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Insight into the genetic diversity of Mycobacterium bovis isolated from cattle in Malawi

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Abstract:	We describe the genetic diversity and phylogenetic relationships of Mycobacterium bovis, isolated from cattle in Malawi. Deletion analysis, spoligotyping, and MIRU-VNTR typing were used to genotype the isolates. Combined with a larger dataset from neighboring countries, the overall M. bovis diversity in Southern Africa was contextualized. From the southern and northern regions of Malawi, 24 isolates were confirmed as M. bovis. We pooled data for the central region (60 isolates) from our recent publication to conceptualize the genetic and phylogenetic relationships of M. bovis in Malawi. European 1 was the dominant M. bovis clonal complex, with 10 unique spoligotype patterns, and SB0131 was ubiquitous. High genetic diversity, a low clustering rate, and many singletons, coupled with a low mutation transmission index, infer a low level of recent transmission, and suggest an endemic status of bovine tuberculosis (bTB) in Malawi. M. bovis isolates from Zambia, Mozambique, and South Africa were genetically related to Malawian isolates, whereas Tanzania isolates were distantly related. The diversity and phylogenetic analysis suggest earlier introductions and maintenance of M. bovis by constant reinfection from reservoir animals. These findings are fundamental to understanding the source and route of infection in order to establish alternative management strategies for bTB.
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Response to Reviewers:	Point-to-point responses to Reviewer #1's comments Comment 1: I would like to thank the authors for taking into account my comments and going back to the data to further analyse the samples with the 10 MIRU VNTR approach. I will add comments to the responses given by the authors in the following lines: Line 253 and 254: "Furthermore, this hypothesis gained support and validation through the examination of the 10-MIRU-VNTR analysis (Fig. 4)." I find that the direct comparison between both analyses is very difficult given that the color codes and tree structures are completely different. I think the readers would appreciate a little more cohesion between the two figures, maybe even including as two subfigures within the same figure. I would also like to suggest that the authors use the same type of tree and the same color codes. I think that a cladogram structure as in figure 4 would be better suited, as the genetic relationships are resolved much easier than in figure 3. In addition, I would like to suggest that the authors give a little more information regarding the similarities and differences between trees and combinations of alleles, not only a brief overview. Response: We have revised the manuscript as suggested, we have changed the Fig 3 and combined with Fig 4 to form Fig 3A & B with revised figure legends. Further we have added information regarding the similarities and differences between trees and combinations of alleles (Lines 196-202, 210-213 and 277-290). Please find them with read letters. Further, we have added a column for allelic diversity for 5 MIRU-VNTR loci (see Table 2 with red letters) Comment 2: Line 245: Particularly, the strain SB0120 demonstrates dominance among Zambian cattle and wildlife. I believe that the term strain is not adequate here, since there may be multiple strains within the same spoligotype. The same applies for SB0425 (line 278). Please correct. Response: We have corrected accordingly. Please find them at lines 245 and 270 with red letters, respectively Comment 3: What criteria was used to establish the genetic clades? Response: The criteria was based on clustering of isolates. Comment 4: What was the method used for the definition of cluster? This should be indicated in the text. Response: We added this L112-114. Please find it with read letters.

Cover Letter

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Dr. Paolo Pasquali
Co-Editors-in-Chief
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Dear Prof. Ward,

Please find our revised manuscript entitled "**Insight into the genetic diversity of *Mycobacterium bovis* isolated from cattle in Malawi**" by Kapalamula et al and point-to-point responses for reviewer #1's comments.

I believe that the revised manuscript deserves publication in your journal.

Our paper contains original research and has not been submitted or published in any journal and is not being considered for publication elsewhere.

I believe that this manuscript fits to the scope of the journal.

We declare that we have no conflict of interest to disclose.

And hope to hear good news from you soon.

