IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 25, Issue 1 Ser. 4 (January. 2026), PP 22-27
www.iosrjournals.org

Bioinformatics Profiling Of Mycobacterium Ulcerans Strains In Southwest, Nigeria

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Abstract
Background: *Mycobacterium ulcerans infection (Buruli ulcer) is a debilitating disease characterized by extensive and severe destruction of the skin and subcutaneous tissue resulting in the formation of large ulcers. This necrotizing neglected tropical disease whose mode of transmission is still unknown was regarded as an emerging infectious disease by WHO in 1998. Index cases of BU were first reported in Ibadan (now in Oyo State), south-west, Nigeria in 1975.*
Objective*: The study aimed to identify cases of BU in five states of SouthWest Nigeria and carry out a phylogenetic analysis of the strains encountered.*
Method*s: Here we processed 256 samples from the south-west, Nigeria for IS2404 of Mycobacterium ulcerans by Nested PCR and RT-qPCR techniques. Randomly selected PCR-positive samples were subjected to gene sequencing protocols to identify and determine the strains in the region using the molecular and bioinformatics tools of modern population genomics. The genomes sequenced were deposited in the NCBI Data Bank, assigned accession numbers and then were mapped to some reference sequences by BLAST analysis and Phylogenetic analyses using two different approaches were also done.*
Result*: All the sequenced genomes can be designated as belonging to Clade A of classical lineage. Overall high concordance and higher genetic similarity to reference strains from Africa was observed.*
Conclusion: *This corroborates its strong geographical diversity with intra-continental strains being genomically extremely closely related.*

Date of Submission: 17-01-2026 Date of Acceptance: 27-01-2026

I. Introduction

Buruli ulcer (BU), which is caused by *Mycobacterium ulcerans* is a necrotizing disease of the skin occurring in tropical and subtropical regions of the world. BU has been reported in 34 countries in tropical and sub-tropical regions of the world particularly in Africa, Asia, Australia, South America and the Pacific. It is most prevalent in Central and West Africa while the latter is now regarded as the epicenter of the disease which is regarded as one of the world's neglected tropical diseases [1, 2]. The mycolactone, a macrocyclic polyketide toxin (which is cytotoxic and immunosuppressive in nature), produced by the causative pathogen is responsible for the characteristic chronic necrosis of tissue and skin, usually with undermined edges [3, 4]. *Mycobacterium ulcerans* is closely related to the mycobacteria that cause tuberculosis and leprosy. BU is the third most common mycobacteriosis in immunocompetent humans after tuberculosis and leprosy, but in some communities in West Africa, BU has overtaken both tuberculosis and leprosy to become the most common [5]. While the mode of transmission of *M. ulcerans* is poorly understood, awareness of the disease among the populace and healthcare professionals is equally poor, thus making diagnosis a challenging exercise [6, 7].

BU usually begins as a painless nodule or papule (pre-ulcerative form) on the skin, which, if left untreated, over times, progresses to massive ulcer ulcerations (ulcerative form) most especially on the body

extremities [8]. When not treated, it can lead to permanent disfigurement, functional impairment and disability with attendant stigmatization in the society [9, 10]

II. Methodology

Being a neglected and obscure disease, community-based BU awareness and sensitization sessions were carried out prior to the active case search for suspected BU cases in the target states. Swab and Fine Needle Aspiration (FNA) samples were collected from suspected lesions. Nested PCR and qRT- PCR techniques were deployed to confirm BUD among the participants. Amplicons of eight of the samples positive to IS*2404* of *M. ulcerans* were randomly selected and subjected to genomic Sanger sequencing on ABI 3500XL sequencer platform, generating sequences results for the 8 samples.The chromatograms of the sequences were viewed using FinchTV analysis software and manual base calling was carried out for regions of ambiguities. The nucleotide sequences were thereafter subjected to BLASTn analysis to identify the organism and subsequently submitted to GenBank (ID-2207789) after which Accession Numbers were obtained. In determining the genetic relatedness of the strains from this study and some other previously sequenced strains, two slightly different approaches of molecular phylogenetic analysis were carried out and subsequently, phylogenetic trees were generated.

