Infection, Genetics and Evolution 137 (2026) 105869

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Genomic insights into *Mycobacterium orygis* in wild ungulates in Chennai, India

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ARTICLE INFO

Keywords:
Mycobacterium orygis
Mycobacterium tuberculosis complex (MTBC)
Tuberculosis
Wild ungulates
Zoonosis

ABSTRACT

Bovine tuberculosis (bTB) represents a significant global threat to livestock, wildlife, and human health, yet wildlife tuberculosis (wTB) remains underreported in India. This study aimed to investigate the presence of *Mycobacterium tuberculosis* complex (MTBC) species in wild ungulates at Guindy National Park, Chennai. Post-mortem tissue samples and fecal pellets were collected from spotted deer, sambar deer, and blackbuck, and analyzed using culture, molecular diagnostics, histopathology, and whole genome sequencing (WGS). *Mycobacterium orygis* was isolated from tissue samples of four animals, while molecular assays detected MTBC DNA in nine fecal samples. Drug resistance was identified in three fecal samples. Histopathology revealed characteristic granulomatous lesions, and WGS confirmed *M. orygis* in all tissue-derived isolates. Pangenome analysis identified 4222 genes with an estimated γ value of 0.0086, suggesting an open pangenome with novel genes accumulating at a slow rate. Comparative genomic analysis, including insertion sequence (IS) profiling and SNP analysis, revealed limited overall diversity but unique SNPs in Chennai isolates, suggesting local genomic differentiation. These findings highlight the dynamic nature of *M. orygis* within MTBC and highlight the importance of functional studies to understand host specificity and adaptability and the urgent need for systematic wTB surveillance and One Health-based interventions in India.

1. Introduction

Bovine tuberculosis (bTB) is a chronic infectious disease that primarily affects cattle; however, its host range extends to numerous mammals, including domestic and wild animals. It is caused by members of the *Mycobacterium tuberculosis* complex (MTBC), a group of closely related mycobacteria that are pathogenic to animals and humans (World Organisation for Animal Health (WOAH), 2022). The MTBC species that cause tuberculosis (TB) in animals are diverse, with each species having a particular host preference, although cross-species transmission is common. *Mycobacterium bovis*, traditionally associated with cattle and the conventional definition of zoonotic TB, also infects wildlife, such as badgers, deer, and wild boars, contributing to the persistence of the disease in livestock populations. *Mycobacterium caprae* has been reported to cause TB primarily in goats and deer in Europe, whereas *M. orygis*, initially described in oryxes, has been isolated from a range of wildlife, including antelopes and deer in South Asia (Refaya et al., 2022; Sharma et al., 2023). *Mycobacterium pinnipedii*, responsible for TB in marine mammals such as seals, and *M. microti*, which predominantly affects small rodents, further exemplify the broad host spectrum of MTBC (Malone and Gordon, 2017). *Mycobacterium tuberculosis*, primarily a human pathogen, has also been documented in cattle, wild ungulates, elephants, dogs, and non-human primates where cases of zooanthroponotic transmission have been definitively proved (Mätz-Rensing et al., 2015; Mukherjee et al., 2018; O'Halloran et al., 2021; Palaniyandi et al., 2019; Rajbhandari et al., 2023; Zachariah et al., 2017).

India faces a significant TB burden, with 26 % of the country's population affected and the highest number of human TB cases reported globally (World Health Organization (WHO), 2024). However, there is

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https://doi.org/10.1016/j.meegid.2025.105869
Received 12 August 2025; Received in revised form 28 November 2025; Accepted 18 December 2025

limited data on bovine TB (bTB) and wildlife TB (wTB) since the focus has primarily been on controlling TB in humans. While there are sporadic reports on the prevalence of wTB in India, the causative organism is seldom identified using gold standard methods (Ramanujam and Palaniyandi, 2024). This lack of data is concerning given the potential for zoonotic transmission of MTBC species from animals to humans, as well as reverse zoonotic transmission from humans to animals. The One Health approach, which emphasizes the interconnectedness of human, animal, and environmental health, is critical for addressing this gap. Surveillance of wTB is essential for understanding the full extent of the TB epidemic in India and developing strategies to mitigate its impact (Ramanujam and Palaniyandi, 2023).

Wildlife TB represents a complex and heterogeneous zoonotic challenge, with *Mycobacterium bovis* and other members of the *M. tuberculosis* complex circulating across diverse hosts and ecosystems (Conteddu et al., 2024; Reis et al., 2021; Silva-Pereira et al., 2024). *Mycobacterium bovis* infections have been well documented in European badgers (*Meles meles*) in the UK and Ireland, which serve as wildlife reservoirs and perpetuate bTB in cattle herds (Kelly et al., 2021). Meta-analyses indicate that TB prevalence can reach up to 21.9 % in wild boar, 13.7 % in red deer, and 11.8 % in European badgers reflecting notable geographic and host-specific variation (Reis et al., 2021). In Africa, the African buffalo (*Syncerus caffer*) is a known wildlife reservoir for *M. bovis* and has significant implications for wildlife conservation and public health (Lakin et al., 2022). Wild boar (*Sus scrofa*) in Spain and brush tail possums (*Trichosurus vulpecula*) in New Zealand are other well-known wTB reservoirs (Santos et al., 2015; Walter et al., 2012).

Several studies have reported the isolation of MTBC from animals belonging to the Cervidae family. Recent genomic and ecological studies in Europe have demonstrated that *M. bovis* transmission among deer and wild boar is maintained through complex multi-host interactions across ecological corridors, as shown in Portugal where the European 3 clonal complex circulates between livestock and wildlife with frequent spillover events (Pereira et al., 2024a, 2024b). In Spain, red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*) have been found to be infected (García-Bocanegra et al., 2012; Martín-Hernando et al., 2010; Santos et al., 2022). In Northern Ireland, a province-wide survey identified *M. bovis* infection in 2.5 % of culled deer, revealing limited but detectable interspecies transmission, with infected cattle remaining the primary source of infection and deer acting mainly as spillover hosts(Allen et al., 2025). Similarly, in North America, white-tailed deer (*Odocoileus virginianus*) have been identified as reservoirs for *M. bovis*, complicating efforts to control this disease in cattle (VerCauteren et al., 2018). In China, wTB in deer has been estimated at an overall prevalence of 16.1 %, with higher rates up to 26.5 % in certain provinces (Li et al., 2024). In India, *M. orygis* has been reported in various wildlife species, including spotted deer (*Axis axis*) and black buck (*Antelope cervicapra*) (Refaya et al., 2022; Sharma et al., 2022). Additionally, other MTBC species have been identified in gazelles *(Gazelle bennettii*), barking deer (*Muntiacus vaginalis*), and Nilgai (*Boselaphus tragocamelus*) (Ramanujam and Palaniyandi, 2024).

Across Asia, several countries in the WHO's Southeast Asia Region (SEAR) and Western Pacific Region (WPR), including Bangladesh, India, Indonesia, Myanmar, Nepal, Sri Lanka, Thailand, China, Japan, and Malaysia, have officially reported *M. bovis* infections in animals, intimating the widespread yet under documented presence of MTBC in Asian livestock and wildlife (Singh et al., 2024). Despite well-characterized systems in Europe and Africa, zoonotic tuberculosis remains largely underreported in Asia, where diagnostic and surveillance capacities are limited (de Macedo Couto et al., 2022; Singh et al., 2024). Within this regional context, India occupies a crucial position, where emerging evidence suggests that *M. orygis* may be more frequently associated with zoonotic infections than *M. bovis* (Hugh et al., 2025; Ramanujam and Palaniyandi, 2024; Rani et al., 2025) Despite the acknowledged importance of wildlife in the epidemiology of wTB, data on the prevalence and strain diversity of MTBC in Indian wildlife are scarce. Given the prevalence of MTBC species in Indian wildlife, we conducted an exploratory study in the Guindy National Park, Chennai, India. Our aim was to determine the presence of MTBC species in spotted deer, sambar deer, and black buck, and to evaluate the genetic diversity of these MTBC strains.

MTBC members are well recognized for their high level of nucleotide similarity with an average nucleotide identity >99 % as evidenced by comparative genomics studies (Jia et al., 2017; Riojas et al., 2018). Despite this, they exhibit unique traits in regard to phenotype, virulence, pathogenicity and host tropisms (Ramanujam and Palaniyandi, 2024). In the recent years, genomic tools such as Regions of difference (RDs), insertion sequences like IS6110 and IS1081 and SNP analysis profiling have proven crucial in understanding the evolutionary dynamics within MTBC.

Among these members, *M. orygis* has increasingly been isolated from wildlife, cattle and humans particularly in the south east Asian regions (Ramanujam and Palaniyandi, 2023). Interestingly, these isolates do not cluster into distinct clonal complexes, as the SNP differences between them typically span only a few hundred base pairs, indicating limited genetic divergence.

In this study, we conducted a comprehensive genomic comparison of newly isolated *M. orygis* strains alongside previously reported ones. Our analysis included RDs, insertion elements and SNPs to explore their genetic diversity and evolutionary relationships. Additionally, we performed a pangenome analysis to examine the complete gene repertoire of *M. orygis* and gain broader insights into its genomic architecture.

2. Materials and methods

2.1. Study site description

The Guindy National Park (GNP) in Chennai, India, is a 270 ha area of land, which is a National Park of India that extends from the grounds of Raj Bhavan, the residence of the Governor of Tamil Nadu. For ex situ conservation, 8.9 ha was enclosed as the Guindy Children's Park. The Park has a total of 35 enclosures housing 16 species of mammals, including a special area for blackbuck (an endangered species in India), spotted deer, and sambar deer (Wildlife Conservation and Management in Tamil Nadu, 2016). The national park is adjacent to the Indian Institute of Technology, Madras (IIT-M) campus, the Central Leather Research Institute (CLRI), and Anna University, where these animals can roam freely. These places have a high population of wild ungulates, where humans and animals coexist, making them hotspots for zoonotic TB transmission.

2.2. Sample collection

Spotted deer, sambar deer, and black bucks are protected in India under the Wildlife Protection Act of 1972 (The Wild Life (Protection) Act, Government of India (GOI), 1972)). This Act prohibits invasive sampling methods, such as nasal swabs or blood collection from these species, due to their protected status, thereby necessitating alternative sampling approaches. As this was an initial exploratory study, formal sample size estimation and statistical comparison were not applicable.