Yours sincerely,

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Highlights

- The first report of nationwide molecular epidemiological survey in Malawi
- The overall *M. bovis* diversity in Southern Africa was contextualized.
- *M. bovis* from Malawi related to those from Zambia, Mozambique, and South Africa
- *M. bovis* from Malawi distantly related to those from Tanzania
- The diversity suggested earlier introductions and maintenance of *M. bovis* in Malawi

Fig. 1

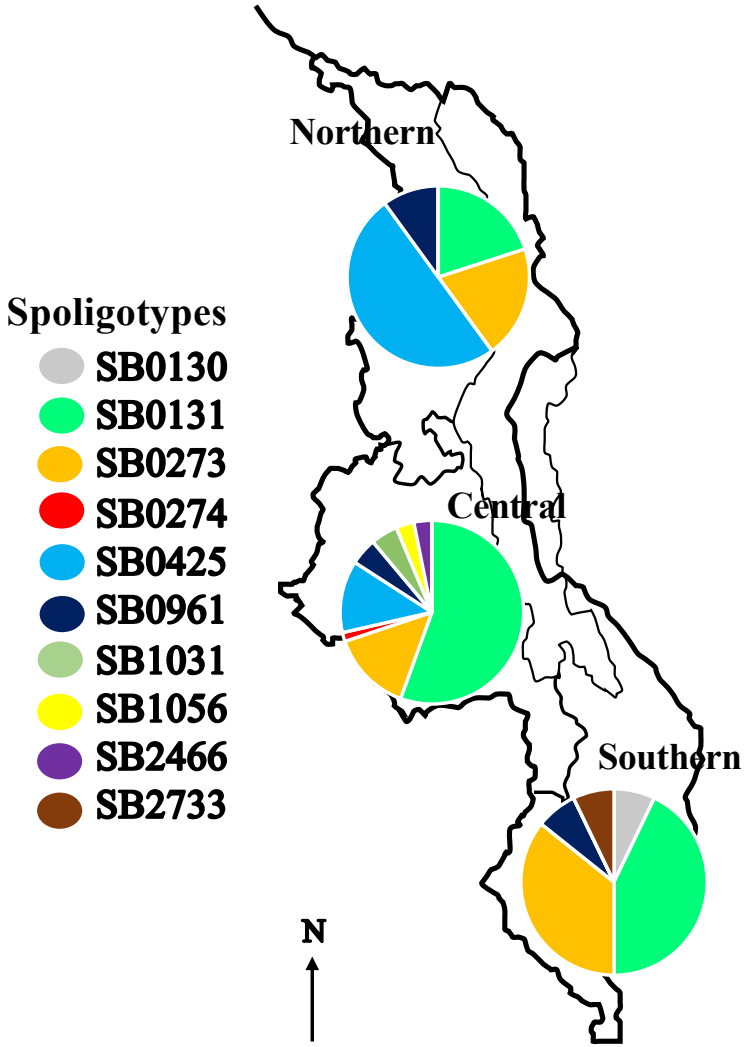
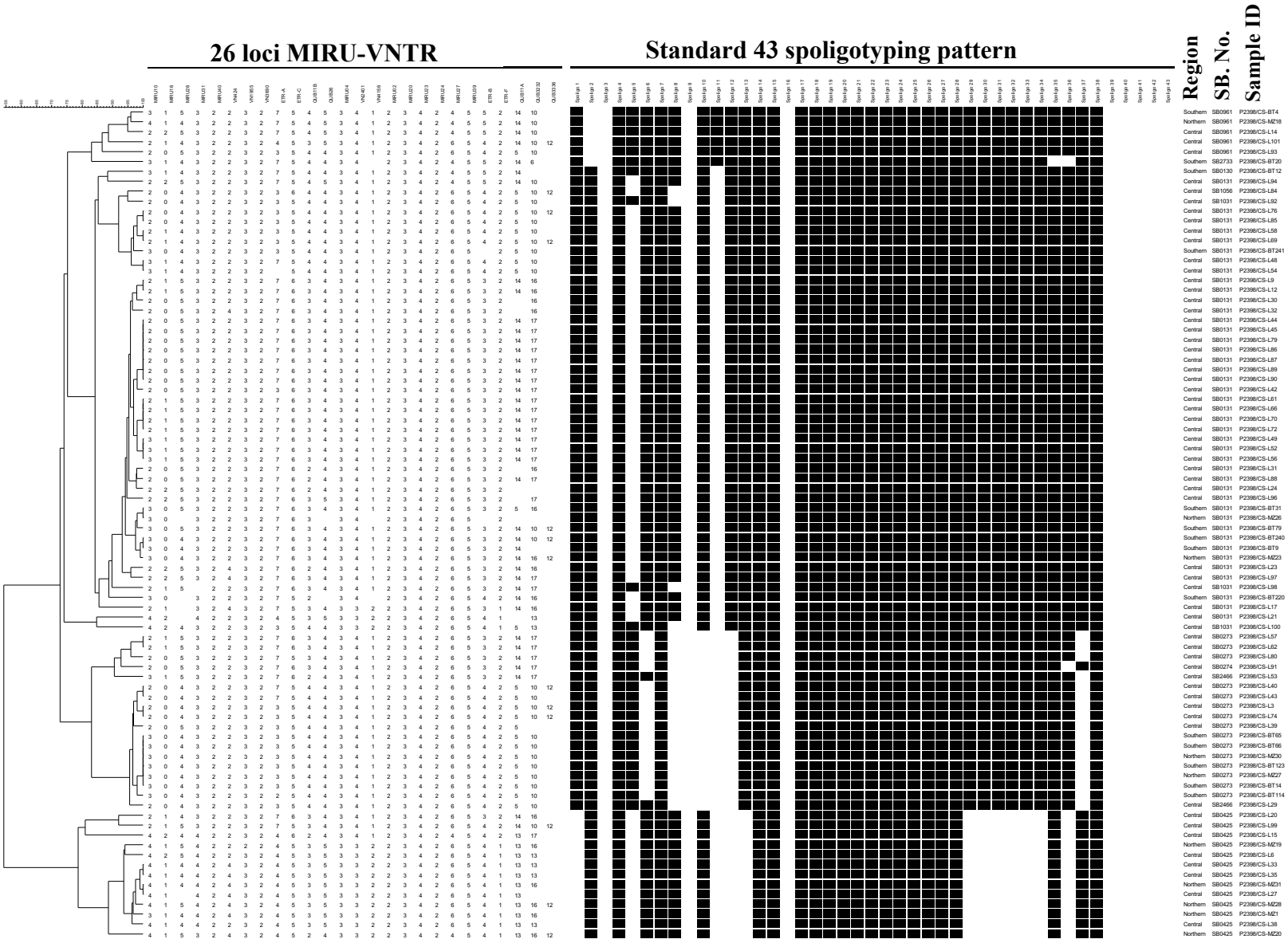
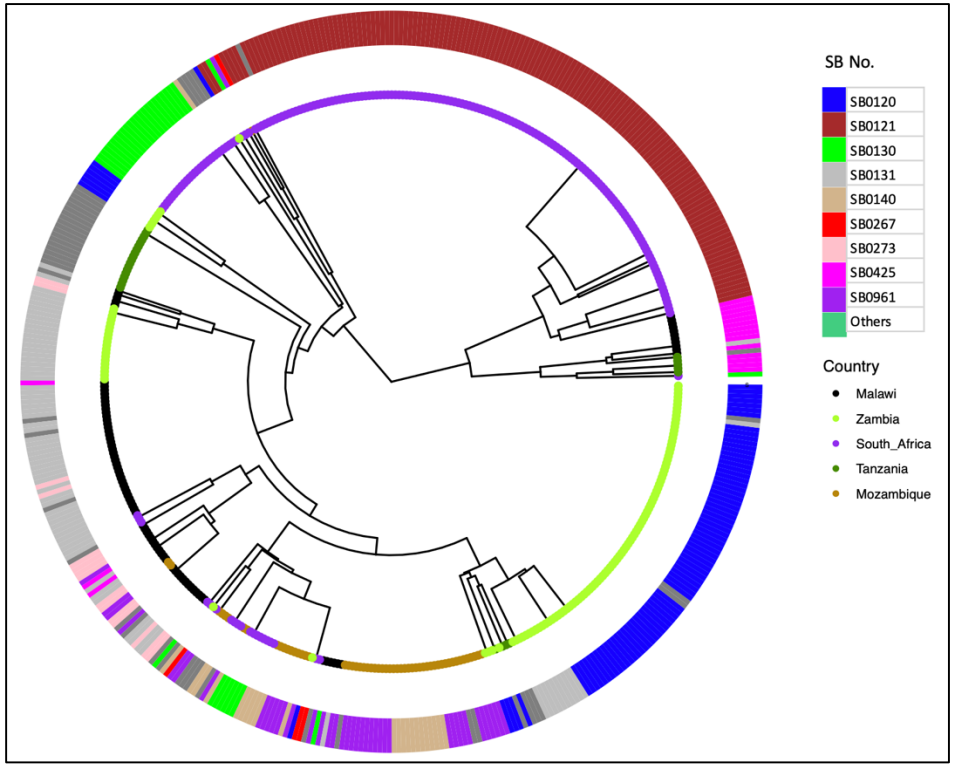