APPROACH 1 FOR PHYLOGENETIC ANALYSIS: A phylogenetic tree was created using the deposited sequences and reference strains sequences. FASTA versions of all the strains used were obtained from the NCBI data bank (https://www.ncbi.nlm.nih.gov/). The FASTA version of each of the strain was transferred to Notepad application (Microsoft Windows Version 1803, 2018 USA) and manually corrected. CLustalX 2.1 Bioinformatics Application [11] was used to analyze the corrected nucleotide sequences. A TreeviewX version 0.5.0 application was then used to create different format of phylogenetic tree including Rectangular Cladogram, Slanted Cladogram and Phylogram styles of the tree. For the Rectangular Cladogram a Bootstrap of N-J Tree at 2000 values (Number of Boostrap Trials = 2000) was employed to show statistical significance which implies that 2000 values of the bootstrap indicate likelihood of significance by up to 95%. MS-Paint was used to indicate the strains that show more high significance with each other.

APPROACH 2 FOR PHYLOGENETIC ANALYSIS: Due to high number of target sequences that were all identical to one another within the genome (orthologues), 8 representative sequences from each genome were therefore used. They were labelled as follows:

CP000325 – Fragment 1 to Fragment 8
AP017624.1 – Fragment 1 to Fragment 8
AP017635.1 – Fragment 1 to Fragment 8
LR135168 – Fragment 1 to Fragment 8

For diversity, the following IS*2404* sequences from NCBI were added (These were not part of whole genome sequences, they were deposited individually):

EF164897 - *Mycobacterium marinum* strain ITM 00-1026 insertion sequence IS*2404*-like transposase gene, partial cds
KM459601 - *Mycobacterium ulcerans* clone MNS2_G_PG3 insertion sequence IS*2404*, complete sequence
KM459600 - *Mycobacterium ulcerans* clone S11LB_G_PG3 insertion sequence IS2404, complete sequence
KM459599 - *Mycobacterium ulcerans* clone W1LU_G_PG3_436 insertion sequence IS2404, complete sequence

The alignment was done between the sample sequences by using the Multiple Sequence alignment tool in CLC Bio (v8) at 160 bp. There were 105 constant sites, 55 variable site and 17 parsimoniously informative sites within the multiple sequence alignment.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model [12], with 1000 bootstrap replicates. The tree with the highest log likelihood (-527,87) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 44 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There was a total of 159 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [13].The Phylogenetic tree was constructed in the following way:

The alignment was subjected to model testing. The correct Phylogenetic model applied was: **Jukes-Cantor model**

Phylogenetic analysis was performed with the model, as well as 1000 bootstrap replicates Bootstraps >50% were indicated at branch nodes

The IS*2404* sequence for *M. marinum* was used as an outgroup to root the tree.

III. Results

Among the total number of 256 patients with BU-suspected lesions 157 were confirmed BU positive using Nested PCR and RT-qPCR techniques. Plate 1 and Plate 2 show lesions from among the confirmed positive cases.