Carcasses of wild ungulates that had died naturally between 2020 and 2023 in GNP, Chennai, were transported to the Madras Veterinary College for post-mortem examination, immediately upon location of the animal carcass. Following necropsy, lung and mediastinal lymph node samples were collected (within 48 h of the estimated time of death) based on gross pathological observations (hard calcified pale-yellow TB like lesions) from eight animals, including two blackbucks, five spotted deer and one sambar deer. The liver from one barking deer (that lives in the animal enclosures of the Guindy Children's Park) showing TB like lesions was also sampled. Extensive calcification of mesenteric lymph nodes observed in several carcasses indicated possible systemic involvement and prompted the subsequent collection of fecal samples from live animals to investigate potential shedding of MTBC organisms. In total, 14 fecal samples were collected in 2023 from animal enclosures within GNP, comprising six from sambar deer, four from blackbuck, and four from spotted deer. All samples were placed in sterile screw-cap tubes, transported on ice to the ICMR–National Institute for Research in Tuberculosis (NIRT), and stored at −80 °C until further processing.

2.3. *Tissue sample processing*

Samples were processed within 5 days after receipt at ICMR-NIRT using the saline dilution method (Ramanujam et al., 2024). Briefly, frozen tissues were thawed on ice, trimmed to remove surrounding fat, and approximately 5 g of each sample was fixed in 10 % formal saline for histopathological analysis. Formalin-fixed tissues were embedded in paraffin, sectioned into 5-μm slices, and stained with hematoxylin and eosin. The resulting sections were examined microscopically, and granulomatous lesions were categorized into stages I–IV based on structural characteristics (Palmer et al., 2022).

The remaining portion of each tissue sample was subjected to surface decontamination using a 1:200 dilution of 8.25 % sodium hypochlorite for 15–30 min, followed by thorough rinsing with sterile saline. The tissues were then homogenized using a FastPrep-24 5G instrument (MP Biomedicals, Singapore) at 5 m/s for four 40-s cycles per 5 g of tissue. Direct smears were prepared from the homogenates and stained by the Ziehl–Neelsen method to detect acid-fast bacilli. The remaining homogenate was subsequently decontaminated with 4 % NaOH, neutralized with phosphate-buffered saline (pH 7.4), and used for mycobacterial culture.

Decontaminated samples were inoculated into BACTEC 960 Mycobacteria Growth Indicator Tubes (MGIT) supplemented with BBL MGIT PANTA™ antibiotic mixture (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) tubes, and onto Lowenstein-Jensen (LJ), LJ with sodium pyruvate (LJ-SP), and Selective Kirchner's (SK) media. Smears were Ziehl-Neelsen stained. The MGIT cultures were incubated for seven weeks, LJ and LJ-SP for eight weeks, and SK for six weeks, followed by decontamination and reinoculation onto LJ and LJ-SP for an additional eight weeks. When colonies appeared on solid media or when growth was detected in MGIT tubes, a commercially available immunochromatographic assay (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) was used to confirm if these colonies were MTBC organisms (Abe et al., 1999).

2.4. *Drug susceptibility testing (DST)*

Drug susceptibility testing was performed using the BD BACTEC MGIT 960 SIRE Kit (Becton Dickinson Diagnostic Systems, USA) for first-line anti-TB drugs. Streptomycin (1.0 μg/mL), isoniazid (0.1 μg/mL), rifampin (1.0 μg/mL), and ethambutol (5.0 μg/mL) were prepared in sterile distilled water and added to MGIT tubes. Cultures were diluted (1,5) with saline, and 500 μL of the diluted suspension was inoculated. Growth was assessed by comparing drug-containing tubes with growth controls per the manufacturer's guidelines.

2.5. *DNA extraction from Fecal and tissue isolates*

Fecal DNA was extracted from 15 g of sample using the QIAamp® DNA Stool Kit (Qiagen, Germany) following standard protocols. DNA from cultured mycobacterial colonies was extracted via the cetyltrimethylammonium bromide-sodium chloride (CTAB-NaCl) method. Cultures were lysed with lysozyme (10 mg/mL), SDS (10 %), and Proteinase K (10 mg/mL), followed by CTAB/NaCl treatment. DNA was precipitated with isopropanol at −20 °C, washed with 70 % ethanol, air-dried, and dissolved in TE buffer.

2.6. *Molecular characterization*

DNA was extracted from cultured isolates obtained during post-mortem examination as well as directly from fecal pellets, and both were independently subjected to PCR targeting the *MPT64* gene (240 bp) to confirm the presence of MTBC. Spoligotyping analysis was conducted on culture-positive isolates derived from tissue samples to determine lineage, using biotinylated primers Dra and Drb. The PCR products were hybridized to Biodyne C membranes with immobilized oligomeric spacers from *M. tuberculosis* H37Rv and *M. bovis* BCG, detected using an enhanced chemiluminescence kit.

2.7. *Line probe assay (LPA) and GeneXpert MTB/RIF ultra*

DNA isolated from fecal samples was used for MTBC detection and drug susceptibility testing, as culturing fecal material is often hindered by heavy microbial contamination. The GenoType MTBDRplus 2.0 (Hain Lifescience GmbH, Nehren, Germany) and GeneXpert MTB/RIF Ultra (Cepheid, Sunnyvale, CA, USA) assays were performed according to the manufacturers' instructions. The MTBDRplus assay identifies MTBC and mutations in *rpoB*, *katG*, and *inhA* associated with rifampicin and isoniazid resistance, while the GeneXpert MTB/RIF Ultra targets *rpoB*, IS1081, and IS6110 regions to detect MTBC DNA and rifampicin resistance using an automated real-time PCR platform.

2.8. *Targeted gene sequencing for non-tuberculous mycobacteria (NTM) identification*

Among the five cultured isolates obtained from tissue samples, one isolate (KL668) exhibited acid-fast bacilli upon Ziehl–Neelsen staining of culture smears, but tested negative in the immunochromatographic assay for MTBC, indicating NTM (Ramanujam et al., 2025). The DNA from the cultured isolate KL668 was amplified for *hsp65* sequencing using primers ACCAACGATGGTGTGTCCAT and CTTGTCGAACCGCATACCCT (Telenti et al., 1993). PCR products were visualized on a 1.5 % agarose gel, purified using the Qiagen PCR Purification Kit, and sequenced using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Species identification was performed via BLASTn analysis against NCBI's NTM database.

2.9. *Whole genome sequencing (WGS) and phylogenetic analysis*

Paired-end libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) on the HiSeq 2500 platform in high-output mode generating 2 × 150 bp reads. Taxonomic classification and contamination screening were performed using Kraken2 (ver. 2.1.1). The raw reads were processed using the vSNP pipeline (https://github.com/USDA-VS/vSNP3) which integrates read alignment, variant detection, filtering and phylogenetic reconstruction. The reads were independently aligned to *M. tuberculosis* H37Rv (NC_000962) and *M. orygis* 51,145 (CP063804) using BWA-MEM (ver. 0.7.17.1). Variant calling were performed using Freebayes (ver. 1.3.1) Garrison and Marth, 2012 and low quality SNPs were filtered with VCF filter (QUAL $\geq$20, minimum coverage $\geq$10× and minimum mapping quality $\geq$30). The filtered VCF files were compiled to construct SNP matrices, from which RAxML (ver. 8.2.4) generated phylogenetic tree using the GTRCATI substitution model by default. Sequencing and alignment quality metrics including read length, coverage depth, genomic coverage depth and unmapped reads are automatically reported (Hicks et al., 2024). The SNPs generated were further inspected and validated using the Integrated Genomics Viewer (IGV) (Robinson et al., 2011) and phylogenetic trees were visualized and annotated using iTOL (ver. 6.5.1) (Letunic and Bork, 2019). To quantify genetic distances and infer potential transmission clusters, a pair-wise SNP distance matrix was computed from the multi-FASTA SNP alignment generated by vSNP using the snp-dist tool (https://github.com/tseemann/snp-dists).

2.10. *Insertion sequence (IS) mapping*

The ISMapper pipeline (version 2.0) (Hawkey et al., 2015) (https://github.com/jhawkey/IS_mapper) was used to map the IS6110 and IS1081 insertion sites within short-read sequences using *M. tuberculosis* H37Rv (NC000962.3) and *M. orygis 51,145* (CP063804) as reference genomes. The presence of insertions were manually visualized using Integrative Genomics Viewer (IGV), leveraging BAM files generated by ISMapper 2.0 (Robinson et al., 2011). To annotate the functional implications, protein sequences from upstream and downstream genes near IS6110 and IS1081 insertion sites, along with those from genes containing the insertions, were analyzed. Functional annotation was performed using eggNOG-mapper (version emapper—2.1.9), which is based on eggNOG orthology data (Cantalapiedra et al., 2021).

2.11. *Regions of difference analysis*

The classification of MTBC species is primarily based on SNPs and large polymorphic deletions within RDs, which are genomic loci representing evolutionary events such as insertions or deletions unique to specific lineages. These RDs play a pivotal role in determining the host range, virulence, and other lineage-specific traits, providing valuable insights into strain differentiation (Bespiatykh et al., 2021). In this study, RDscan (https://github.com/dbespiatykh/RDscan) was employed to identify deletions in animal lineage-associated RDs in all the globally available *M. orygis* genomes.

2.12. *Pangenome analysis*

De novo assembly of the sequence reads was performed using SPAdes (Prjibelski et al., 2020) and assembly quality was assessed with QUAST (Gurevich et al., 2013). Of the 212 genome sequences initially considered, four were excluded due to poor assembly quality, and the final analysis was carried out on 208 high-quality genomes. The resulting contigs were annotated using Prokka (Seemann, 2014). The generated GFF3 files were used for pangenome analysis with Panaroo v1.33 (Tonkin-Hill et al., 2020) in *strict* mode to determine the core and accessory genome composition. The pangenome_reference.fa file generated by panaroo was used as a query to perform a blast search against the *M.orygis* CP063804.1 reference genome. The resulting blast output and the gene_presence_absence_roary.csv from panaroo was used as inputs for the ref_blast_matches.R available at https://github.com/kmceres/Mbovis_pangenome/tree/main/Rscripts (Ceres et al., 2022) to identify the functional status of each gene. The gene_presence_absence.Rtab file was manually curated based on the output files generated by the script and this cleaned Rtab file was used for downstream analysis. To evaluate the openness of the pangenome, Heap's law analysis was conducted using a python script from the PanGenomeAnalysis tool (https://github.com/vsmicrogenomics/ PanGenomeAnalysisTool) with the curated gene_presence_absence.Rtab file. A phylogenetic tree was constructed from the curated file based on Jaccard distance using the neighbor-joining algorithm implemented in the Vegan and Ape packages in R. The resulting tree was visualized and annotated in iTol.