Fig. 2



A



B

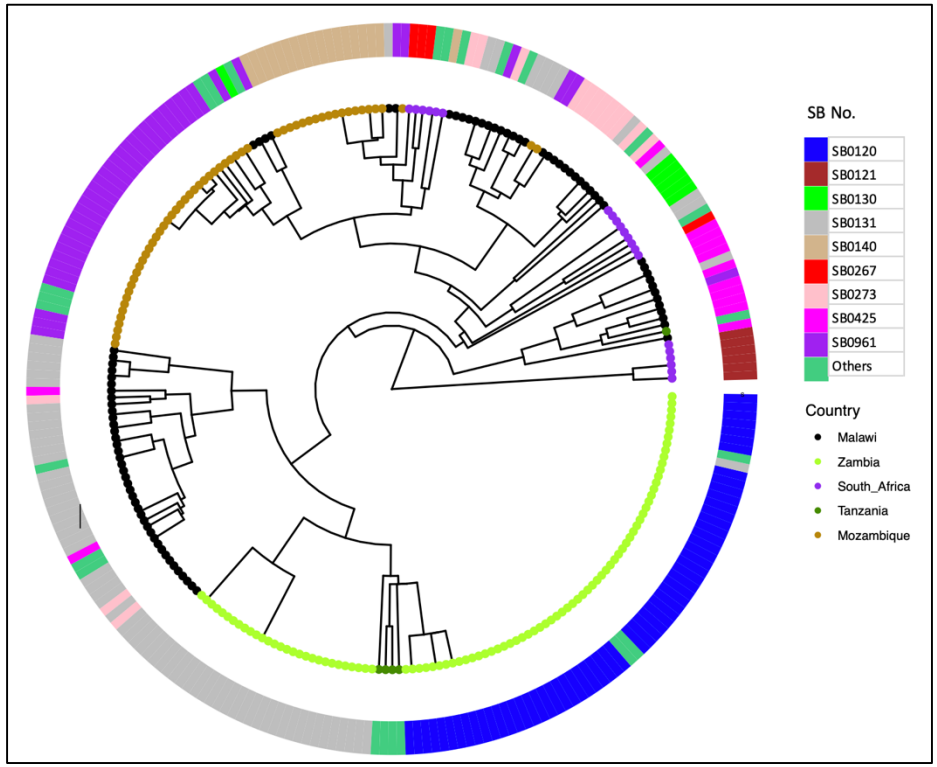


Fig 3: Phylogenic trees showing strains from different countries constructed using unweighted pair group method with arithmetic mean (UPGMA) based on (A) 5 MIRU-VNTR loci and (B) 10 MIRU-VNTR loci. The key shows the SB numbers and country where the strains were isolated from illustrated by different colors. The trees were constructed by MIRU VNTR Plus online software (https://www.miru-vntrplus.org/) and visualized with R-studio (https://posit.co/products/open-source/rstudio/)

Sup Table 1

Insight into the genetic diversity of *Mycobacterium bovis* isolated from cattle in Malawi

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Abstract

We describe the genetic diversity and phylogenetic relationships of *Mycobacterium bovis*, isolated from cattle in Malawi. Deletion analysis, spoligotyping, and MIRU-VNTR typing were used to genotype the isolates. Combined with a larger dataset from neighboring countries, the overall *M. bovis* diversity in Southern Africa was contextualized. From the southern and northern regions of Malawi, 24 isolates were confirmed as *M. bovis*. We pooled data for the central region (60 isolates) from our recent publication to conceptualize the genetic and phylogenetic relationships of *M. bovis* in Malawi. European 1 was the dominant *M. bovis* clonal complex, with 10 unique spoligotype patterns, and SB0131 was ubiquitous. High genetic diversity, a low clustering rate, and many singletons, coupled with a low mutation transmission index, infer a low level of recent transmission, and suggest an endemic status of bovine tuberculosis (bTB) in Malawi. *M. bovis* isolates from Zambia, Mozambique, and South Africa were genetically related to Malawian isolates, whereas Tanzanian isolates were distantly related. The diversity and phylogenetic analysis suggest earlier introductions and maintenance of *M. bovis* by constant reinfection from reservoir animals. These findings are fundamental to understanding the source and route of infection in order to establish alternative management strategies for bTB.

