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Molecular epidemiology of tuberculosis in Tetouan, Morocco: insights into strain diversity and transmission dynamics

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Article

Keywords: MIRU-VNTR, Mycobacterium tuberculosis complex, Beijing lineage, Mycobacterium africanum, molecular epidemiology, genetic diversity, transmission dynamics, Morocco, North Africa

Posted Date: August 4th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10308539/v1

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Additional Declarations: No competing interests reported.

Abstract

Background

Tuberculosis (TB) remains a major public health challenge in Morocco, with marked regional disparities shaped by social and environmental conditions. We investigated the molecular epidemiology of Mycobacterium tuberculosis complex (MTBC) strains circulating in Tétouan, Morocco, focusing on strain diversity, lineage distribution, transmission dynamics, and associated sociodemographic factors using 24-locus MIRU-VNTR genotyping.

Methods

In this prospective cross-sectional study, 103 MTBC isolates from bacteriologically confirmed pulmonary TB patients diagnosed in Tétouan between 2020 and 2023 were analyzed using the 24-locus MIRU-VNTR scheme. Genetic diversity was assessed using the Hunter–Gaston Discriminatory Index (HGDI) and locus-level allelic diversity (*h*). Phylogenetic relationships were analyzed using UPGMA dendrograms and minimum spanning tree (MST) analysis *via* the MIRU-VNTR*plus* platform and recent transmission was estimated using the n − 1 method. Associations between clonal complex (CC) assignment and sociodemographic variables were evaluated using binary logistic regression. To explore the evolutionary context of the locally dominant Beijing and LAM strains, their profiles were compared to representative global datasets.

Results

The 24-locus MIRU-VNTR scheme demonstrated excellent discriminatory power (HGDI = 0.9896). Among the 103 isolates, 52 (50.5%) were assigned to established lineages: LAM (29.1%), Beijing (9.7%), Ghana (3.9%), S (2.9%), Haarlem (1.9%), West African 1 (1.9%), and West African 2 (1.0%). UPGMA analysis identified 17 (16.5%) isolates grouped into five identical-profile clusters, translating to a recent transmission rate of 11.6%. MST analysis revealed 10 CCs encompassing 40 (38.8%) isolates. Residence in the Old Medina slum area was the only independent predictor of CC membership (*p*=.001).

Conclusion

The MTBC population in Tétouan is characterized by high genetic diversity and limited evidence of recent transmission, with residence in informal urban settlements (slums) emerging as a potential predictor of transmission. The first reported detection of Ghana, West African 1, and West African 2 lineages in Morocco suggests recent strain introductions, while the phylogeographic structure of Beijing and LAM strains reflects multiple historical dissemination routes involving Northern Eurasia, the Mediterranean basin, Africa, and possibly South America, followed by local diversification. Our findings support strengthening molecular surveillance through high-resolution genomic approaches and implementing geographically targeted public health interventions to interrupt TB transmission in lower-middle-income countries such as Morocco.

Introduction

Tuberculosis (TB) remains a major global public health threat and the leading cause of death from a single infectious agent. In 2024, an estimated 10.7 million new cases and 1.23 million deaths were reported worldwide, disproportionately affecting low- and middle-income countries (LMICs) [1]. Although TB is preventable and curable, progress toward elimination remains insufficient. The WHO End TB Strategy aims to achieve a 50% reduction in incidence by 2025 compared with 2015 levels [2], though the global decline reached only approximately 12–15% by 2024, falling substantially short of this milestone [1], highlighting persistent transmission as a major obstacle towards the End TB targets [3].

In Morocco, TB remains a major public health concern, ranking first in North Africa with an estimated incidence of 98 cases per 100,000 inhabitants in 2024 [1, 4]. Although the National Tuberculosis Control Program has achieved relatively high case detection (83%) and treatment success (86%) rates, consistent with WHO 2015 targets [5], incidence has declined slowly, from 115 per 100,000 inhabitants in 2000 to 92 per 100,000 in 2023 [2, 6, 7].

Drug-resistant TB further complicates control efforts, particularly among previously treated patients, where multidrug-resistant TB (MDR-TB, defined as resistance to at least isoniazid [INH] and rifampicin [RMP]) is estimated at 8.8% [8]. Alarmingly, 19% of worldwide MDR/RR-TB cases in 2023 showed fluoroquinolone (FQ) resistance — meeting the criteria for pre-extensively drug-resistant TB (pre-XDR-TB) — while additional resistance to bedaquiline or linezolid defines extensively drug-resistant TB (XDR-TB), associated with severely limited treatment options and higher mortality [9].

TB distribution in Morocco shows marked regional disparities. In 2024, four out of twelve regions accounted for 72% of reported cases nationally, including the northern Tangier–Tétouan–Al Hoceima region, which reported an incidence of 111 per 100,000 inhabitants [4, 7]. These

disparities likely reflect unequal socioeconomic conditions, overcrowding, precarious housing, urbanization, internal and transnational mobility, and differences in circulating *Mycobacterium tuberculosis* complex (MTBC) strains [3, 10]. Northern Morocco additionally represents a major migratory interface between sub-Saharan Africa and Europe, potentially influencing local MTBC diversity and transmission dynamics [11, 12].

Molecular epidemiology plays a critical role in understanding MTBC population structure and transmission patterns. Whole-genome sequencing (WGS) is increasingly regarded as the gold standard for TB genotyping and drug-resistance profiling [13], and its implementation is expanding in LMICs through initiatives such as the WHO Global TB Programme's sequencing guidance and decreasing costs of portable nanopore platforms [14, 15]. However, WGS implementation in Morocco remains constrained by limited local bioinformatics capacity, lack of standardized pipelines for real-time surveillance, and the need for sustained funding to maintain sequencing infrastructure. Consequently, 24-locus Mycobacterial Interspersed Repetitive Units – Variable Number of Tandem Repeats (MIRU-VNTR) remains a practical high-resolution genotyping method in settings such as Morocco.

To our knowledge, despite the high TB burden in northern Morocco, no dedicated molecular epidemiological study has been conducted in the Tangier–Tétouan–Al Hoceima region. Previous Moroccan studies were geographically restricted, included limited representation from the north, and were conducted more than a decade ago [16–19]. Moreover, no prospective study has applied the complete 24-locus MIRU-VNTR scheme within a geographically defined high-burden province in this region.

To address these gaps, we conducted a prospective, cross-sectional molecular epidemiological study in a high-burden urban-slum setting in northern Morocco (Tangier–Tétouan–Al Hoceima region). We applied 24-locus MIRU-VNTR genotyping to pulmonary TB isolates collected in Tétouan city between 2020 and 2023 to describe MTBC lineage distribution, investigate associated sociodemographic factors, and generate baseline data to support geographically targeted TB control strategies.

Materials and Methods

Study Design and Setting

A prospective cross-sectional molecular epidemiological study was conducted between January 2020 and December 2023 at the Provincial Diagnostic Center for Tuberculosis in Tétouan, Morocco. This center serves as the sole reference facility for TB diagnosis, treatment, and surveillance across Tétouan Province, in accordance with the National TB Control Program (PNLAT). Tétouan is a Mediterranean coastal city located in northwestern Morocco (35.57°N, 5.37°W), with a population of 611,928 inhabitants, according to the 2024 census, representing approximately 1.7% of the national population. The province comprises the urban municipality of Tétouan and 20 surrounding rural communes, characterized by high population density (~ 319 inhabitants/km²) **(Additional file 1, Table S1)**. The geographical location and administrative organization of the study area are shown in Fig. 1.