3. Results

3.1. *Culture and drug susceptibility testing*

Post mortem lung and lymph node samples were collected from eight animals in total. The animals appeared emaciated upon arrival and visible lesions were seen on the lungs and lymph nodes (Fig. 1). Four out of eight samples were positive by phenotypic assays, while one sample was annotated as NTM. The DST revealed that all the positive samples were sensitive to the first line anti TB drugs. The results of the culture and phenotypic assays are given in Table 1.

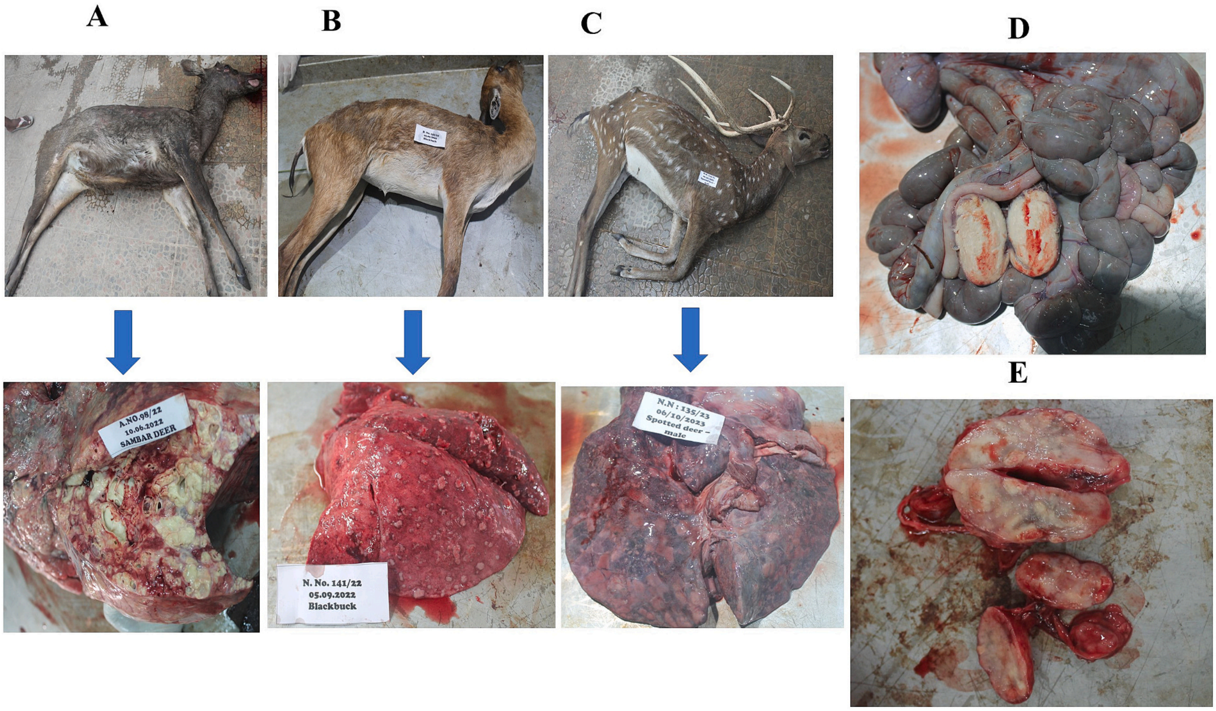

Fig. 1. Macroscopic calcified lesions observed post-mortem in wild ungulates from Guindy National Park, Chennai, India. The upper panels show the animal carcasses before necropsy, and the lower panels display the corresponding organs exhibiting tuberculous lesions; arrows indicate the connection between the carcass and the sampled organ. (A) Sambar deer, (B) black buck, (C) spotted deer, (D) mesenteric lymph node of black buck, representative of similar lesions observed in mesenteric and mediastinal lymph nodes of several carcasses, and (E) bronchial lymph node of black buck.

Table 1
Phenotype results for the post mortem samples.

S.no	Sample type	Sample number	Age/ Sex	ZN staining	Culture Results			Drug sensitivity				Final Result
					LJ	LJ-SP	MGIT	SR	H	RIF	E	
Black buck												
1.	Lung	KL503	5Y/F	Neg	Neg	Neg	Neg	NA	NA	NA	NA	Neg
2.	Lung	KL292	4Y/F	Pos	Pos	Pos	Pos	S	S	S	S	Pos
Spotted deer												
3.	Lung	KL521	3Mo/M	Neg	Neg	Neg	Neg	NA	NA	NA	NA	Neg
4.	Lung	KL522	3Mo/M	Neg	Neg	Neg	Neg	NA	NA	NA	NA	Neg
5.	Lung	KL668	4Y/M	Pos	NTM	Neg	Neg	NA	NA	NA	NA	NTM
6.	Mediastinal LN	KL732	8Y/F	Pos	Pos	Pos	Pos	S	S	S	S	Pos
7.	Lung	KL759	4Y/M	Pos	Pos	Pos	Pos	S	S	S	S	Pos
Sambar deer												
8.	Mediastinal LN	KL426	15Y/F	Pos	Pos	Pos	Pos	S	S	S	S	Pos
Barking deer												
9.	Liver	KL611	2Y/M	Neg	Neg	Neg	Neg	NA	NA	NA	NA	Neg

Note: LN- Lymph node, Y- Years, Mo- Months, M- Male, F- Female, ZN staining- Ziehl–Neelsen staining, MGIT- Mycobacteria Growth Indicator Tubes, SR - Streptomycin, H - Isoniazid, RIF- Rifampicin, E – Ethambutol, S - Sensitive and R – Resistant, Pos- Positive, Neg- Negative, NA- Not Applicable; NTM- Non-tuberculous mycobacteria.

3.2. *Histopathology*

Histopathological examination revealed well-developed granulomatous lesions consistent with advanced stages of tuberculosis. A Stage III granuloma was observed in the lung of the blackbuck (KL292), characterized by a central necrotic core surrounded by macrophages, lymphocytes, and multinucleated giant cells, with evidence of partial mineralization and encapsulation by a dense fibrous capsule. In contrast, Stage IV granulomas were observed in the lungs of the sambar deer (KL426) and two spotted deer (KL732 and KL759). These lesions displayed coalescing necrotic centers with extensive mineralization and prominent fibrous encapsulation, indicative of chronic infection and prolonged host–pathogen interaction (Fig. 2). Such variation in granuloma stage and organization between species may reflect differences in host immune responses to *M. orygis* infection (Palmer et al., 2022).

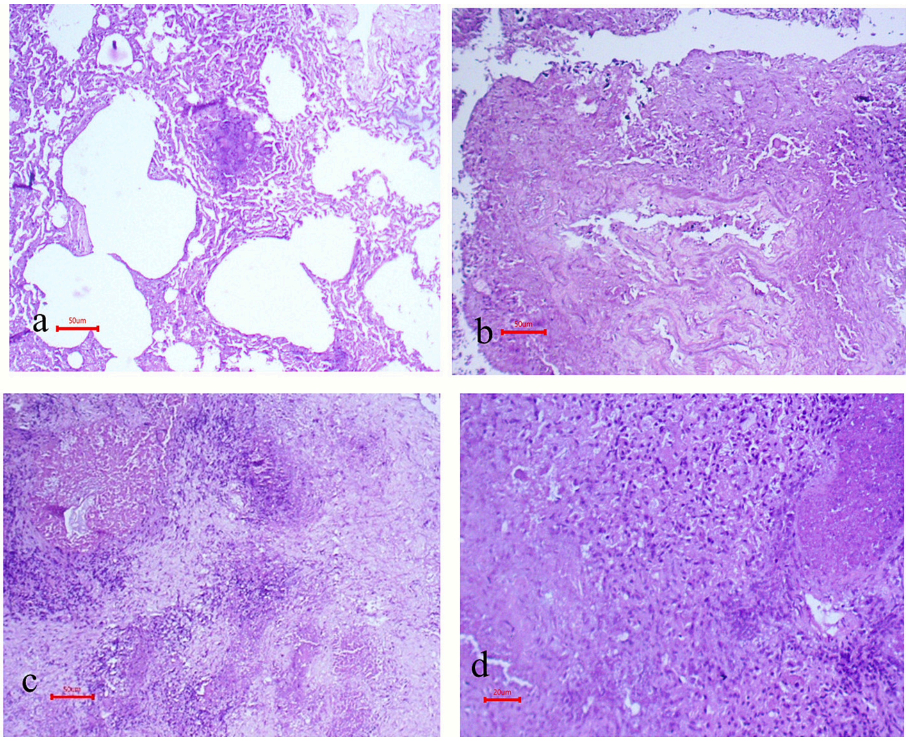

Fig. 2. Histopathological features of granulomatous lesions in wild ungulates infected with *Mycobacterium orygis* (hematoxylin and eosin staining; scale bar = 50 μm).
(a, b) Lung of blackbuck (KL292) showing a Stage III granuloma with a central necrotic core surrounded by epithelioid macrophages, lymphocytes, and multinucleated giant cells, partially mineralized and enclosed by a fibrous capsule.
(c, d) Lung of spotted deer (KL759) showing a Stage IV granuloma with coalescing necrotic centers, extensive mineralization, and dense fibrous encapsulation.

3.3. Molecular assays

For the isolates obtained from tissue cultures, DNA was extracted using the CTAB–NaCl method. PCR amplification targeting the *MPT64* gene produced a 243 bp amplicon in four isolates (KL292, KL732, KL759, and KL426), confirming their identity as members of the MTBC. Spoligotyping of these PCR-positive isolates revealed the characteristic pattern of spoligotype 587 (ST587), corresponding to *M. orygis* (Brudey et al., 2006; van Ingen et al., 2012).