Keywords

Mycobacterium bovis; bovine tuberculosis; genetic diversity; deletion analysis; spoligotyping; MIRU-VNTR

Introduction

Mycobacterium bovis, a member of the *Mycobacterium tuberculosis* complex (MTBC), is the primary cause of bovine tuberculosis (bTB) in cattle (Smith et al., 2006). Furthermore, *M. bovis* potentially infects other mammalian hosts, including wildlife and humans (OIE, 2021). The implications of bTB extend far beyond the rural livelihood socioeconomic and animal health direct impacts to issues encompassing wildlife health and management and public health, diagnosis, and treatment efforts (OIE, 2021; Zinsstag et al., 2006). Even though bTB is found world-wide, Africa suffers the greatest burden (OIE, 2021). Unfortunately, there is a lack of monitoring and reporting for bTB, leading to an underestimate of the disease's real impact. In industrialized countries, bTB has almost been eliminated in cattle by testing and slaughtering of the reactors (FAO, 2015). However, due to financial constraints, this method cannot be used in developing countries like Malawi. In such situations, spatial patterns, flows, and pathogen diversity become crucial information for determining the source and route of infection and developing alternative disease management techniques such as animal movement control (Egbe et al., 2017).

Previously, we reported highly diversified and clonally expanding *M. bovis* strains circulating in cattle from the central regions of Malawi (Kapalamula et al., 2021). These isolates showed genetic similarities to those reported in neighboring countries, suggesting possible transmission links, wide spread of the disease, and a lapse in bTB mitigating efforts (Kapalamula et al., 2021). However, these findings may not be generalizable to the entire country but rather shed more light on what might be going on in terms of bTB molecular epidemiology and transmission dynamics in Malawi.

We therefore collected granulomatous bTB-like lesions from cattle slaughtered at major abattoirs in the northern and southern regions of Malawi and genotyped the isolates. Additionally, we combined the dataset from the central region (Kapalamula et al., 2021) (based on deletion analysis, spoligotyping patterns, and MIRU-VNTRs) that allowed us to conceptualize the *M. bovis* genetic diversity in Malawi. Furthermore, we combined Malawian isolates with a larger dataset from neighboring countries to contextualize *M. bovis* phylogenetic networks in Southern Africa.

Materials and methods

Study design: A cross-sectional study and basic purposive sampling method were utilized to collect granulomatous bTB-like lesioned tissues during routine abattoir cattle examination at major regional abattoirs in Blantyre (Southern region) and Mzuzu (Northern region) between August 2019 and February 2020. These abattoirs are supplied by animals from districts within the respective region as observed on the accompanied livestock movement permits. Sample size was estimated at ~1000 based on a 50% prevalence assumption at 95% confidence interval and 5% absolute precision since there were no previous studies on bTB epidemiology in the area. Sample collection, preparation and culturing have been described in detail in our previous publication (Kapalamula et al., 2021). Genomic DNA was extracted by the boiling method involving heating at 95^{o}C for 15 minutes and immediate cooling at -20^{o}C for 30 minutes.

Mycobacterial identification and confirmation: Isolates were identified as MTBC, by a specific PCR targeting the insertion sequence 6110 (*IS6110*) with slight modifications (Kapalamula et al., 2021). The PCR products were run on 1.5% agarose gel stained with gel red (Biotium, Inc., CA, USA) for 18 min at 100V with an expected band size of 123bp. A multiplex PCR targeting the region of difference 4 (RD4) was performed to identify *M. bovis* as described previously (Bakshi et al., 2005). The expected PCR products were 168 bp for *M. bovis* and 337 bp for *M. tuberculosis*.