(**A**) National map of Morocco showing the location of the Tangier-Tétouan-Al Hoceima region (highlighted in red) in the northwest of the country. (**B**) Administrative map of the Tangier-Tétouan-Al Hoceima region, with its provinces and prefectures. (**C**) Detailed map of Tétouan Province showing the 20 rural communes and the urban Municipality of Tétouan, the site of the reference tuberculosis diagnostic laboratory where the study was conducted. Commune boundaries are shown in blue; neighbouring administrative units are labelled for geographic orientation. The north arrow indicates map orientation [20].

Study population and sample collection

A total of 1,434 patients with bacteriologically confirmed pulmonary TB were registered at the Provincial Diagnostic Center (2020–2023) **(Additional file 2)**. Sputum specimens were prospectively collected from 609 consecutive patients with pulmonary TB and tested using GeneXpert MTB/RIF *Ultra*® (Cepheid, USA), covering bacterial load categories from low to high. Samples were processed immediately or stored at 4°C and cultured within 48 hours. Sociodemographic and clinical data were collected from the provincial tuberculosis surveillance registry. Of these, 450 (73.9%) yielded viable MTBC-positive cultures. DNA extraction was performed for all culture-positive isolates, and 175 (38.9%) had sufficient DNA quality and quantity (DNA ≥ 10 ng/µL with 260/280 ratio ≥ 1.8) for reliable 24-locus MIRU-VNTR genotyping. Among them, 103 isolates (58.9%) produced complete and interpretable profiles across all 24 loci and were included in the final analysis. The remaining 72 isolates were excluded due to incomplete profiles caused by absent or ambiguous migration bands despite repeated PCR amplification and electrophoresis. The workflow for this study is shown in Fig. 2.

Drug susceptibility testing

All sputum samples with confirmed TB were initially tested using the Xpert MTB/RIF *Ultra*®assay (Cepheid, USA) to detect MTBC and assess RMP susceptibility. Samples identified as RMP-resistant were subsequently analyzed using the Xpert MTB/XDR assay (Cepheid, USA) to determine resistance to additional anti-TB drugs, including INH, FQs, Ethionamide (ETH), and second-line injectable agents, enabling identification of MDR-TB, pre-XDR-TB, and XDR-TB. This classification was based on Xpert MTB/RIF *Ultra*®and Xpert MTB/XDR results only.

Sputum processing and mycobacterial culture

Specimen decontamination and culture were performed using the modified Petroff method [21]. Briefly, sputum was inoculated onto Löwenstein–Jensen and Coletsos media and incubated at 37°C for up to 8 weeks. Positive cultures were suspended in Tris–EDTA buffer, heat-inactivated (95°C for 45min.) and stored at -80°C until further use. Inactivated isolates were shipped on dry ice (Biological Substance, Category B) to the University of Warsaw, Poland, for molecular analysis.

DNA Extraction

Colonies were suspended in Tris–EDTA buffer and lysed by heat treatment at 95°C for 45 min. Cellular debris was removed by centrifugation, and the resulting crude DNA extract (supernatant) was stored at -20°C.

24-locus MIRU-VNTR genotyping

Genotyping followed the standardized 24-locus MIRU-VNTR protocol [22]. Briefly, PCR amplification was carried out using established primer pairs (**Additional file 1. Table S2**) in 96-well microplates. Cycling conditions included 95°C for 3min; 40 cycles of 95°C (30s), 50°C (30s), and 72°C (90s); and final extension at 72°C for 7min.

Amplicons were resolved on 1.5% agarose gels and visualized under UV light. *M. tuberculosis* H37Rv was used as the positive control. Alleles were assigned using standardized sizing tables. All profiles were independently read by two investigators, with repeat testing for ambiguous results.

MIRU-VNTR data analysis: The 24-locus allelic profiles were entered into a standardized database and analyzed using the MIRU-VNTR*plus* web platform (http://www.miru-vntrplus.org; accessed in 2024) for lineage assignment and phylogenetic analysis [23]. Strain lineages were assigned by comparison with validated reference strains using a maximum distance threshold of 0.17. Phylogenetic relationships were evaluated using both an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram and a minimum spanning tree (MST).

UPGMA-based clustering and recent transmission estimation

Isolates sharing identical 24-locus MIRU-VNTR profiles (zero allelic differences under the UPGMA criterion) were considered clustered, whereas unique profiles were classified as singletons. The clustering rate was calculated as the proportion of clustered isolates among all genotyped isolates. Recent transmission was estimated using the $n - 1$ method [24]

Minimum spanning tree analysis

A minimum spanning tree (MST) was constructed from 24-locus MIRU-VNTR profiles to visualize phylogenetic relationships and infer transmission patterns among isolates. Clonal complexes (CCs) were defined using a maximum threshold of two allelic differences (DLV ≤ 2), encompassing identical profiles, single-locus variants (SLV), and double-locus variants (DLV). Isolates differing by ≥ 3 loci were considered unlikely to belong to the same transmission chain. To better discriminate transmission chains from broader evolutionary relatedness, MST analysis was additionally performed using the stricter SLV criterion (≤ 1 allelic difference) [25]. This conservative approach reduces the risk of falsely linking unrelated strains through convergent evolution or shared ancestral polymorphisms.

Allelic diversity and discriminatory power

Allelic diversity at each MIRU-VNTR locus was calculated using the allelic diversity index (h), and the overall discriminatory power was assessed using the Hunter–Gaston discriminatory index (HGDI) [26]. Both h and HGDI range from 0 (no discrimination) to 1 (complete discrimination). Allelic diversity was classified as low ($h < 0.3$), moderate ($0.3 \leq h < 0.6$), or high ($h \geq 0.6$).

Statistical Analysis

Descriptive statistics were used to summarize the study population and genotyping data. Frequencies and percentages were calculated for qualitative variables, while quantitative variables were described using means and standard deviations. Associations between clonal complex membership and demographic/clinical variables were assessed using binary logistic regression. Adjusted odds ratios (AORs) with 95% confidence intervals were reported. Statistical significance was set at $p<.05$. Analyses were performed using IBM SPSS v26 Statistics.

A detailed description of laboratory procedures and MIRU-VNTR genotyping conditions, including PCR protocols, primer sequences, electrophoresis settings, allele assignment, allelic diversity and discriminatory power calculations, is provided in **Additional file 3**.

Results

Demographic and clinical characteristics: A total of 103 MTBC isolates from bacteriologically confirmed pulmonary TB patients were genotyped using the 24-locus MIRU-VNTR scheme. The cohort was predominantly male (76.7%; n = 79), with a mean age of 43.1 ± 17.5 years. The largest age group was 31–50 years (36.9%; n = 38) **(**Table 1**)**. Most patients resided in urban areas (78.6%; n = 81), including 32.1% (n = 26) in the Old Medina; while 19.4% (n = 20) lived in rural settings and 1.9% (n = 2) were incarcerated. Newly diagnosed TB cases represented 89.3% (n = 92), while 10.7% (n = 11) were relapses. Treatment outcome data were available for 98 (95.1%) patients: treatment success was achieved in 85.7% (n = 84), 12.2% (n = 12) were lost to follow-up, and 2.0% (n = 2) died **(**Table 1**)**.