DNA isolated from fecal samples using the QIAamp® DNA Stool Kit (Qiagen, Germany) was also subjected to *MPT64* PCR. A total of 14 fecal samples were examined—six from sambar deer, four from blackbuck, and four from spotted deer. Four animals (three blackbuck and one spotted deer) were positive for MTBC by PCR. To further confirm detection and assess drug susceptibility, the GeneXpert MTB/RIF Ultra and GenoType® MTBDRplus version 2.0 (Hain Lifescience GmbH, Nehren, Germany) assays were performed on these fecal samples. Nine samples (four sambar deer, four blackbuck, and one spotted deer) tested positive for MTBC with GeneXpert, and an equal number were positive by line probe assay—five sambar deer, three blackbuck, and one spotted deer. Drug-resistance profiling revealed that two blackbuck samples were resistant to isoniazid, and one blackbuck sample was resistant to both isoniazid and rifampicin. A summary of these results is presented in Table 2.

3.4. Identification of NTM species

Targeted gene sequencing identified isolate KL668, that was classified as NTM as *Mycobacterium* sp. strain 79_MI18_10584 (GenBank accession number OR672024).The analysis yielded a query coverage of 99 % and an E value of 0.0, with a high sequence identity of 98.40 %. The species was reported by Gomez-Buendia et al. in March 2024 and was isolated from cattle that tested positive for intradermal skin test from an officially TB-free herd (Gomez-Buendia et al., 2024). The same NTM species was also identified in tissue samples of slaughtered cattle in Chennai, India (Ramanujam et al., 2025).

3.5. Whole genome sequencing analysis

On average, approximately 4.3 million paired-end reads were generated per sample, with a range spanning from 1.9 to 6.5 million read pairs. The mean read length was 166 bp, yielding an average coverage depth of 125.72× (range: 56–190×; median: 128×) following alignment to the *M. tuberculosis* H37Rv reference genome. Taxonomic profiling of the isolates, conducted using Kraken, indicated minimal contamination with non-MTBC sequences, with contamination levels consistently below 2 %.

Processed reads were analyzed using the vSNP pipeline, and independent alignments were performed against *M. tuberculosis* H37Rv, the standard reference genome for MTBC analyses, and *M. orygis* 51,145, which serves as the representative reference for *M. orygis* studies. The resultant genomic coverage ranged between 92 and 98 % for the H37Rv-aligned data and 94–99 % for the *M. orygis* 51,145-aligned data (Appendix B, Table 2.1; Appendix C, Table 3.1). All four sequenced isolates were classified as *M. orygis*.

Two independent phylogenetic trees were constructed: one using *M. tuberculosis* H37Rv and another using *M. orygis* 51,145 as the reference genome. The four *M. orygis* isolates recovered from wild ungulate tissue samples were compared with all the 208 publicly available *M. orygis* genomes in NCBI SRA, including 99 human, 96 animal, and 13 unknown-origin isolates from 10 countries (Fig. 3; Appendix B, Table 2.4; Appendix A, Fig. S1).

Comparative SNP analyses and IS analyses were also performed independently using both reference genomes. Results obtained using the *M. tuberculosis* H37Rv as reference are presented in the main text, whereas the corresponding analyses based on the *M. orygis* 51,145 as reference are provided in Appendix A.

3.6. Comparative SNP analysis

The four *M. orygis* isolates recovered in this study showed pairwise SNP differences ranging from 54 to 144, which is well above the 3–15 SNP cutoff used to infer recent transmission (Walker et al., 2013), thereby excluding the possibility of direct transmission among the sampled deer. To explore their genetic relatedness within the regional context, these isolates were compared with 27 previously reported *M. orygis* isolates from cattle and wild ungulates in the Chennai region (total = 31 isolates). This analysis identified 12 distinct transmission clusters, each with pairwise SNP distances within the accepted 3–15 SNP threshold, indicating close genetic relationships and suggesting localized interspecies circulation of *M. orygis* between cattle and wild ungulates in the area (Appendix B, Table 2.2).

Subsequently, a broader SNP analysis was performed using the four study isolates together with 208 publicly available *M. orygis* genomes (total = 212 genomes) to assess global diversity. The reads were aligned to the *M. tuberculosis* H37Rv reference genome, and a total of 9797 SNPs were identified, including 5157 nonsynonymous and 2900 silent mutations (Appendix B, Table 2.3). The highest SNP variation was observed at locus NC_000962.3:729114 (GGC → GGT) within *echA3* (enoyl-CoA hydratase), where 92.9 % ($n = 197$) of analyzed sequences differed from the reference, encompassing 100 % ($n = 99$) of human-origin and 98 % ($n = 98$) of animal-origin isolates. Notably, 217 SNPs were unique to the

Table 2

Results of Xpert MTB- RIF Ultra GenoType® MTBDRplus version 2.0 (Hain Lifescience GmbH, Nehren, Germany) line probe assay for the fecal samples collected from the enclosures of live animals.

S.no	Animal	Lab number	MPT64 PCR	Xpert MTB- RIF Ultra		LPA		
				MTBC presence	Rif resistance	MTBC presence	H resistance	Rif resistance
1	Sambar deer	KW1	N	N	NA	P	S	S
2	Sambar deer	KW2	N	Detected Very low	N	N	NA	NA
3	Sambar deer	KW3	N	Detected Very low	N	P	S	S
4	Sambar deer	KW4	N	Detected Very low	N	P	S	S
5	Sambar deer	KW5	N	N		P	S	S
6	Sambar deer	KW6	N	Trace detected	Very low	P	S	S
7	Black Buck	KW7	N	Trace detected	Indeterminate	P	R	S
8	Black Buck	KW8	P	Detected Very low	Indeterminate	P	R	R
9	Black Buck	KW9	P	Trace detected	N	P	R	N
10	Black Buck	KW10	P	Trace detected	Indeterminate	N	NA	NA
11	Spotted deer	KW11	P	Trace detected	Indeterminate	P	S	S
12	Spotted deer	KW12	N	N	NA	N	NA	NA
13	Spotted deer	KW13	N	N	NA	N	NA	NA
14	Spotted deer	KW14	N	N	NA	N	NA	NA

Note: MTBC- *Mycobacterium tuberculosis* complex; Rif- Rifampicin; H- Isoniazid; LPA- Line probe assay; P- Positive; N- Negative; NA- Not applicable.

Table 3
Details of IS insertions among all *M. orygis* isolates as compared with *M. tuberculosis* H37Rv.

R. No	Orientation	X	Y	Region of insertion (Left/ Right)	Number of Sequences
IS6110 insertions					
1	R	369,119	369,124	Rv0304c*^{Ch}	18
2	R	419,061	419,068	Rv0349*	29
3	R	481,253	481,267	Rv0402c*	27
4	R	832,930	832,933	Rv0740 / Rv0742*	29
5	R	890,375	890,379	Rv0794c / Rv0797*	26
6	R	937,094	937,101	Rv0840c*^{Ch}	28
7	R	1,299,995	1,300,000	Rv1169c*	25
8	F	1,415,819	1,415,825	Rv1266c*	27
9	R	1,527,923	1,527,926	Rv1358*	29
10	R	1,540,588	1,540,593	Rv1367c*	28
11	F	1,889,678	1,889,683	Rv1664*	27
12	R	1,906,339	1,906,576	Rv1681 / Rv1682*	28
13	F	1,981,207	1,981,791	Rv1752 / Rv1753c*	26
14	R	1,987,762	1,988,988	Rv1754c / Rv1758	27
15	F	1,996,169	1,997,395	Rv1762c / Rv1765c	29
16	F	2,365,482	2,366,708	Rv2104a / Rv2107	23
17	R	2,430,176	2,431,402	Rv2166c / Rv2169c	25
18	F	2,550,082	2,551,308	Rv2277c / Rv2281	29
19	F	2,635,645	2,636,871	Rv2353c / Rv2357c	29
20	R	2,784,674	2,785,900	Rv2478c / Rv2481c	26
21	R	2,884,945	2,884,949	Rv2565*	29
22	F	2,972,177	2,973,403	Rv2647 / Rv2650c	30
23	R	3,120,583	3,121,809	Rv2813 / Rv2816c	26
24	F	3,123,541	3,123,547	Rv2813 / Rv2816c*	29
25	F	3,317,962	3,317,967	Rv2964 / Rv2965c*	27
26	R	3,366,531	3,366,776	Rv3007c / Rv3008*	25
27	F	3,551,298	3,552,525	Rv3183 / Rv3188	25
28	R	3,552,586	3,552,708	Rv3183 / Rv3188*	23
29	F	3,710,449	3,711,676	Rv3324c / Rv3327	26
30	R	3,711,736	3,711,740	Rv3324c / Rv3327*	23
31	R	3,795,117	3,796,343	Rv3379c / Rv3382c	27
32	F	3,890,847	3,892,073	Rv3473c / Rv3477	29
33	R	4,197,849	4,197,854	Rv3749c*	27
IS1081 insertions					
34	F	1,169,326	1,170,656	Rv1045 / Rv1047	29
35	R	1,341,350	1,342,638	Rv1198 / Rv1200	28
36	R	2,828,571	2,829,785	Rv2512c	24
37	F	2,983,087	2,983,837	Rv2665 / Rv2667*	28
38	R	3,381,333	3,382,660	Rv3022A / Rv3024c	26

Note: * refers to novel insertions. *Ch referes to novel insertions identified only in strains isolated from Chennai region.

31 Chennai isolates, of which 116 occurred in annotated genes (Fig. 4) and 101 in unannotated genomic regions (Appendix B, Table 2.4), indicating region-specific evolutionary signatures within the local *M. orygis* population.

3.7. *Insertion sequence analysis*

The presence and distribution of two crucial insertion sequences, IS6110 and IS1081, across the genome of the 4 study isolates along with 27 other *M. orygis* strains isolated from the animals in Chennai region were analyzed, with reference genomes of *M. tuberculosis* H37Rv and *M. orygis* 51,145. The IS regions present at the loci that are present in the reference genome are annotated as 'known insertions,' while those that are not present in the reference, but only in the query sequence are termed as 'novel insertions.' Among the 31 sequences analyzed, the median number of IS6110 insertions was 36, ranging from 2 to 54 copies. Of these sequences, 96.8 % ($n = 30$) were classified as high-copy (HC) strains, while 3.2 % (n = 1) were classified as low-copy (LC) strains (Fomukong et al., 1998). The analysis of IS1081 insertion regions revealed a median of 5 insertions, ranging from 0 to 6 (Appendix B, Table 2.5). Unlike IS6110, classifications into HC or LC strains are not defined for IS1081, and hence not reported here.