Genotyping of *M. bovis* isolates: DNA of all confirmed *M. bovis* isolates was used for genotyping experiments. The isolates were characterized by (a) deletion typing based on a PCR targeting chromosomal deletions of RDAf1 (African 1 complex), RDAf2 (African 2 complex) and RDEu1 (European 1 complex) (Kapalamula et al., 2021), (b) spoligotyping (Kamerbeek et al., 1997) and (c) MIRU-VNTR typing based on 26 loci (Kapalamula et al., 2021). *M. bovis* spoligotype database was used for referencing (https//: www.mbovis.org).

Data management and statistical analysis: Data was saved in Excel spreadsheet and imported to Bionumerics software package version 7.6 (Applied Maths, Sint-Martens-Latem, 2016) and MIRU-VNTR plus online software (https://www.miru-vntrplus.org/).

Phylogenetic and genetic diversity analyses: An unweighted pair group average linkage algorithm (UPGMA) and Neighbor Joining (NJ) trees were used to analyze isolates' genetic relationships based on distance matrices, with minimum phylogenetic distances set at a single locus variation (SLV). Spoligo- and MIRU-VNTR typing discriminatory power was estimated by the Hunter Gaston Discriminatory Index (HGDI) (Hunter & Gaston, 1988). Additionally, HGDI was used to estimate *M. bovis* genetic diversity for each region. In this study, a cluster was defined as comprising two or more isolates that share similar spoligotype or MIRU-VNTR patterns and are grouped within the same branch of a phylogenetic tree.

Transmission estimates: To reflect on the extent of *M. bovis* transmission, two transmission indices were computed and compared among the regions. The RTI was computed using the formula (Tanaka & Francis, 2005),

RTI = (sample size-total number of unique genotypes)/(the sample size-1) (1)

The TMI, described as the measure of the number of mutations caused by a transmission event was computed by

$$TMI = \tilde{\mu}\left(\frac{n-g+v_1}{v_1}\right) \quad (2)$$

where $\tilde{\mu}$ is an independent estimate of the mutation rate of the genetic marker (Tanaka & Francis, 2005). A composite mutation rate for MIRU-VNTR (0.0027) per loci per year was used as calculated in previous studies (Egbe et al., 2017). And *g* is the number of unique genotypes, whereas v_1 is the number of single-step mutation events inferred from the data. These indices range from 0 (the least recent transmission) to 1(maximum genotypes transmission).

M. bovis clustering rate (CR) was estimated using the following formula:

CR= (number of clustered isolates-number of clusters) / total number of isolates) (3)

The diversity and phylogenetic relationships of *M. bovis* in Malawi: The phylogenetic networks of *M. bovis* were determined by integrating spoligotyping and MIRU-VNTR typing data using the UPGMA tree. Data from this study was combined with recent published (60 isolates) (Kapalamula

et al., 2021) to compute the diversity and phylogenetic relationships of *M. bovis* in Malawi. Isolates were classified to *M. bovis* clonal complexes based on the criteria published previously (Berg et al., 2011; Müller et al., 2009; Smith et al., 2011).

Contextualizing the diversity of *M. bovis* isolates between Malawian isolates and those from neighbouring countries (Southern Africa): We constructed a phylogenetic network using *M. bovis* isolates reported in neighboring countries to contextualize the diversity of *M. bovis* in Southern Africa. Using literature, a large data set (481isolates) were used; South Africa (193 isolates (Musoke et al., 2015 (n=31); Hlokwe et al., 2013 (n=131); Hlowe., et al 2011 (n=31)), Zambia (120 isolates) (Squarre et al., 2021 (n=42); Malama et al., 2014 (n=25); Hang'ombe et al., 2012 (n=53)), Tanzania (27 isolates (Katale et al., 2017 (n=5); Berg et al., 2011 (n=8); Müller et al., 2009 (n=14)), Mozambique (57 isolates) (Machado et al., 2018) and Malawi (Kapalamula et al.,2021 (60 isolates)) and (24 isolates) this study). Phylogenetic network was constructed based on spoligotyping data and 5 VNTR loci (ETR A, B, C, D and E) found common among the studies. Furthermore, an extended analysis was conducted, encompassing a broader spectrum of loci with a direct comparison to the previous dataset comprising only 5 MIRU-VNTR loci. The primary objective of this expanded analysis was to augment the study's resolution, thereby enabling the identification of potential hidden relationships considering that using 5 MIRU-VNTR loci may have limitations of masking the diversity. In the pursuit of this goal, our inclusion criteria were structured around two key parameters: (1) the utilization of datasets specifically from within southern African countries, and (2) the incorporation of isolates featuring no more than 3 missing loci data points.