Comparison of the final genotyped cohort (n = 103) with the registered population (n = 1,434) and culture-positive isolates (n = 450) showed no significant differences in age distribution, sex ratio, or urban/rural residence **(Additional file 1, Table S3)**.

Drug susceptibility

Rifampicin susceptibility was detected in 97.1% (n = 100) of isolates. Three (2.9%) isolates were RMP-resistant, including one classified as a pre-XDR-TB case with additional resistance to INH and FQs. No XDR-TB cases were identified.

Lineage distribution

Molecular classification identified 97.1% (n = 100) of isolates as *M. tuberculosis sensu stricto* and 2.9% (n = 3) as *M. africanum*. Overall, 52 (50.5%) isolates were assigned to established lineages, while 51 (49.5%) remained unassigned (Unknown). Latin American Mediterranean (LAM) was the predominant lineage (29.1%; n = 30), followed by Beijing (9.7%; n = 10), Ghana (3.9%; n = 4), S (2.9%; n = 3), Haarlem (1.9%; n = 2), West African 1 (1.9%; n = 2), and West African 2 (1.0%; n = 1) **(**Table 1**)**.

Table 1
Demographic, clinical, and molecular genotypic characteristics of pulmonary tuberculosis patients, Tétouan Province, Morocco, 2020–2023 (n = 103)

Variable	Category	n (%)
Age group (years)	≤ 18	3 (2.9)
	19–30	28 (27.2)
	31–50	38 (36.9)
	51–64	18 (17.5)
	≥ 65	16 (15.5)
Gender	Male	79 (76.7)
	Female	24 (23.3)
Residential setting *(n = 103)*	Urban	81 (78.6)
	Rural	20 (19.4)
	Incarcerated	2 (1.9)
Urban district *(n = 81)*	New Medina	55 (67.9)
	Old Medina (Slums)	26 (32.1)
TB status	New case	92 (89.3)
	Relapse	11 (10.7)
Treatment outcome *(n = 98)*	Cured	68 (69.4)
	Treatment completed	16 (16.3)
	Lost to follow-up	12 (12.2)
	Died	2 (2.0)
Molecular lineage	Unassigned	51 (49.5)
	LAM (Latin American Mediterranean)	30 (29.1)
	Beijing	10 (9.7)
	S	3 (2.9)
	Ghana	4 (3.9)
	Haarlem	2 (1.9)
	West African 1	2 (1.9)
	West African 2	1 (1.0)
Clustered isolates *	Yes	17 (16.5)
	No	86 (83.5)
Patients in transmission chains (CCs)**	Yes	40 (38.8)
	No	63 (61.2)

Data are presented as absolute frequencies and percentages [n (%)]. Percentages are calculated relative to the total study population (n = 103) unless a specific sub-denominator is indicated in parentheses alongside the variable heading. Treatment outcome data were available for 98 (95.1%) patients; five patients were excluded due to incomplete records. Urban district distribution is reported only for the 81 patients residing in urban areas. Treatment success is defined as the combined proportion of patients classified as cured or having completed treatment. Molecular lineage was determined by comparison of 24-locus MIRU-VNTR copy-number profiles against validated reference strains in the MIRU-VNTR*plus* database (www.miru-vntrplus.org) using a maximum distance threshold of 0.17; isolates with no match within this threshold were classified as unassigned.

*Clustered isolates: isolates sharing strictly identical 24-locus MIRU-VNTR profiles (0 allelic differences), as identified by UPGMA-based analysis.**CCs: Clonal complexes, defined as groups of isolates differing by no more than two allelic loci (Double Locus Variant, DLV threshold)

in the Minimum Spanning Tree (MST) analysis.

Allelic diversity and discriminatory power

The 24-locus MIRU-VNTR scheme showed excellent discriminatory power (HGDI = 0.9896). Fifteen of 24 *loci* exhibited high allelic diversity ($h > 0.6$), seven showed moderate diversity ($0.3 \leq h < 0.6$), and MIRU04 locus displayed low discriminatory power ($h = 0.28$) **(**Table 2**)**.

Table 2
Allelic diversity and discriminatory power of 24 MIRU-VNTR *loci* assessed in 103 *Mycobacterium tuberculosis* complex isolates from pulmonary TB patients, Tétouan Province, Morocco, 2020–2023.

Locus	Alias	*h*	Discriminatory Power
154	MIRU02	0.52	Moderate
424	Mtub04	0.75	High
577	ETRC	0.70	High
580	MIRU04	0.28	Low
802	MIRU40	0.79	High
960	MIRU10	0.61	High
1644	MIRU16	0.57	Moderate
1955	Mtub21	0.68	High
2059	MIRU20	0.40	Moderate
2163b	QUB11b	0.74	High
2165	ETRA	0.57	Moderate
2347	Mtub29	0.58	Moderate
2401	Mtub30	0.70	High
2461	ETRB	0.52	Moderate
2531	MIRU23	0.70	High
2687	MIRU24	0.46	Moderate
2996	MIRU26	0.65	High
3007	MIRU27	0.61	High
3171	Mtub34	0.62	High
3192	MIRU31	0.68	High
3690	Mtub39	0.77	High
4052	QUB26	0.74	High
4156	QUB4156	0.52	Moderate
4348	MIRU39	0.71	High
	Overall (HGDI)*	**0.9896**	**Excellent**

Loci are listed in ascending order of chromosomal position. The allelic diversity index (*h*) was calculated for each locus, using the MIRU-VNTR*plus* web platform tools. *The overall discriminatory power of the complete 24-locus panel was assessed using the Hunter-Gaston Discriminatory Index (HGDI). Per-locus allelic diversity was classified as: High ($h \geq 0.6$), Moderate ($0.3 \leq h < 0.6$), or Low ($h < 0.3$). Locus nomenclature follows the standardized system [22].

UPGMA clustering and recent transmission

Among 103 isolates, UPGMA analysis identified 91 distinct MIRU-VNTR patterns, with five identical-profile clusters comprising 17 (16.5%) isolates and 86 (83.5%) singleton profiles. Four clusters were exclusively composed of LAM isolates (n = 7, 4, 2, and 2), while one cluster included two Beijing isolates **(**Fig. 3**)**.

The four Ghana lineage isolates occupied the uppermost position of the dendrogram, forming a compact clade with short inter-isolate branch lengths. Immediately adjacent, the two Haarlem isolates clustered together, separated from the Ghana clade by a moderate branch length. The Beijing lineage isolates (n = 10) formed a well-supported, cohesive clade with consistently short internal branches. The three S lineage isolates grouped as a discrete group. The LAM clade was the largest and most internally diverse, comprising 30 isolates distributed across multiple sub-branches with variable branch lengths. The two West African 1 isolates appeared as a proximate pair in the lower region of the dendrogram. The 51 unassigned isolates were dispersed throughout the lower portion of the tree across multiple scattered branches, with long inter-isolate distances **(**Fig. 3**)**.