A total of 38 common IS6110 insertion loci were identified, with 18 classified as novel and 13 matching loci in *M. tuberculosis* H37Rv. Two novel insertion loci unique to strains isolated from Chennai were also identified. For IS1081, five common insertion loci were identified, with one novel locus and the remaining four corresponding to loci already present in *M. tuberculosis* H37Rv.The specific regions of these insertion sites are detailed in Table 3 and visually illustrated in phylogenetic trees constructed using the maximum likelihood method in Fig. 5.

To ascertain the functional implications of IS events, all 83 genes containing IS6110 or IS1081 insertions were subjected to annotation using the eggNOG 5.0 tool, which successfully mapped 76 genes to functional categories. Of these, 19 genes were associated with 394 Gene Ontology (GO) terms—comprising 265 Biological Processes (BP), 83 Molecular Functions (MF), and 33 Cellular Components (CC) (Appendix B, Table 2.6). The distribution of GO terms across annotated genes is provided in Appendix B, Table 2.7; no annotations were available for 12 genes. KEGG pathway mapping revealed that 19 genes were linked to 36 distinct pathways, primarily related to metabolic functions, including lipid and carbohydrate metabolism, energy production, and intermediary biochemical processes (Appendix B, Table 2.8).These functional categories are consistent with prior reports indicating that IS6110 and IS1081 insertions frequently occur near genes involved in lipid metabolism, transcriptional regulation, and oxidative stress response, influencing virulence and survival within host macrophages (Gonzalo-Asensio et al., 2018; Roychowdhury et al., 2015).

Insertion Sequence analysis was also performed on all the 208 globally available *M. orygis* strains. The results are presented in Appendix A: Section 2 and Appendix B: Tables 2.9–2.13, Appendix C: Tables 3.9–3.13).

3.8. *Regions of difference analysis*

The four *M. orygis* isolates obtained in this study exhibited the characteristic genomic signature of the species, defined by deletions in RD7, RD8, RD9, RD10, RDOryx_1, RDOryx_4, RD12Oryx, RD301, and RD315, along with the presence of RD1 and RD4, which collectively serve as unique lineage markers of *M. orygis* (Bespiatykh et al., 2021). A heat map was constructed using all available *M. orygis* isolates, along with the study isolates (Fig. 6). *M. orygis* recovered from *Cynomolgus Macaques* shipped from Southeast Asia and the *M. orygis* sequences isolated from humans in Canada (also of Southeast Asian origin), showed a partial presence of RDOryx_1 (Islam et al., 2023; Swisher et al., 2024). RD^{can} deletion was also observed in all samples because this larger region overlapped with the RD12_Oryx region. RD^{sur} deletion is

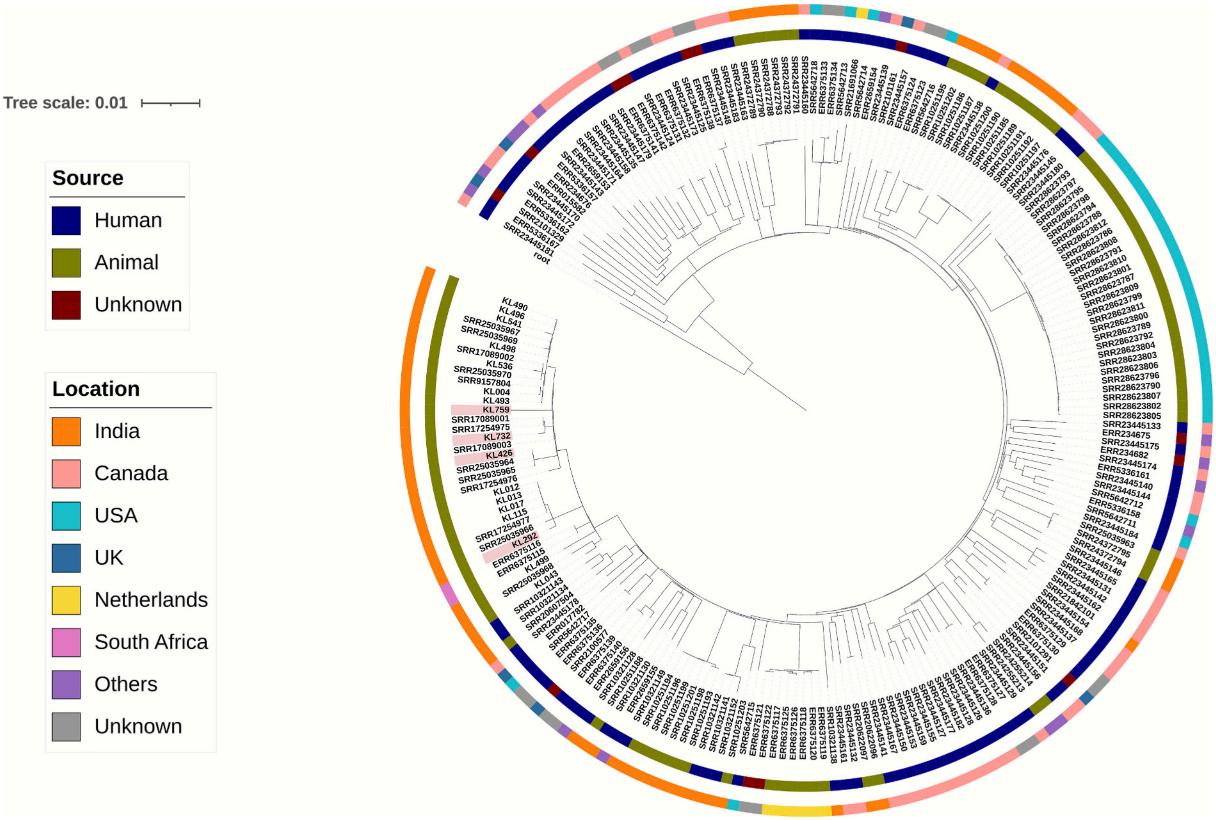

Fig. 3. Interpretative phylogenetic tree combining four study isolates (labelled pink) along with 208 *M. orygis* sequences downloaded from NCBI SRA. The inner band and outer band represent the isolates' source and geographic location respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

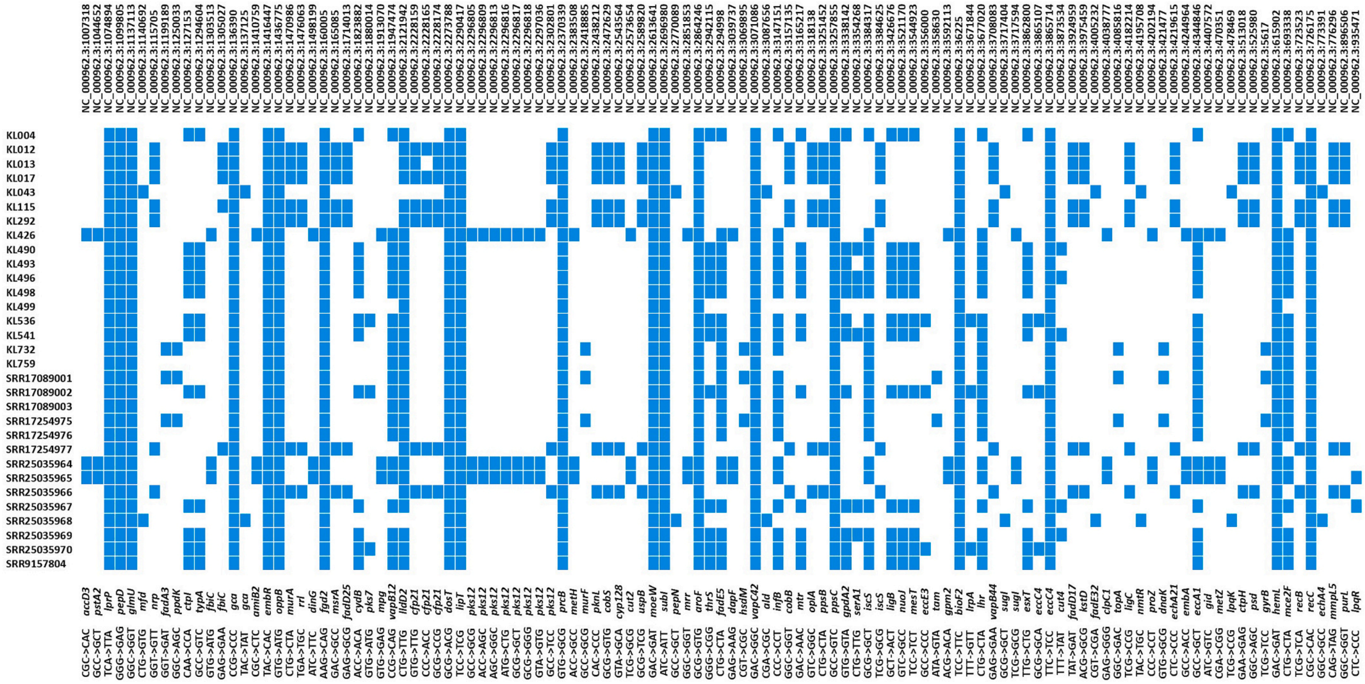

Fig. 4. Present (represented by blue) Absent Matrix for the common SNPs observed across the 31 *M. orygis* strains isolated from Chennai. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