Results

Animal characteristics summary: A total of 587 cattle were examined during the study period as follows: 313 from the southern region and 274 from the northern region. Out of these, 32/313 (southern region) and 18/274 (northern region) had bTB-like lesions in various tissues and samples were collected. A total of 31 isolates were identified as MTBC of which 24 were confirmed as *M.*

bovis. It is noteworthy to mention that the remaining seven isolates (identified as MTBC) were not further investigated to other MTBC species because it was out of scope of the present study.

Geographical distribution and diversity of *M. bovis* spoligotypes: Out of 24 isolates, 21 (88%) had RDEu1 deletion hence belonged to the European 1 (Eu1) clonal complex. We did not find isolates that belonged to African 1 or African 2 clonal complexes. Three isolates (12%) were categorized as having 'BCG-like' spoligotype pattern for lacking additional spacers in addition to the absence of *M. bovis* spacers makers 3, 9, 16, and 39–43. Spoligotyping for the northern and southern regions revealed a total of six types SB0131, SB0130, SB0273, SB0425, SB0961 and SB2733. Compared to the global database (http://www.mbovis.org), only one had not been previously reported and was assigned spoligotype number SB2733 which was characterized by the absence of spacers 2, and 35-36. Combining with data from the central region (Kapalamula et al., 2021), ten spoligotypes were revealed and SB0131, SB0273 and SB0961 were ubiquitous. (Fig 1). Interestingly, SB0425 was only found in the northern and the central regions. SB0274, SB1031 and SB2466 were exclusively found in central region whereas SB0130 and SB2733 were found in Southern region (Fig 1). The distribution of spoligotypes in each region is summarized (Supplementary table 1).

Fig. 1 | Spatial distribution of spoligotype diversity and dominance in the three regions of Malawi. Ten spoligotypes were revealed and spoligotype SB0131 was dominant in Malawi.

Genotype diversity of *M. bovis* in Malawi: The unique spoligotypes recovered from the northern and southern regions were further differentiated into 16 distinct 'genotypes' (8 singletons and 8 clusters) by a 26 loci MIRU-VNTR typing. Combined with data from the central region (Kapalamula et al., 2021), high genetic diversity was observed in the central region (0.980) followed by the southern region (0.971) and the northern region (0.933). Using the combined analysis of Spoligo- and MIRU-VNTR typing, the 84 isolates (60 from a previous study (Kapalamula et al., 2021) and 24 from this study) revealed 56 genotypes (39 singletons and 17 clusters) with a

discrimination index of 0.985 and clustering rate of 33% (Fig. 2). The largest cluster comprised of seven isolates, followed by four isolates, three isolates and two isolates (Fig. 2).

Fig. 2: A UPGMA phylogenetic tree showing the relationships of spoligotypes from cattle slaughtered in major regions of Malawi. The tree is constructed based on a combined spoligotyping and a 26 loci MIRU-VNTR analysis.

The transmission rates of *M. bovis* within the three regions of Malawi were estimated using the parameters from empirical molecular data (Table 1) using formulae 1 & 2. We observed low RTI and TMI in all regions (Table 1)

Table 1: Attributes used for the comparative analysis for the calculation of recent transmission index and transmission mutation index of *M. bovis* within the three regions in Malawi

	Region		
Attributes	**Southern**	**Central**	**Northern**
Number of isolates (n)	14	63	10
Number of unique genotypes (g)	11	43	7
Number of clusters and size	3 (2,2,2)	11(2,2,2,2,2,2,2,3,3,4,7)	3 (2,2,2)
Single locus variation genotypes (v_1)	10	38	3
RTI	0.231	0.3226	0.3333
TMI	0.0035	0.0041	0.0054

Contextualizing the diversity of *M. bovis* isolates between Malawian isolates and those from neighboring countries (Southern Africa)

Using 5-MIRU VNTR loci, we constructed phylogenetic median networks based on a comprehensive dataset of 481 isolates from the literature. This analysis aimed to elucidate hypothetical linkages and evolutionary relationships among isolates in Southern Africa. The results indicate a significant genetic relatedness among isolates from the Southern African region (Fig. 3A). Specifically, Malawian isolates appear to share a closer genetic affinity with Zambian and

Mozambican isolates, as evidenced by their common spoligotypes SB0131 and SB0961 (Fig. 3A). The clustering rate for this analysis was determined to be 90.64% (Table 2).