The recent person-to-person transmission rate (RTR) estimated by the *n − 1* method was 11.6%. None of the Ghana, Haarlem, S, West African 1, West African 2, or unassigned isolates formed identical-profile clusters. This is consistent with the long branch lengths connecting these isolates to the remainder of the tree.

The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm applied to pairwise genetic distance matrices derived from 24-locus MIRU-VNTR copy-number profiles. Each terminal node represents one clinical isolate, identified by its study number. Node background color indicates lineage assignment as determined by the MIRU-VNTR*plus* reference database: dark blue = Beijing; orange = Ghana; pink/magenta = Haarlem; red = LAM; yellow = S; olive green = West African 1; dark green = West African 2; pale yellow = Unassigned (-?-). The allele copy-number matrix to the right of the dendrogram displays the 24 MIRU-VNTR locus values for each isolate. Isolates sharing identical 24-locus profiles (0 allelic differences), and therefore designated as clustered, are visually identifiable as terminal branches merging at a node distance of zero. The horizontal scale bar represents a genetic distance of 0.1 (10% allelic difference across the 24 loci). The dendrogram was generated using the MIRU-VNTR*plus* web platform (www.miru-vntrplus.org).

Minimum Spanning Tree and Clonal Complex Analysis

MST analysis was performed using both SLV (≤ 1 allelic difference; Fig. 4A) and DLV (≤ 2 allelic differences; Fig. 4B) thresholds. The two panels share a similar global topology, confirming the structural robustness of the clustering signal independent of the threshold chosen.

Under the SLV criterion, 10 CCs were identified encompassing 33 isolates (32%), including a Beijing cluster (n = 5), five LAM clusters: one of 7 isolates, one of 4 isolates, two of 2 isolates each, and one additional LAM pair on the tree periphery. One Ghana pair also formed a discrete SLV-CC. The remaining isolates (n = 70, 68%) were singletons separated by 6–13 allelic differences, reflecting the high background genetic diversity **(**Fig. 4A**)**. Applying the DLV threshold expanded the encompassing isolates to 40 (38.8%) distributed over 10 CCs, while 63 (61.2%) isolates remained as singletons. The largest CC comprised 10 LAM isolates with a hub-and-spoke architecture, anchored by 7 isolates sharing an identical central profile with three additional LAM profiles connected at 1–2 allelic differences. The second-largest CC contained 8 Beijing isolates, with 5 SLV-linked isolates expanded by three additional isolates falling within the DLV boundary. Four additional smaller LAM clusters (comprising two 4-isolate clusters and two 2-isolate clusters), one Ghana CC (n = 2), and one unassigned CC (n = 2) were also identified **(**Fig. 4B**).** The two West African 1 isolates remained singletons in both panels, with a mutual genetic distance exceeding the DLV threshold **(**Fig. 4**)**, supporting independent introduction events from distinct source chains rather than descent from a common local founder.

Overall, MST analysis showed that transmission networks in Tétouan were mainly structured around LAM and Beijing lineages, while the high proportion of singletons supported a predominantly reactivation-driven epidemic.

Each node represents a distinct 24-locus MIRU-VNTR profile. The border color of each node indicates lineage affiliation according to the color key: navy blue = Beijing; yellow-gold = Ghana; green = Haarlem; red = LAM; purple = S; grey-mauve = West African 1; magenta = West African 2; olive = Unassigned. The node fill reflects the number of clinical isolates sharing that exact profile: pale blue = 2 isolates; blue = 4 isolates; dark blue = 7 isolates; white = 1 isolate (unique singleton profile). Numbers on branches denote the genetic distance (number of allelic differences) between adjacent profiles. Clonal complexes (CCs), delineated by rectangular or rhombus-shaped boxes, group profiles connected within the defined threshold. In Panel A, CCs are defined at the SLV threshold (≤ 1 allelic difference); in Panel B, CCs are defined at the DLV threshold (≤ 2 allelic differences). Singletons falling outside any CC boundary are displayed as isolated, unlabeled nodes. The MST was generated using the MIRU-VNTR*plus* web platform (www.miru-vntrplus.org).

Global phylogenetic context of Beijing and LAM lineages

To place the Beijing and LAM isolates from Tétouan within a broader phylogenetic framework, additional comparative analyses were performed using previously published global datasets. Comparison of the 10 Beijing isolates from Tétouan with representative Beijing genotype profiles from Russia/FSU, China, and South Africa [27–30] showed that one isolate (isolate 145) was positioned distantly from the remaining Beijing strains **(Additional file 5)**. The other nine isolates were distributed within two major Beijing genotype clusters. Three isolates (isolates 32, 140, and 151) were located within the 100 − 32 cluster and differed from the ancestral 100 − 32 profile by a single MIRU-VNTR locus each. Six additional isolates were associated with the 94 − 32 cluster. Two isolates (isolates 38 and 152) displayed the exact 94 − 32 profile, whereas four others represented closely related variants within the same cluster **(Additional file 5)**.

Phylogenetic analysis of the 30 Moroccan LAM isolates, performed together with 259 representative global LAM profiles [31–34], revealed substantial heterogeneity within this lineage **(Additional file 6)**. Nineteen isolates belonged to the RD115 sublineage, of which 18 clustered within the RD115/LAM-RUS branch of the dendrogram **(Additional file 6, panel A)**. Two isolates were assigned to the RD-Rio sublineage and clustered together with strains from Saudi Arabia and Brazil. Six isolates belonged to the SIT33 sublineage **(Additional file 6, panel B)**, while the remaining isolates were distributed among other branches of the LAM family.

Factors associated with clonal complex membership: Old Medina residency was the only factor independently associated with clonal complex membership (AOR = 6.020; 95% CI: 2.158–16.683; p = .001), with residents nearly six times more likely to belong to a potential transmission cluster than those in the New Medina. Sex, age, overall urban/rural setting, and TB case type showed no significant associations **(**Table 3**)**.

Table 3
Association between DLV-defined clonal complex membership and demographic, geographic, and clinical characteristics of pulmonary tuberculosis patients (n = 103), Tétouan, Morocco, 2020–2023.

Variable	Category	CC Isolates n (%)	Singletons n (%)	AOR (95% CI)	p-value
Age group (years)	≤ 18	0 (0.0)	3 (100.0)	—	NS
	19–30	8 (28.6)	20 (71.4)	—	
	31–50	17 (44.7)	21 (55.3)	—	
	51–64	9 (50.0)	9 (50.0)	—	
	≥ 65	6 (37.5)	10 (62.5)	—	
Gender	Male	32 (40.5)	47 (59.5)	1	0.485
	Female	8 (33.3)	16 (66.7)	0.96 (0.366–2.534)	
Residential setting	Urban	33 (39.8)	50 (60.2)	1	0.790
	Rural	7 (35.0)	13 (65.0)	0.816 (0.427–3.057)	
Urban district	New Medina	15 (27.3)	40 (72.7)	1	**0.001**
(n = 81)	**Old Medina (Slums)**	**18 (69.2)**	**8 (30.8)**	**6.020 (2.158–16.683)**	
TB status	Relapse	2 (18.2)	9 (81.8)	1	0.166
	New case	38 (41.3)	54 (58.7)	3.056 (0.630–14.816)	

Data are presented as absolute frequencies and percentages [n (%)], calculated relative to each subgroup row total. Multivariable binary logistic regression was performed with clonal complex (CC) membership as the binary dependent variable (CC member = 1; singleton = 0). Adjusted odds ratios (AOR) with 95% confidence intervals (CI) were estimated by multivariable binary logistic regression, with all variables entered simultaneously. Reference categories are indicated as (1). Age was assessed as a categorical variable; as no age group reached statistical significance, AORs were not computed (—). Urban district (New Medina vs Old Medina) was evaluated in a separate multivariable logistic regression model restricted to urban residents (n = 81). Statistical significance was defined as $p < 0.05$; statistically significant results are shown in bold.