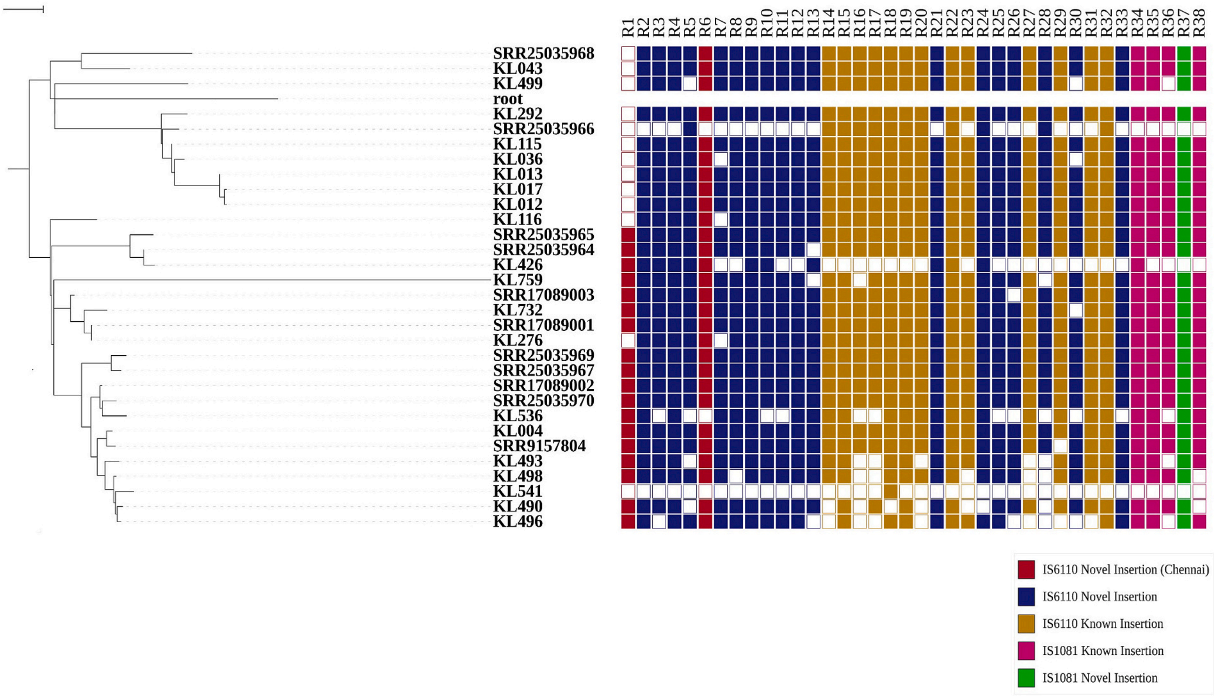

Fig. 5. The presence or absence of insertion sequences is depicted in the contextual phylogenetic tree combining the 31 *M. orygis* strains isolated from Chennai constructed using whole genome SNPs obtained after alignment with *M. tuberculosis* H37Rv as reference genome. The regions R1 to R38 are mentioned in Table 3.

also commonly observed because this region overlaps with the RDOryx_1 region.

3.9. Pangenome analysis

Genome assembly using SPAdes failed for 4 out of 212 isolates due to poor read quality. The remaining 208 assemblies were assessed for quality using QUAST, and high-quality contigs were subsequently annotated using Prokka. The resulting 208 GFF3 files were used for pangenome analysis with Panaroo, which initially identified a total of 4765 genes including 3435 core genes present in >99 % of the genomes, 408 soft core genes present in 95–99 % of the genomes, 230 shell genes present in 15–95 % of the genomes, and 692 cloud genes representing the fraction of genes present in <15 % of genomes. The genes identified as pseudogenes and the cloud genes that did not match with the reference genome were manually curated from the gene_presence_absence. Rtab file resulting in a total of 4, 222 genes with 3309 as core, 516 as soft core, 254 as shell and 143 cloud genes. The Heap's law estimate of the pangenome fitness yielded a γ value of 0.0086, indicating an open pangenome structure (Fig. 7), which contrasts with previous reports suggesting a closed pangenome for *M. orygis* (Karthik et al., 2023). However, the low γ value suggests that novel genes continue to be discovered at a slow rate as additional genomes are incorporated. A phylogenetic tree was generated using Jaccard distance from the curated data set showing the distribution of pangenome components – core, soft core, shell and cloud genes across 208 *M. orygis* genomes (Fig. S6).

4. Discussion

Tuberculosis poses significant health risks not only to humans, but also to wildlife and domestic animals. The presence of wTB complicates efforts to eradicate the disease, and animals can serve as reservoirs for infection, leading to its transmission to livestock and humans. The zoonotic potential of MTBC organisms underscores the significant threat posed by wTB, necessitating robust surveillance and control strategies. This study confirmed the presence of *M. orygis* in free-ranging wild ungulates, such as sambar deer, spotted deer, and black buck in GNP, Chennai.

Controlling wTB is essential for several reasons. First, wild animals can harbor MTBC, which can be transmitted to humans and domestic livestock, creating a complex web of infections that complicates TB eradication. The zoonotic potential of wTB is of particular concern in regions where wildlife and livestock coexist. Infected wildlife can serve as a reservoir for bTB, thereby perpetuating the disease cycle. Moreover, the ecological consequences of wTB should not be overlooked. Wildlife populations infected with wTB may experience population decline due to disease-related mortality. This is particularly concerning for endangered species, which may already be threatened by habitat loss and other factors. Managing wTB is essential not only for animal health but also for maintaining biodiversity and ecosystem stability.

The deer population in GNP is reported to be declining owing to factors such as roadkill, jackal attacks, plastic ingestion diseases, and natural causes (Gautham, 2022). *Mycobacterium orygis* has been previously reported in wild ungulates in India (Sharma et al., 2023), specifically in Chennai (Karthik et al., 2023; Refaya et al., 2022; Refaya et al., 2020). This study identified *M. orygis* infection in black buck, spotted deer, and sambar deer, as well as drug-resistant MTBC in fecal samples from black bucks in Chennai. India's declining forest cover and the increase of urban encroachment into forest areas along with the 'protected' nature of deer in India necessitates initiation of proper surveillance for wTB.

Mycobacteria are often shed into the environment via secretions (oronasal, bronchial-alveolar secretions, fecal, and/or urinary excretions). In 1998, Lugton et al. isolated *M. bovis* from red deer in 4/53 nasopharyngeal tonsils, 4/53 oropharyngeal swabs, 1/53 tracheal and nasal swabs, and 1/46 fecal samples, but not from any urine specimens collected from the Castlepoint and Hauhungaroa Range in New Zealand (Lugton et al., 1998). In 2015, Santos et al. confirmed the presence of MTBC in 48/88 hunter-harvested red deer carcasses segregated into 9/22 oronasal, 29/36 bronchial-alveolar, and 9/30 fecal samples. In fact, red deer were categorized as super shedders, and it was hypothesized that the removal of these super shedders could significantly reduce horizontal disease transmission (Santos et al., 2015).

Shedding pathogens from infected individuals significantly increases

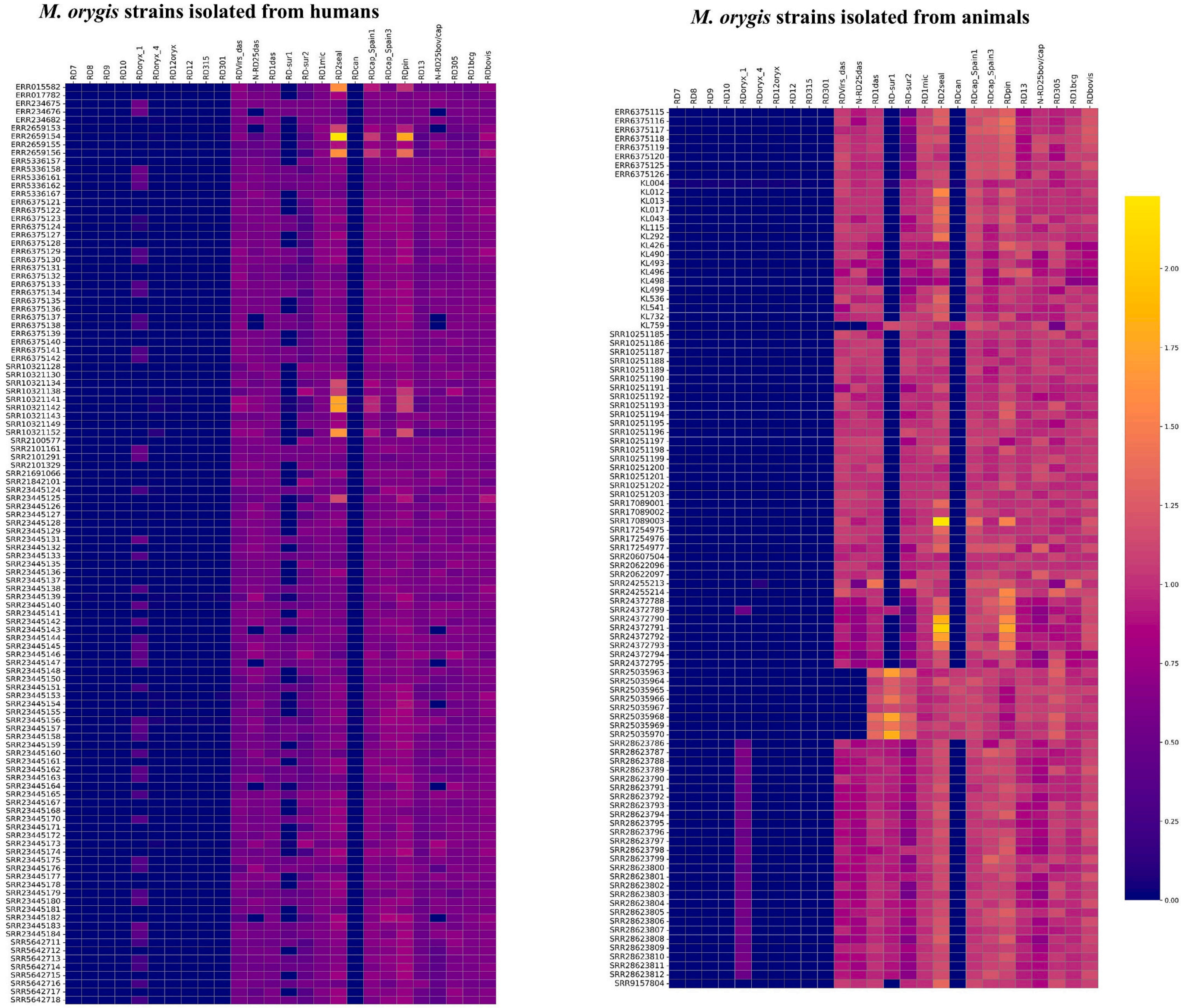

Fig. 6. Heatmap showing the extent of RD deletions among 212 *M. orygis* isolates from animal (left panel) and human (right panel) sources. Each column represents one of 26 RDs associated with animal-adapted MTBC lineages. The analysis, performed using RD-Analyzer with *M. tuberculosis* H37Rv as the reference, displays a colour gradient from dark purple (lowest) to bright yellow (highest), reflecting the normalized proportion of each RD retained across isolates. Isolates are ordered alphabetically within each panel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the risk of disease transmission via environmental pathways. The experimental validation of environmental transmission was demonstrated by Palmer et al. (2004) through a study involving white-tailed deer and crossbred calves. The deer contracted *M. bovis* after being placed in enclosures previously occupied by calves that had been orally infected with the pathogen. Conversely, uninfected calves became infected after consuming food remnants from pens that had housed infected deer (Palmer et al., 2004). Given that Mycobacteria were initially soil dwellers *M. tuberculosis, M.bovis* and *M.canettii* can survive in the soil for about 12 months and remain virulent and are capable of causing infection in mice, there is an inexcusable need to address the prevalence of wTB in Chennai.