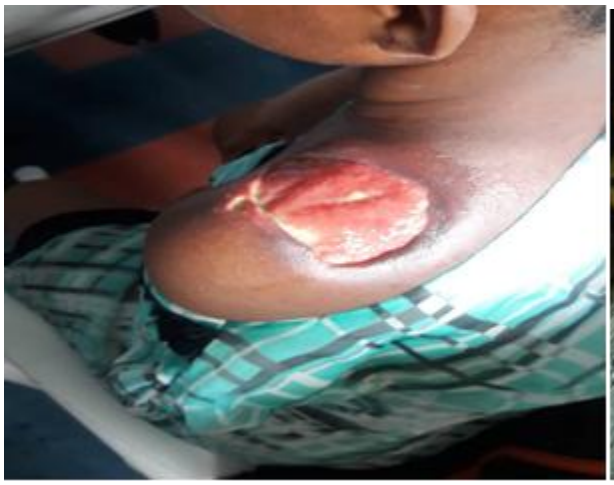

Plate 1. Confirmed BU Lesion at the shoulder of a 10-year old girl

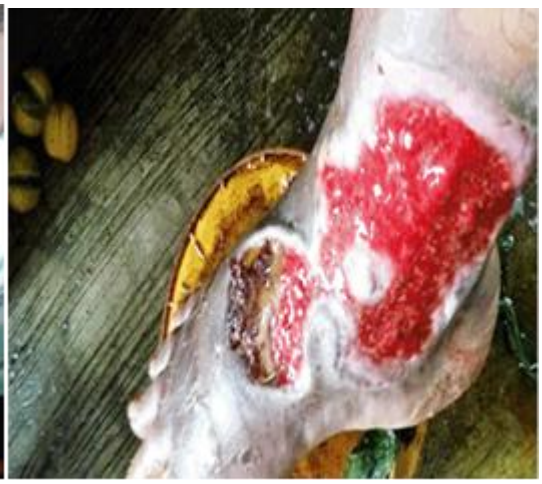

Plate 2. Multifocal Category III BU Lesion on a 43yr-old woman's right lower limb

Figure 1 shows the chromatograph of one of the sequences as viewed using Finch TV software package. The accession numbers allotted for the generated sequences by GenBank with the submission ID-2207789 are shown in Table 1 below.

Figures 2A and 2B depicts the corrected nucleotide sequences obtained in the Approach 1 after alignments.

Figure 3 and Figure 4 are the Phylogenetic trees generated from Approach 1 and Approach 2 respectively.

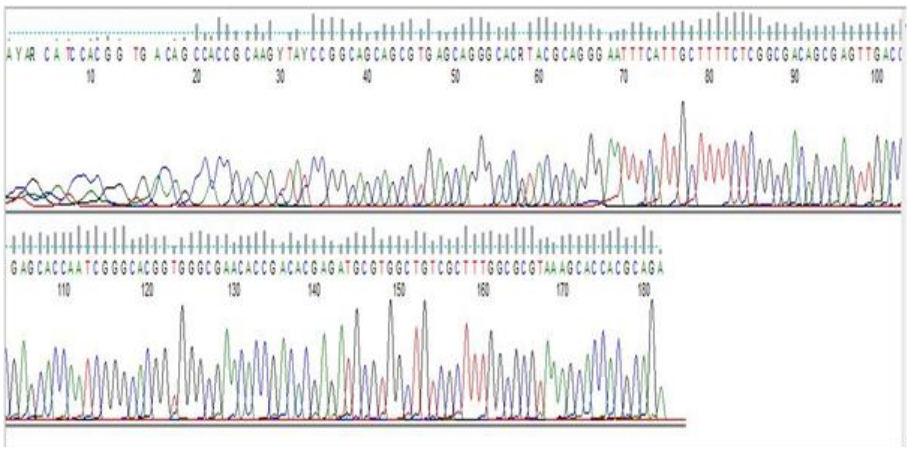

Figure 1. Chromatograph from Sanger sequencing as viewed by the FinchTV software package

Table 1. The GenBank accession numbers for the nucleotide sequences

S/N	Sample ID	Sequences	Accession Numbers
1.	1923	Sequence 1	MK764762
		Sequence 2	MK764763
2.	2011	Sequence 3	MK764764
		Sequence 4	MK764765
3.	2030	Sequence 5	MK764766
		Sequence 6	MK764767

4.	2031	Sequence 7	MK764768
		Sequence 8	MK764769
5.	2032	Sequence 9	MK764770
		Sequence 10	MK764771
6.	2033	Sequence 11	MK764772
		Sequence 12	MK764773
7.	2061	Sequence 13	MK764774
		Sequence 14	MK764775
8.	2075	Sequence 15	MK764776
		Sequence 16	MK764777