Abbreviations: NS: not significant; CC: clonal complex (groups of isolates differing by ≤ 2 allelic loci in the Minimum Spanning Tree, MST, Double Locus Variant threshold, DLV); AOR: adjusted odds ratio; CI: confidence interval; New Medina; modern urban residential district; Old Medina historically dense overcrowded urban district (Slums).

Discussion

This study provides the first detailed molecular epidemiological description of Mycobacterium tuberculosis complex (MTBC) strains circulating in Tétouan, northern Morocco, using the standardized 24-locus MIRU-VNTR scheme. The genotyping method showed excellent discriminatory power (HGDI = 0.9896), confirming its continued utility for population-level surveillance in settings where WGS is not yet routinely available.

The study population was predominantly male (76.7%) and middle-aged (mean age of 43.1 years), consistent with global [35], national [36] and provincial [37, 38] TB epidemiology and reflecting greater occupational and social exposure among adult males [39]. Most (78.6%) patients were urban residents, including a third from the overcrowded Old Medina, highlighting the established role of socioeconomic conditions in TB transmission [40–42].

Most (89.3%) cases were newly diagnosed, and treatment success was high (85.7%), consistent with national programmatic performance [4]. Rifampicin resistance was found sporadically (2.9%), including one pre-XDR-TB case. Although higher (*ca*. 1%) than the national MDR-TB prevalence [36], direct comparison is limited because national estimates include both pulmonary and extrapulmonary TB, and are derived from different surveillance methods. This rate may reflect improved detection after the introduction of GeneXpert MTB/RIF in 2017 [4] rather than a true increase in drug-resistant TB, although systematic surveillance with phenotypic and molecular second-line DST is needed for confirmation.

High allelic diversity was observed at 15 of the 24 MIRU-VNTR loci, including nine that demonstrated high discriminatory power in the last nationwide study (Mtub04, MIRU40, MIRU10, Mtub21, QUB11b, MIRU23, MIRU26, Mtub39, and QUB26) [16]. Among these, MIRU10, MIRU40, MIRU23, and MIRU26 were consistently identified as the most discriminative loci across several Moroccan settings [16, 17, 19, 43]. In contrast, MIRU04 again showed low discriminatory performance (*h* = 0.28), confirming its limited epidemiological utility in this context [16, 17, 19, 44].

These findings support the implementation of a reduced, country-adapted MIRU-VNTR panel composed of the most informative loci as a first-line tool for routine MTBC surveillance, enabling efficient cluster detection and strengthening TB control activities. However, validation against the full 24-locus scheme and local WGS is required before implementation. The complete 24-locus scheme could be reserved for higher-resolution investigations, including sub-cluster analysis and confirmation of transmission chains, in line with stepwise hierarchical genotyping strategies and locally adapted frameworks described in the literature [45–47]. Such an approach would optimize resource use by reducing costs and laboratory workload while preserving substantial discriminatory power, which is particularly relevant for TB surveillance in high-burden, resource-constrained settings, such as Morocco [48–50].

At the phylogenetic level, all but three isolates were *M. tuberculosis sensu stricto*. Those three represented *M. africanum*, a species rarely reported in Morocco [16–19, 44]. Almost a third of isolates belonged to LAM lineage, consistent with its known dominance in North Africa and previous Moroccan studies [16, 19, 44, 51–53]. The large and internally diverse LAM clades observed in UPGMA and MST analyses are consistent with long-term endemic circulation and progressive diversification within local transmission networks, highlighting the need for continued monitoring since this lineage has been linked with high prevalence of drug resistance nationally [44, 53]. The S and Haarlem lineages, also commonly reported in Morocco [16–19], were present at much lower (below 3%) frequencies.

The Beijing lineage was detected at a higher (9.7%) frequency in our study than in previous Moroccan reports, where it was rare or absent, with frequencies ranging from 0% to 3% [16, 17, 43]. This is epidemiologically relevant, as Beijing lineage has been associated with higher transmissibility, greater virulence, clonal expansion, and MDR-TB emergence [27, 32, 54–57]. Its clustering pattern and internal connectivity may indicate local circulation and possible onward transmission, warranting strengthened surveillance.

Global phylogenetic comparisons placed Moroccan Beijing and LAM isolates within broader international lineage frameworks. Most Beijing isolates clustered within two phylogenetic groups commonly reported in Russia and the former Soviet Union (FSU): the 100 – 32 cluster, corresponding to the epidemic B0/W148 subtype—the second most prevalent Beijing subtype in Russia and strongly associated with multidrug resistance (MDR) [30]— and the 94 – 32 cluster, corresponding to the Beijing Central Asian/Russian subtype [27–29, 58]. These findings suggest possible epidemiological links between Moroccan and Northern Eurasian Beijing strains populations, though the directionality and timing of these connections cannot be determined from MIRU-VNTR data alone. The limited single-locus variation observed among Moroccan isolates within the 100 – 32 cluster may indicate local diversification following introduction; however, multiple independent introductions from closely related sources cannot be ruled out.

The LAM lineage showed greater phylogenetic heterogeneity. Most Moroccan isolates belonged to the RD115/LAM-RUS branch — predominant across Russia, FSU countries, Northern Eurasia and parts of Eastern and Southern Europe [31–34, 59] — a pattern more consistent with shared ancestral history than recent importation. Two isolates fell within the RD-Rio sublineage and clustered with strains from Saudi Arabia and Brazil, pointing to more complex dissemination pathways beyond the putative South American region of origin [60]. Six additional isolates belonged to the SIT33 sublineage, consistent with its known distribution in Morocco [17], across Africa, South America, and the Western Mediterranean basin [31].

Collectively, these findings indicate that the phylogeographic structure of Beijing and LAM strains in Morocco reflects multiple historical dissemination routes involving Northern Eurasia, the Mediterranean basin, Africa, and possibly South America, combined with subsequent local diversification. However, the limited resolution of MIRU-VNTR precludes robust reconstruction of these processes; whole-genome sequencing will be essential to precisely delineate introduction events, transmission pathways, and diversification dynamics.

Of particular note is the first reported detection in Morocco of the Ghana (3.9%), West African 1 (1.9%), and West African 2 (1.0%) lineages, corresponding to Lineages 5 and 6 of the *M. africanum* complex. These lineages are largely confined to West Africa, particularly the Gulf of Guinea [61, 62]. Their presence in Tétouan may reflect recent introductions linked to the city's role as a major migratory transit corridor for sub-Saharan African populations [63, 64], however, without patient-level migration, origin, or travel data, this hypothesis remains speculative and requires prospective validation and comparison with strains from countries of origin. The Ghana clonal complex (n = 2 at SLV threshold) may suggest possible early local transmission, whereas West African 1 and 2 were singletons, supporting independent introductions rather than established local circulation.