Recent advances from Portugal, where wTB persists in multi-host wildlife–livestock systems, provide important ecological and microbiological evidence supporting this environmental pathway. In an endemic TB hotspot in Portugal, Pereira et al. (2023) detected metabolically active or dormant MTBC cells in 54 % of 65 natural substrate samples, with sludge containing up to 2.3×10^4 viable cells per gram, a burden exceeding the estimated minimum infectious dose. A subsequent investigation recovered the first whole-genome sequence of *M. bovis* directly from environmental samples, demonstrating that environmental and animal isolates clustered within the same lineages, confirming active excretion and environmental viability maintenance (Pereira et al., 2024a, 2024b). Spatial mapping further revealed widespread environmental contamination: 49 % of 89 samples collected across 38 field sites tested positive for MTBC DNA, with transmission risk strongly shaped by landscape characteristics such as slope and soil moisture (Ferreira et al., 2024a). When host space-use data were integrated, red deer and wild boar emerged as the species associated with the largest proportion of high-risk areas, with 26.5 % of the landscape classified as high risk in the multi-host model. Complementary camera-trap research in the area additionally demonstrated that indirect wildlife–cattle interactions were more frequent than wildlife–wildlife interactions, and were largely driven by seasonal variation in natural food and water availability (Ferreira et al., 2024b), reinforcing that environmental contamination, host movement ecology, and resource

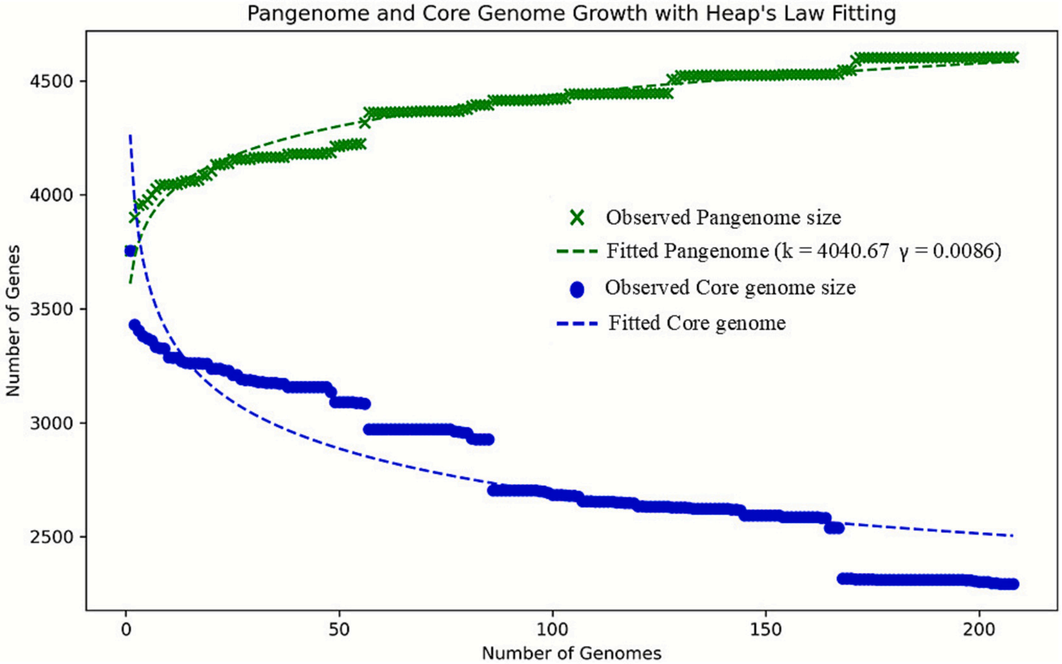

Fig. 7. Heap's law plot depicting the pangenome dynamics of *M. orygis*. The estimated γ value of 0.0086 supports an open pangenome structure, indicating that the gene repertoire continues to expand with the addition of new genomes.

distribution collectively create ecological conditions that favour MTBC persistence and transmission.

Diagnosing wTB involves unique challenges due to the extensive host range, multiple pathogen species involved, and difficulties in sample collection. Immunological assessments, including the tuberculin skin test (TST) and interferon-gamma release assays (IGRAs), are commonly used for bTB screening in cattle and are less practical in wildlife because of the logistical challenges associated with their administration and interpretation of results in free-ranging species. Rapid detection methods such as PCR have revolutionized the identification of MTBC DNA directly from clinical samples, offering timely results. Whole-genome sequencing (WGS) further enhances the diagnostic landscape by facilitating precise species identification and providing epidemiological insights into the transmission dynamics among wildlife and domestic animals (Thomas et al., 2021).

Diagnosing MTBC in ruminants using fecal samples is challenging. As these animals frequently ingest soil along with grass and other forage while grazing, which is a natural reservoir for mycobacteria that primarily contains NTMs, the process becomes more complex. Specifically, in culture, the growth of NTMs often outcompetes that of the MTBC. This competitive overgrowth can hinder the accurate cultivation and isolation of MTBC from fecal samples, rendering cultures less reliable and cumbersome for diagnostic purposes. Consequently, in this study, we used more direct molecular assays, such as PCR, GeneXpert Ultra, and MDR TBplus LPA to overcome these obstacles (Rajendran et al., 2022).

Although these assays target the same organism, variation in results can occur due to differences in their analytical sensitivity, specificity, and sample-processing methods. In conventional PCR, loss of mycobacterial DNA or inhibition due to host-derived contaminants during fecal DNA extraction may occur, potentially leading to false negatives. In contrast, the GeneXpert MTB/RIF Ultra and GenoType MTBDRplus LPA assays are closed, cartridge- or strip-based systems in which fecal suspensions are processed directly, minimizing sample loss and reducing contamination (Osei Sekyere et al., 2019; Shao et al., 2020). GeneXpert Ultra, in particular, has demonstrated higher sensitivity than conventional PCR, detecting as few as 15.6 CFU/mL of *M. tuberculosis* and maintaining high specificity even in paucibacillary samples (Chakravorty et al., 2017). Collectively, these factors may explain the occasional discordance observed among PCR, LPA, and GeneXpert results in this study.

In the 31 *M. orygis* strains that were isolated from Chennai, we observed nonsynonymous SNPs in several genes associated with critical functions, including drug resistance, virulence, and immune mechanisms, such as *embR* (regulator of ethambutol resistance), *eccC4* (a component of the ESX secretion system crucial for virulence), *cfp21* (modulating host cytokine signaling for immune evasion), *mmpL5* (an efflux pump linked to drug resistance and lipid modification for immune evasion), *pepD* (modulating stress response pathways in macrophages), *dosT* (regulating dormancy under hypoxic conditions, aiding in granuloma survival), and *clpC1* (a chaperone involved in protein homeostasis under stress conditions). Despite the introduction of amino acid changes, these mutations did not occur at positions previously characterized as functionally significant, such as resistance-conferring loci or virulence-modulating sites. Additionally, we identified non-synonymous SNPs in DNA repair genes, such as *recC, dinG, ligB, ligC, and mfd*. Mutations in these genes can impair repair mechanisms, leading to an increase in the number of hypermutator strains. These hypermutator strains are known to accelerate the development of drug resistance, highlighting the potential role of DNA repair mutations in adaptive evolution under selective pressure (Cebrián-Sastre et al., 2023; Woodford and Ellington, 2007). Further investigations are necessary to elucidate their biological significance.

Mycobacterium orygis strains exhibited a high copy number of IS6110 insertions with similar common regions (reference genome: *M. orygis* 51,145), as previously reported by Refaya et al. in 2024 (Refaya et al., 2024). When *M. tuberculosis H37Rv* was used as the reference genome, 18 IS6110 insertion regions that were not present in the reference genome were observed. Low copy number insertions of IS1081 were observed in *M. orygis* when compared to both reference genomes. The insertion sequence IS6110 contributes significantly to the evolution of MTBC species. Gonzalo-Asensio et al. (2018) provided detailed insights into the transposition mechanisms of IS6110, revealing the integration and relocation of this element within the MTBC in a lineage-specific manner (Gonzalo-Asensio et al., 2018). Insertions often occur at specific genomic hotspots that can alter the regulatory or coding regions of genes. IS6110-mediated transpositions can disrupt gene function, modulate gene expression, or introduce regulatory elements into previously non-functional regions of the genome. Strains with high-copy IS6110 tend to exhibit greater genomic variability, which may provide a selective advantage under changing environmental conditions, such as drug exposure or host immune responses, whereas strains with low-copy IS6110, such as *M. bovis*, show reduced transpositional activity, which aligns with a more conserved evolutionary trajectory suited to specific host environments (Roychowdhury et al., 2015).

While IS6110 has been extensively studied for its transposition mechanisms and role in genomic plasticity within MTBC, similar detailed studies focusing on IS1081 are limited. Research has primarily utilized IS1081 for diagnostic purposes and strain differentiation due to its presence in MTBC and absence in non-tuberculous mycobacteria (Ren et al., 2023). In a study by van Soolingen et al., the insertion sequence IS1081 was analyzed across various MTBC species. The researchers found that all 99 MTBC strains investigated harboured five or six copies of IS1081 with minimal associated restriction fragment length polymorphisms. Notably, the *Mycobacterium bovis* BCG strain exhibited a distinct 8.0-kb IS1081-containing *PvuII* fragment, which was absent in other MTBC strains, facilitating its differentiation from other members of the complex (Van Soolingen et al., 1992). Further research is warranted to elucidate the functional role of IS1081 in MTBC.