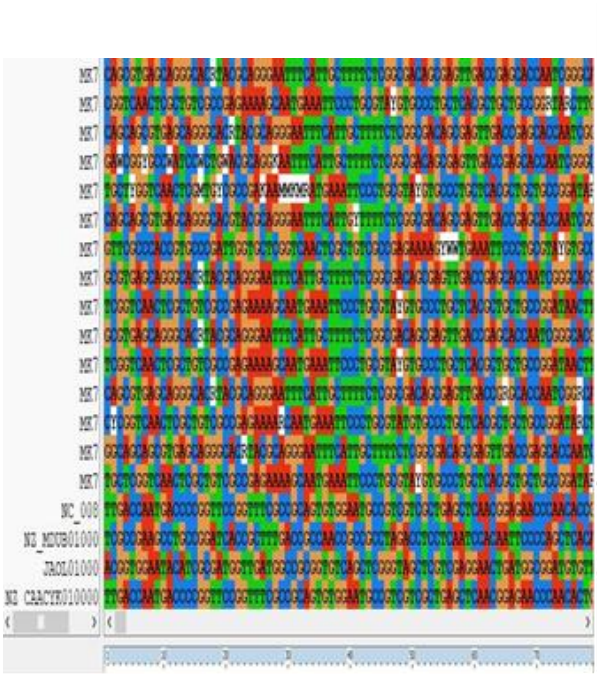

Figure 2A. First Sequence Alignment

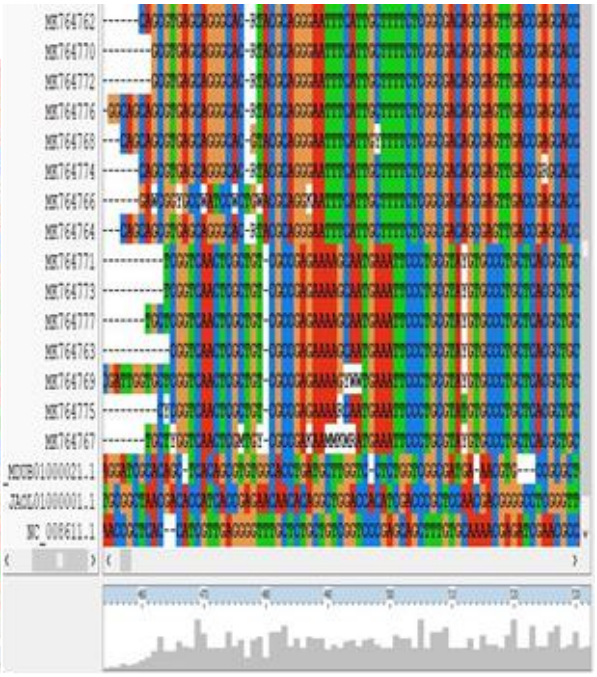

Figure 2B. Sequence Alignment after the procedure

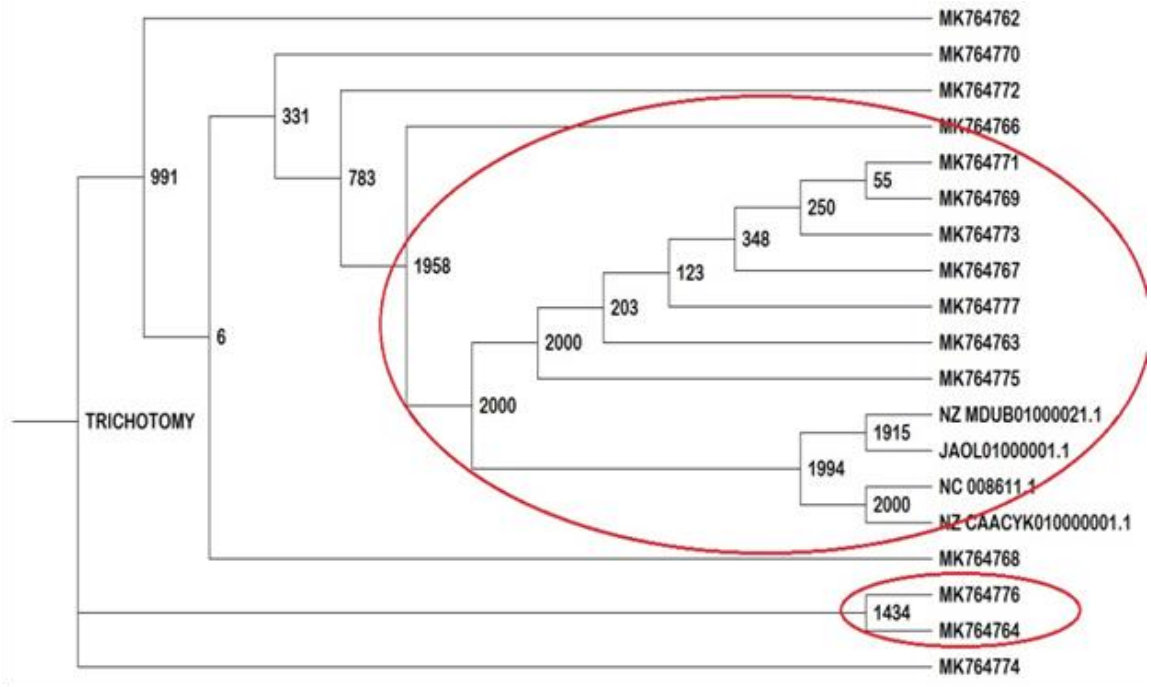

Figure 3. Phylogenetic Tree (Approach 1) - Rectagular Cladogram

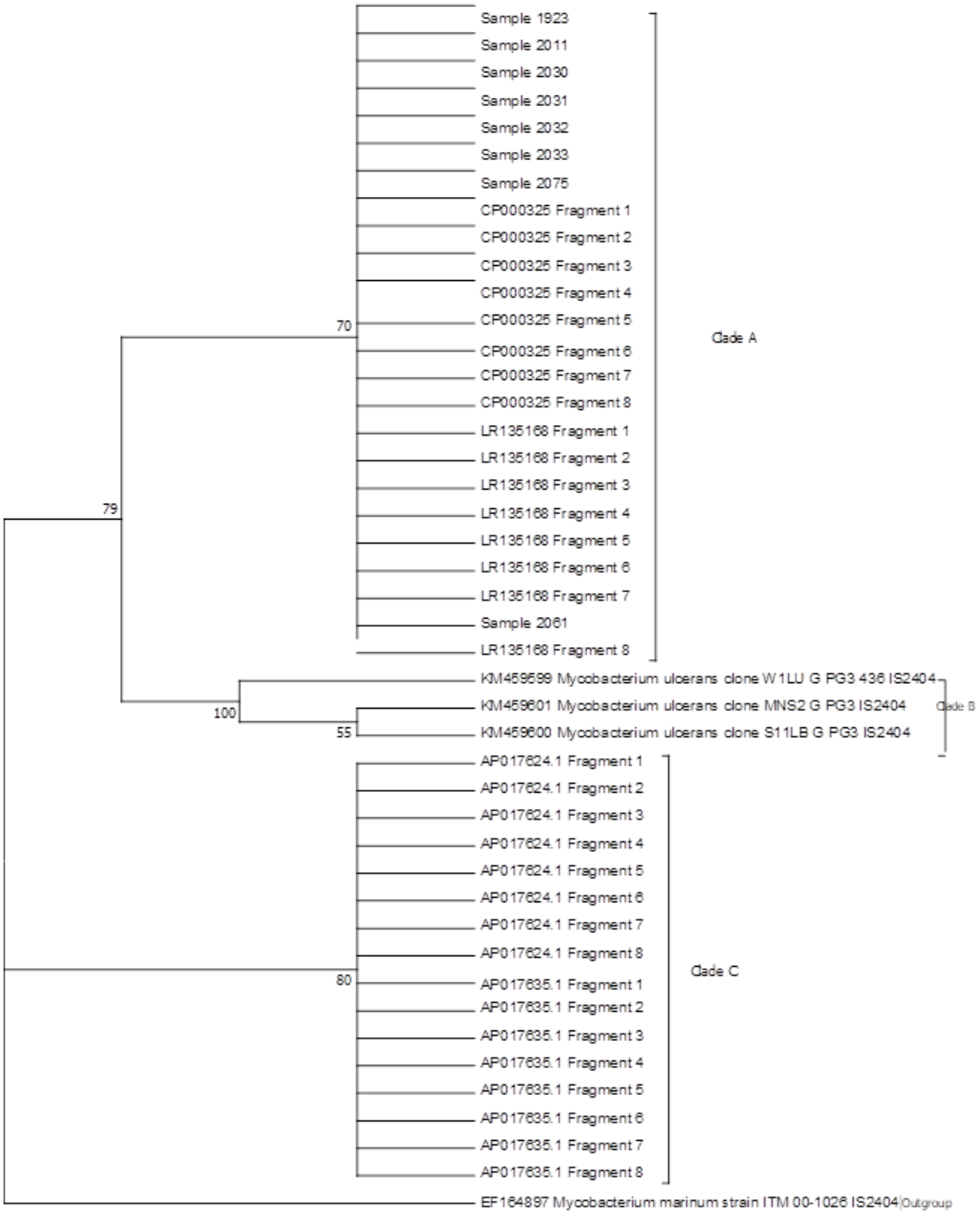

Figure 4. Phylogenetic Tree showing the 3 clades generated using Approach 2.

IV. Discussion

We screened some clinically suspected Buruli ulcer patients using a nested PCR for the confirmation of the presence of an insertion sequence IS*2404* from the genome of *Mycobacterium ulcerans*, the aetiologic agent of Buruli ulcer.

Gene: IS*2404*, which encodes a protein of 327 amino acids, is an insertion sequence from *Mycobacterium ulcerans* genome [14].

In order to evaluate our strains' genetic diversity in comparison with publicly available suitable genome data using comparative genomics, reference genomes of *Mycobacterium ulcerans* were obtained from the NCBI database (Accession