Nearly half (49.5%) of isolates were unassigned, likely due to the limited representation of North African and sub-Saharan MTBC diversity in the MIRU-VNTR*plus* database rather than true genetic novelty [65, 66]. However, unassigned isolates may include previously uncharacterized North African genotypes. Most (96.1%) were singletons, suggesting genetically distinct infections. This highlights gaps in global TB databases and supports expanding WGS-based characterization of North African strains.

Overall, UPGMA clustering yielded 91 distinct genotypic patterns and a clustering rate of 16.5%, lower than the national rate reported in Morocco in 2012 (29.8%) [19] and closer to that of 2015 (14.7%) [16]. The recent transmission estimate was low (11.6%) compared with previous national estimates (22.3% and 20.2%) [17, 19]. MST analysis confirmed this pattern: despite 10 clonal complexes, most isolates remained orphans, and the number of complexes was unchanged between SLV and DLV thresholds, supporting stable epidemiological links rather than threshold-dependent artefacts [67, 68]. Overall, these findings are compatible with a predominantly reactivation-driven epidemic in Tétouan, rather than intense recent transmission, consistent with long-standing endemic reservoirs [69, 70]. However, this interpretation should be made cautiously, as low identical-profile clustering may also reflect high genetic diversity, limited MIRU-VNTR resolution, sampling bias, or rapid progression in a high-transmission setting. Without WGS and epidemiological data, these scenarios cannot be disentangled; thus, we report only limited evidence of recent transmission.

Transmission chains were concentrated in specific high-risk urban settings. Residence in the Old Medina was the only demographic factor independently associated with clonal complex membership (AOR = 6.0; 95% CI: 2.2–16.7; p = .001), with approximately six-fold higher odds of clustering compared with the New Medina. This confirms the link between overcrowding, socioeconomic deprivation, and sustained TB transmission both in Tétouan [71], and globally [40, 72, 73]. These findings support a dual response strategy: intensified active case finding and contact tracing in Old Medina, combined with systematic latent TB infection screening in high-risk groups across the province [74, 75]. Prioritizing these targeted interventions in structurally vulnerable districts such as the Old Medina would not only advance End TB milestones and the Sustainable Development Goals, but also help to break the cycle of infection, poverty and social marginalization [2]. The cost-effectiveness of such targeted approaches, compared with province-wide intensification, should be evaluated through health economic modeling.

Several limitations should be acknowledged. As a single-center study, results may not fully represent MTBC diversity across the wider Tangier–Tétouan–Al Hoceima region. Agarose gel electrophoresis rather than capillary electrophoresis was used for allele sizing, with a higher risk of misclassification, although mitigated by double reading and repeat testing of discordant results. The high proportion of unassigned isolates limited lineage interpretation and reflects incomplete regional representation in reference databases. The lack of epidemiological contact data prevented validation of molecular clusters. Furthermore, since MIRU-VNTR has lower resolution than WGS, the clustering and transmission rate might be overestimated. Finally, substantial stepwise laboratory attrition from initial sampling to complete 24-locus profiles could have introduced selection bias and may limit the representativeness of the final genotyped cohort.

Conclusions

This study provides the first strain-level molecular surveillance data from the Tangier–Tétouan–Al Hoceima region, revealing high MTBC diversity and limited recent transmission based on identical-profile clustering. It confirms the predominance of the LAM lineage and reports, for the first time in Morocco, West African lineages (Ghana, West African 1, and West African 2), as well as a higher frequency of Beijing strains than previously described nationally. Phylogenetic analyses suggest these strains belong to globally distributed sublineages, although MIRU-VNTR resolution limits inference on transmission history.

Residence in socioeconomically deprived slum areas was independently associated with clonal complex membership, supporting geographically targeted interventions, particularly in the Old Medina, alongside intensified case finding, contact tracing, and latent TB screening.

The high proportion of unassigned isolates and methodological constraints of MIRU-VNTR strongly support the implementation of whole-genome sequencing for routine surveillance to resolve transmission, characterize resistance, and track cross-border spread. In the interim, a validated reduced MIRU-VNTR panel may be used for screening, pending prospective validation.

Building on all these findings, future studies integrating WGS with prospective epidemiological contact tracing are underway to achieve higher-resolution transmission mapping in this high-burden, migration-affected setting.

Declarations

Acknowledgments

We sincerely thank the Provincial Delegation of Health and Social Protection of Tétouan for granting access to tuberculosis notification data and strains. We are also grateful to the healthcare professionals in Tétouan Province, and to all members of the Department of Medical Microbiology, University of Warsaw, Poland.

Funding

This research received no specific funding from public, commercial, or not-for-profit funding agencies. The first author received a Short-Term Scientific Mission (STSM) grant from ADVANCE-TB COST Action CA21164.

Author Contributions

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Noureddine Elmtili: Conceptualization, supervision, writing—review and editing.

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Ethics declarations

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. The study protocol was approved by the Institutional Review Board of the Hospital-University Ethics Committee of Tangier (CEHUT; IRB No. AC112JV/2-025). All data were anonymized prior to analysis and used exclusively for research purposes. All patients were referred to the TB reference center by their treating clinicians, and written informed consent was obtained from each participant—or from a legal guardian when applicable—prior to sputum collection and molecular testing.

Clinical trial number: not applicable

Competing Interests

The authors have declared that no competing interests exist.

Data Availability

All data supporting the findings of this study are included within the manuscript and its additional files. **Additional file 1** includes Table S1, describing the administrative and geographical subdivisions of Tétouan Province, and Table S2, listing the oligonucleotide primers used for 24-locus MIRU-VNTR genotyping. **Additional file 2** contains the anonymized dataset of pulmonary tuberculosis cases registered in Tétouan from 2020 to 2023. **Additional file 3** provides the detailed Materials and Methods underlying the results reported herein. **Additional file 4** includes the complete 24-locus MIRU-VNTR allelic profiles, DNA quality metrics, and associated clinical and demographic data for all genotyped MTBC isolates. **Additional file 5** (Figure S1 — 24-Locus Minimum Parsimony Phylogenetic Tree of Beijing-Lineage Mycobacterium tuberculosis Strains) presents the phylogenetic placement of Moroccan Beijing-lineage isolates within a global reference dataset. **Additional file 6** (Figure S2, panels A and B — Global 24-Locus MIRU-VNTR UPGMA Dendrogram of LAM Strains Including Moroccan Isolates) presents the phylogenetic position of Moroccan LAM strains within a global LAM dataset of 259 profiles, divided into two panels (A and B) owing to figure size.