The ability of *M. bovis* to grow preferentially on Löwenstein-Jensen (LJ) media supplemented with sodium pyruvate (SP) is due to a mutation in the *pykA* gene, which encodes pyruvate kinase. This is linked to a well-characterized SNP in *M. bovis*: substitution of glutamic acid (E) with aspartic acid (D) at position 220 (E220D). This mutation impairs pyruvate kinase activity, affecting the ability of the bacterium to metabolize glycerol and necessitating the use of pyruvate as an alternative carbon source (Keating et al., 2005). In *M. orygis* strains isolated from our current study, as well as a previous study conducted in our lab involving *M. orygis* isolates from slaughtered cattle (Ramanujam et al., 2024), we observed a similar preference for LJ media with SP.

Whole-genome sequence analysis of the study isolates along with all *M. orygis* isolates reported globally from diverse geographical locations revealed the same mutation (E220D) in the *pykA* gene in 90 % of the *M. orygis* strains. Additionally, this mutation was also observed in the other animal adapted MTBC lineages such as *M. pinnipedi, M. caprae, M. surricatae, M. microti* as well as *M. africanum* (representative strains from each species were chosen as published by Ramanujam et al., 2024). In 2005, Keating et al., noted that the E220D mutation segregates with RD9 deletion, and reported *pykA* gene mutation in *M. bovis, M. microti* and *M. africanum*. Recent studies have confirmed the absence of RD9 in the animal adapted lineages of MTBC (Bespiatykh et al., 2021; Zwyer et al., 2021). The study further proposed that since lipids are the major carbon source for these organisms in vivo, the mutations observed in *pykA* gene reflect reductive evolution, where mutations aggregate in the genes that are used less and that the pyruvate kinase enzyme is not involved in conferring virulence to these species (Keating et al., 2005). Further analysis with larger datasets of MTBC isolates may provide more insights into many such mutations.

Pangenome analysis of *M. orygis* has provided a comprehensive view of its complete gene repertoire, distinguishing between the core and accessory genomes. The core genome, comprising genes shared by nearly all strains, represent the evolutionary backbone of the species. These genes are essential for fundamental cellular processes and are largely responsible for the conserved phenotypic traits across *M. orygis* isolates. In contrast, the accessory genome which includes soft core, shell, cloud genes and singletons contributes to the genetic variability observed within the species (Tettelin et al., 2005; Vernikos et al., 2015).

A single genome represents only a portion of genetic diversity of the species and relying solely on a single reference genome will not provide a complete view of the gene repertoire. Draft genomes generated from short-read sequencing, often contains gaps and fragmented genes, complicating accurate annotation and characterization of accessory and cloud genes. Although long-read sequencing technologies may improve completeness in the future, current analysis requires careful curation and pangenome approaches to capture the full extent of genomic variation (Salzberg, 2019). To obtain a more comprehensive representation of *M. orygis* diversity, we incorporated publicly available genomes from multiple hosts including humans, cattle, deer and other wildlife. In this study, we observed that several genes classified as core were absent from the *M. orygis* reference genome, which is derived from a human isolate. As a result, all genes consistently present across every genome, were retained as core genes to account for host-specific diversity. Conversely, cloud genes found in fewer than 10 genomes and those that lacking reference matches were excluded from further analysis. Together these steps emphasize the importance of meticulous curation and pangenome approaches in accurately defining the genetic repertoire of multi-host species such as *M. orygis.*

This study provides evidence of an open pan-genome for *M. orygis*, in contrast to the typically conserved genomes of other MTBC members and a previous report suggesting a closed pangenome based on 63 sequences (Karthik et al., 2023). The open nature observed here indicates that the number of accessory genes is likely to increase with the inclusion of more *M. orygis* genomes albeit at a slower rate. This trend is consistent with several recent studies that have also reported open pangenomes within MTBC species (Hurtado-Páez et al., 2023; Negrete-Paz et al., 2023; Periwal et al., 2015; Reis and Cunha, 2021; Yang et al., 2018). However, conflicting reports of closed pangenomes also exist (Dar et al., 2020; Zakham et al., 2021; Ceres et al., 2022; Karthik et al., 2023), likely reflecting differences in study design, genome sampling depth and the pangenome analysis tools and parameters used.

Moreover *M. orygis* is associated with a broad host range, including humans, cattle and wildlife. The accessory genome, particularly cloud genes comprising rare genes and singletons may play a significant role in host adaptation and ecological flexibility, as they are often implicated in the gene gain events. Further functional characterization of these cloud genes is essential to understand their biological relevance and contribution to the adaptive potential of *M. orygis.*

Wildlife TB poses a recognized threat in many countries, prompting the implementation of various strategies for its control and eradication. In New Zealand, efforts to reduce wTB prevalence in brushtail possums include capturing possums near livestock-wildlife interface areas using steel traps, testing them via the tuberculin skin test or interferon-gamma release assay, and culling those that test positive. Additionally, poisoned bait laced with sodium fluoroacetate (1080) is distributed on forest grounds or aerially to control possum populations, with measures in place to minimize non-target species casualties (Nugent et al., 2018; Warburton and Livingstone, 2015). In the USA, wTB control in white-tailed deer involves systematic surveillance and targeted culling in high-prevalence areas. Michigan's multi-phase approach includes culling, hunting regulations, exclusionary fencing, strategic livestock feed and water placement, and livestock protection dogs to reduce deer-livestock interactions (VerCauteren et al., 2018; Walter et al., 2012). In South Africa, the surveillance and regular testing of African buffalo populations are prioritized. Infected buffaloes are culled, whereas others are quarantined to prevent further transmission. In England and Wales, wTB in badgers complicates the control of bTB in cattle (Lakin et al., 2022). The Randomized Badger Control Trials (RBCT) revealed that proactive badger culling marginally reduced bTB in cattle, though reactive culling showed limited efficacy, with perturbation effects dispersing infected badgers. These findings continue to influence controversial badger culling policies (Cassidy, 2019). Additionally, European countries, such as Spain, Portugal and France, have integrated wild boar and red deer culling, fencing, and collaborative research to control wTB and mitigate livestock-wildlife interactions (Payne et al., 2017; Réveillaud et al., 2018). Despite the success of these measures in reducing wTB prevalence globally, similar control initiatives have yet to be undertaken in India. Considering the increasing reports of wTB in Indian wildlife and the significant risks it poses to livestock and public health, implementing systematic surveillance, culling, and habitat management strategies is imperative. Immediate action is required to address wTB in India, leveraging global experiences to develop effective region-specific control measures.

A wTB vaccine offers a sustainable and long-term solution to manage and potentially eliminate the disease, surpassing the practicality and cost-effectiveness of traditional methods, such as culling and wildlife-livestock separation. However, the development of a vaccine for wTB is challenging because of the need to elicit an immune response across diverse species with unique immune systems, diets, and habitats (Palmer and Thacker, 2018). The variability in MTBC strains further complicates the vaccine design. Several studies conducted in the United States, particularly in Michigan, where white-tailed deer form a well-established wildlife reservoir for *M. bovis*, have advanced the field of oral wTB vaccination in cervids. Boggiatto et al., demonstrated that *M. bovis* BCG delivered orally in sodium-alginate spheres elicited antigen-specific cellular immune responses comparable to conventional oral BCG administration, highlighting the feasibility of scalable bait-based vaccination platforms. (Boggiatto et al., 2025). A field deployment study showed that vaccine delivery units (DUs) placed in high-activity agricultural sites were rapidly located and consumed by free-ranging deer, with >50 % uptake at most sites and 100 % consumption at two sites, often within 1–3 days (VerCauteren et al., 2025). Palmer et al. (2023) further showed that prior infection with *M. avium* subsp. *paratuberculosis* (Map) enhanced BCG vaccine efficacy in deer by reducing lesion severity following *M. bovis* challenge, highlighting the importance of host-specific immune backgrounds in shaping vaccine performance (Palmer et al., 2023). Using a mechanistic age-structured transmission model, Pandey et al., demonstrated that pulse vaccination strategies achieving even modest vaccine coverage (30–60 %) can reduce bTB prevalence relatively quickly, particularly when combined with increased harvest. However, complete elimination remained difficult due to the chronic nature of wTB, with long-term repeated vaccination campaigns and improved vaccine uptake identified as critical components for successful disease control (Pandey et al., 2024).

Effective vaccine deployment in remote and inaccessible habitats remains a significant challenge that necessitates innovative delivery approaches. Oral baits containing BCG vaccines have been widely used, offering a non-invasive method suitable for large-scale deployment. Successful examples include vaccination of brushtail possums in New Zealand and European badgers and feed-based formula (Gormley et al., 2017; Palmer et al., 2014; Palmer and Thacker, 2018). Other emerging methods include self-disseminating vaccines using viral vectors, polymer bullets, and plant-based vaccines that deliver antigens via edible plants such as carrots, offering cost-effective and scalable solutions (Murphy et al., 2016; Permyakova et al., 2015; Rhyan and Spraker, 2010). These innovative strategies hold promise in overcoming the logistical challenges of wTB vaccine deployment.

5. Conclusion

The presence of TB in wildlife represents a complex challenge that requires a multifaceted approach. The isolation of *M. orygis* from spotted deer, sambar deer, and blackbuck in Guindy National Park underscores the importance of comprehensive surveillance to understand the dynamics of MTBC transmission in wildlife populations. Enhanced diagnostic techniques, combined with a commitment to the One Health approach, are necessary to bridge knowledge gaps in bTB research in India. With India bearing a significant human TB burden, it is crucial to address the risks posed by wildlife reservoirs of MTBC for both public health and biodiversity conservation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2025.105869.

CRediT authorship contribution statement

Harini Ramanujam: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Mahaprabhu Ramalingam:** Validation, Investigation, Formal analysis. **Ahmed Kabir Refaya:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Priya Rajendran:** Methodology, Investigation. **M. Baskar:** Methodology, Investigation. **Natesan Palanivel:** Methodology, Investigation. **Saraswathi Devarajan:** Methodology, Investigation. **Angayarkanni Balasubramaniam:** Methodology, Investigation. **R. Nithya:** Methodology, Investigation. **Sivakumar Shanmugam:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Kannan Palaniyandi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Informed consent statement

Not applicable.

Institutional review board statement

Not applicable.

Funding

This work was supported by the Indian Council for Medical Research Fellowship/97/2022/ECD-II.

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.

Data availability

The sequence reads of the isolates are available in National Center for Biotechnology Information under Bioproject ID PRJNA1091824.

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