Numbers: LR135168 and CP000325); *M. ulcerans subspecies shinshuense* (Accession Numbers: AP017624 and AP017635). We also obtained sequences of *M. ulcerans* IS2404 gene (Accession Numbers: EF164897; KM459600 and KM459601). The sequences obtained from NCBI were

aligned with the 8 sequences from this study and at an approximately 219bp of the IS*2404* region of the genome which aligned best with the sequences from this study was extracted and used to infer a maximum likelihood tree using as the best-fit model according to Bayesian Information Criterion (BIC).

Basically, the samples in this study are more closely related to *M. ulcerans Agy99* (accession nr CO000325) and *M. ulcerans* (accession nr LR135168); than they are to *M. ulcerans subsp shinshuense* (accession nr AP017624.1 and AP017635.1).

Clade A is separated from Clade B with 79% bootstrap support, and 100% bootstrap support from clade C. These indicate that the clades are significantly different to each other, and the putative identifications assigned to the samples is confident

V. Conclusion

Both approaches showed the similar results. All the samples were identified as M. ulcerans (through BLAST analysis with NCBI).

The grouping of samples/CP000325/LR135168 (Clade A) is separated from other clones of *M. ulcerans* (Clade B) with 79% bootstrap confidence (on the node of the branch). Clade C represents only M. *ulcerans subsp. shinshuense* samples (low relationship to the sequences strains). According to the results, the sequenced strains from the samples are genetically nearly identical and more closely related to *Mycobacterium ulcerans* Agy99 (a reference strain isolated in Ghana) and *Mycobacterium ulcerans* strain SGL03 (reference strain name DRC), than to the *M. ulcerans subsp shinshuense* (a reference strain from Japan). This supports the organism's strong geographical diversity with intra-continental strains being genomically extremely closely related [15]

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