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Figures

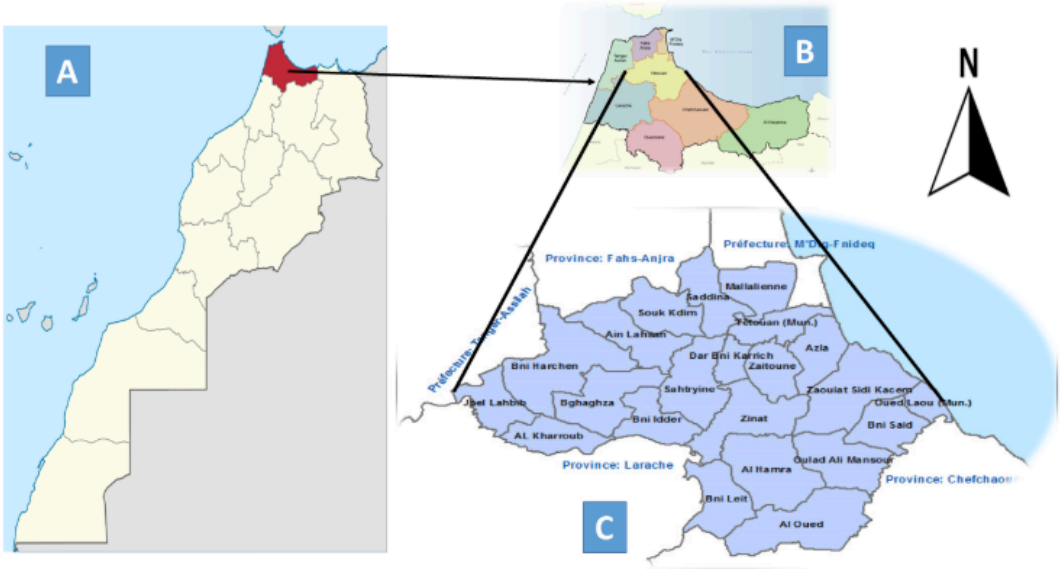


Figure 1

Geographic and administrative context of the study area: Tétouan Province, Tangier-Tétouan-Al-Hoceima Region, Morocco.

(**A**) National map of Morocco showing the location of the Tangier-Tétouan-Al Hoceima region (highlighted in red) in the northwest of the country. (**B**) Administrative map of the Tangier-Tétouan-Al Hoceima region, with its provinces and prefectures. (**C**) Detailed map of Tétouan Province showing the 20 rural communes and the urban Municipality of Tétouan, the site of the reference tuberculosis diagnostic laboratory where the study was conducted. Commune boundaries are shown in blue; neighbouring administrative units are labelled for geographic orientation. The north arrow indicates map orientation [20].

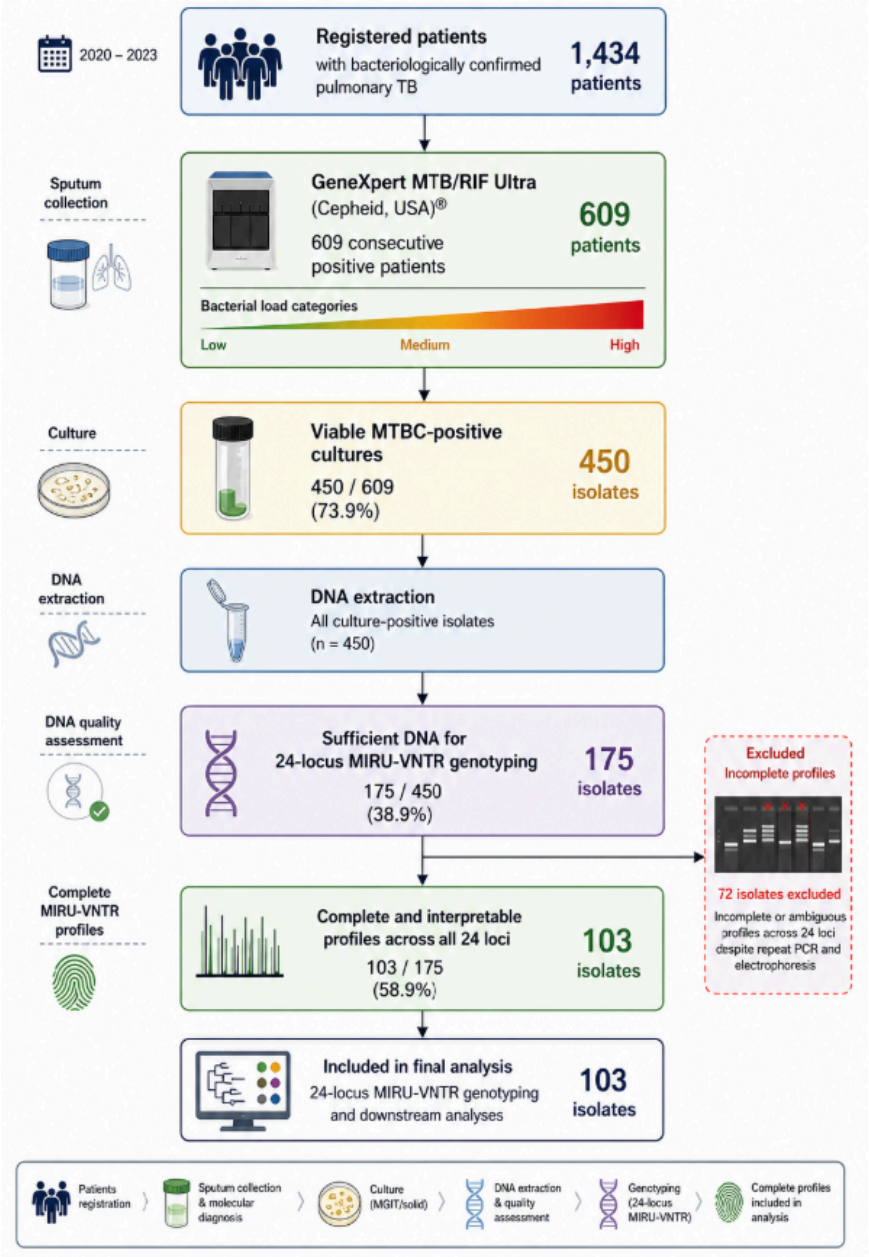


Figure 2

STROBE flow diagram of study participant inclusion and selection of isolates for genotyping.

