats within \( \text{rpoT} \); (5) similar \( \text{G} + \text{C} \) contents; and (6) distant from other mycobacteria. This possibility is supported by our further sequence analysis of 22,400 bp of \( \text{FJ924} \) containing 15 functioning genes and 5 pseudogenes, in which we estimate that the phylogenetic distance between \( M \) \( \text{lepromatosis} \) and \( M \) \( \text{leprae} \) is approximately 10 million years, much longer than between \( M \) \( \text{tuberculosis} \) and \( M \) \( \text{bovis} \), between \( M \) \( \text{avium} \) and its subspecies, and between \( \text{Mycobacterium ulcerans} \) and \( \text{Mycobacterium marinum} \) (X.Y. Han, F.J. Silva, E.J. Thompson, et al, unpublished data, 2008). Thus, genome sequencing of \( M \) \( \text{lepromatosis} \), along with comparative genomics in the future, may address this issue and probe its relationship with human history and migration, particularly the endemicity of DLL in western Mexico and the Caribbean.

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