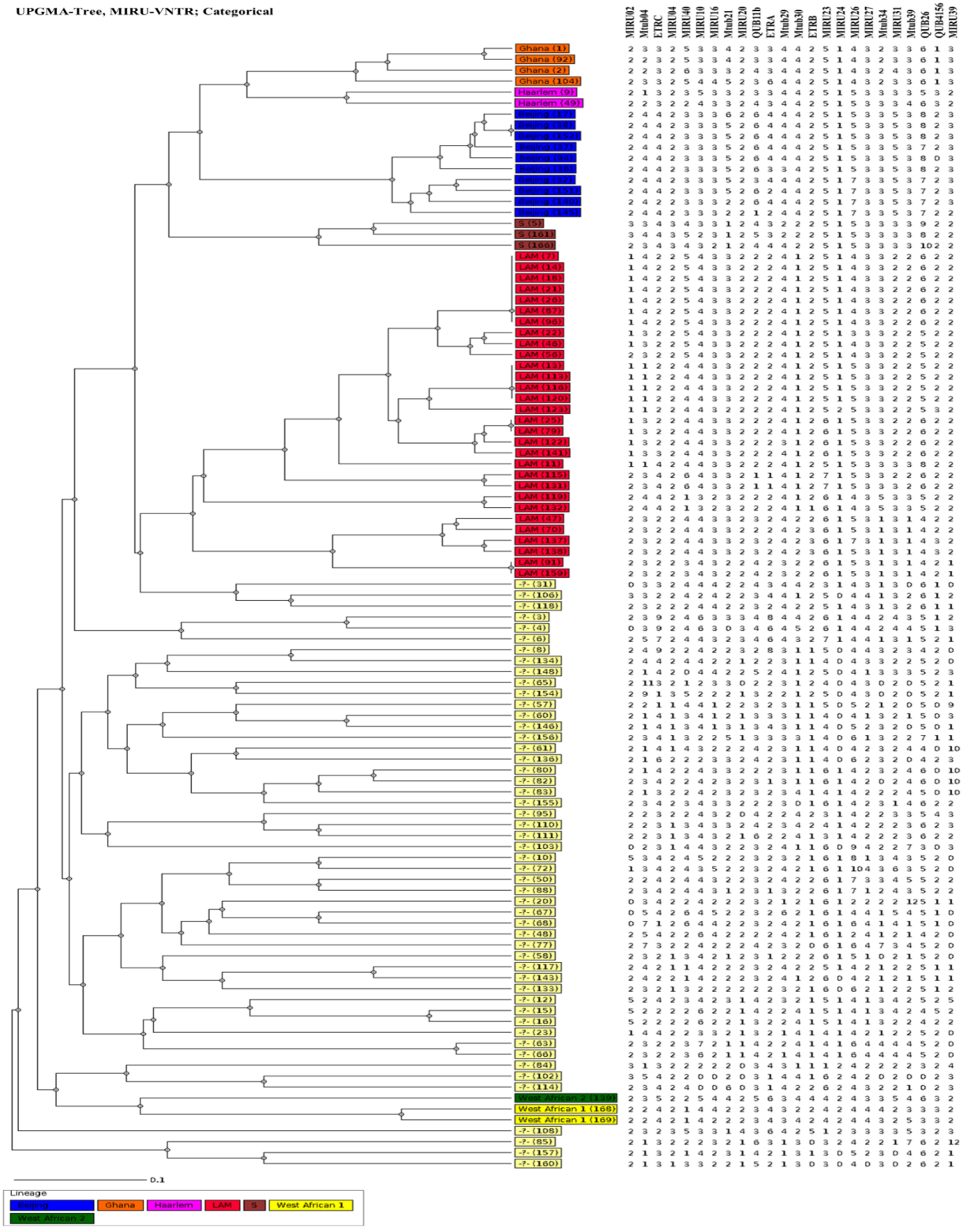


Figure 3

UPGMA dendrogram of 103 *Mycobacterium tuberculosis* complex isolates from pulmonary TB patients in Tétouan, Morocco (2020–2023), based on 24-locus MIRU-VNTR profiles.

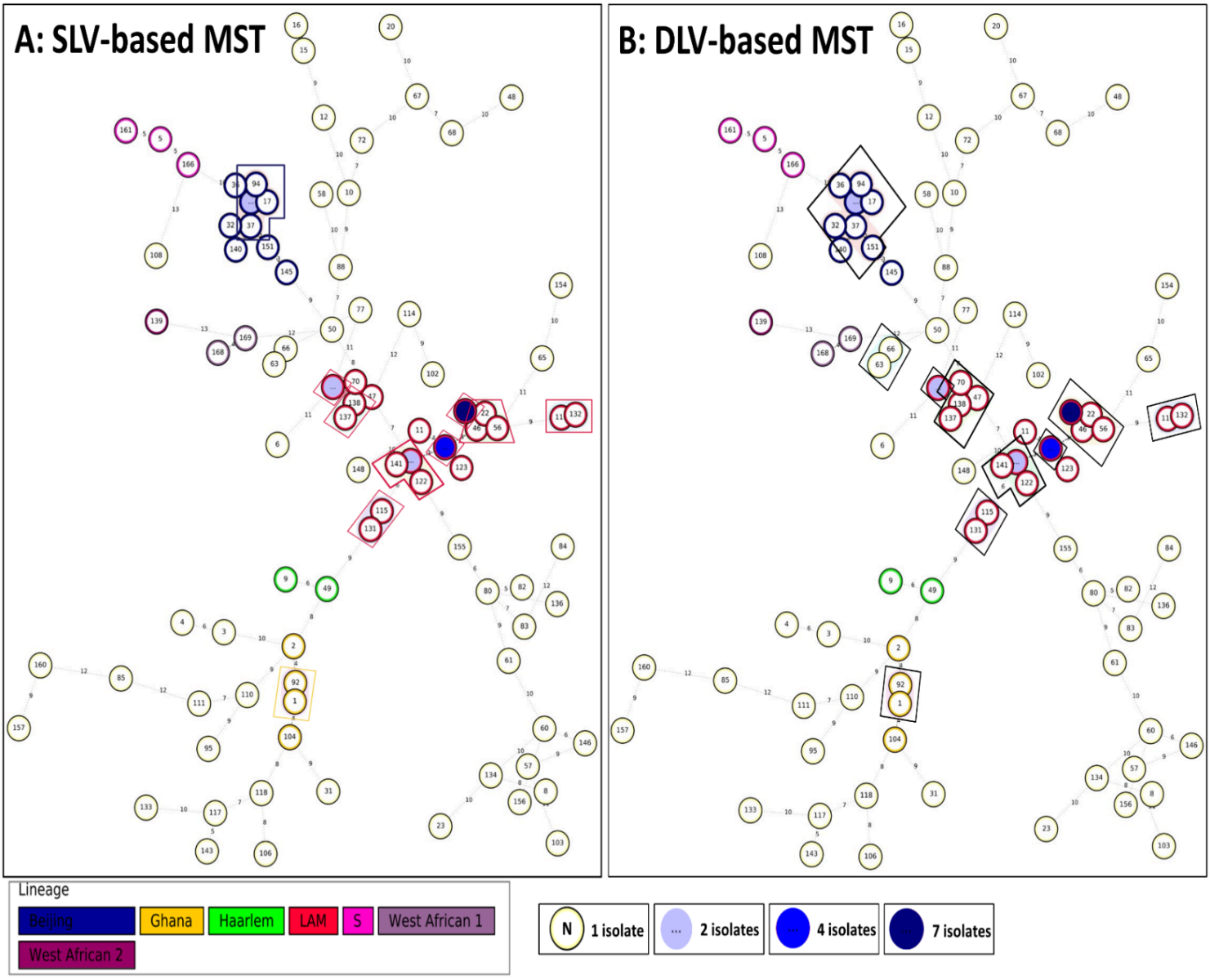


Figure 4

Minimum Spanning Tree (MST) analysis of 103 *Mycobacterium tuberculosis* complex (MTBC) isolates from pulmonary TB patients in Tétouan, Morocco (2020–2023), based on 24-locus MIRU-VNTR profiles. (A) SLV-based MST — Clonal complexes defined by Single Locus Variant threshold; (B) DLV-based MST — Clonal complexes defined by Double Locus Variant threshold.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1tableS1tableS2tableS3.docx
- Additionalfile3SupplementaryMethods.docx
- Additionalfile4MIRUVNTRgenotypingresults.xlsx
- Additionalfile6AFigS2A.jpg
- Additionalfile6AFigS2Ad1.jpg
- Additionalfile6BFigS2B.jpg
- Additionalfile7agarosegelsprints.pdf
- additionalfile220202023DEMODATATBTETOUAN.xlsx
- additionalfile5FigS1.jpg