Furthermore, an additional analysis was conducted to evaluate as many loci as possible, aiming to enhance the study's resolution and facilitate the detection of hidden relationships. It was discovered that a ten-loci approach was prevalent among the southern African isolates, leading to the establishment of a sample population of 259 isolates. Phylogenetic median networks were constructed to elucidate the hypothetical linkages and evolutionary relationships among these isolates, and these results were compared with the findings from the 5-MIRU-VNTR loci analysis. Malawian isolates were closely related to Zambian and Mozambican isolates sharing SB0131 spoligotype (Fig. 3B). The 10-MIRU VNTR loci showed high allelic diversity with the clustering rate was 68.26% (Table 2). Furthermore, eight loci (QUB11a, ETR-B, MIRU16, MIRU26, QUB11b, ETR-A, MIRU10, ETR-C) seem suitable for the Southern African context offering the potential to optimize costs in future research endeavors (Table 2).

Fig 3: Phylogenic trees showing strains from different countries constructed using unweighted pair group method with arithmetic mean (UPGMA) based on (A) 5 MIRU-VNTR loci and (B) 10 MIRU-VNTR loci. The key shows the SB numbers and country where the strains were isolated from illustrated by different colors. The trees were constructed by MIRU VNTR Plus online software (https://www.miru-vntrplus.org/) and visualized with R-studio (https://posit.co/products/open-source/rstudio/)

Table 2: Allelic diversity of the 10 and 5 MIRU-VNTR loci analysis on the regional strains and their clustering rate.

	Allelic Diversity	
Loci	**10 MIRU-VNTR loci**	**5 MIRU-VNTR loci**
MIRU10	0.90	
ETR-B	0.83	0.75
MIRU26	0.79	
MIRU16	0.78	
ETR-A	0.76	0.64
QUB11b	0.74	
QUB11a	0.74	
ETR-C	0.63	0.70
MIRU04 (ETR-D)	0.49	0.47
MIRU31 (ETR-E)	0.47	0.41
Clustering rate (%)	**68.26**	**90.64**

Discussion

Since the major control and/or eradication technique of bTB in cattle, i.e., testing and slaughter of bTB-positive cattle, is financially constrained in most developing countries, e.g., Malawi, the application of population-based molecular markers of bTB is critical in developing alternative mitigating strategies. Such techniques leverage various host population-driven combinations of pathogen genotypes, clustering, diversity, and geographic distribution to predict contact activity, potential transmission events, and, most importantly, disease hotspots (Egbe et al., 2017). We report the genetic diversity and phylogenetic relationships of *M. bovis* isolated from cattle slaughtered at major regional abattoirs in Malawi.

The European 1 (Eu1) clonal complex was detected in the vast majority among the *M. bovis* isolates from Malawi. Eu1 clonal complex has been reported in Mozambican (Machado et al., 2018) and South African cattle (Michel et al., 2008) and identified the latter as the Eu1 clonal complex's transit source to Africa. The presence of the Eu1 complex in Malawian cattle suggests that these strains

have spread throughout the Southern African region. We discovered a small proportion of "BCG-like" spoligotype pattern strains with an intact spacer 11 and an absence of spacer 2 (Fig. 2). These strains appear to have evolved from the spoligotype SB0120 through the loss of spacer 3, in addition to spacers 3, 9, 16, and 39–43 (Ghebremariam et al., 2018). Particularly, spoligotype SB0120 demonstrates dominance among Zambian cattle and wildlife (Hang'ombe et al., 2012). Its prevalence extends across various regions in Africa and Europe (Ghebremariam et al., 2018) and has previously been categorized as European 3 (Eu3) complex (Almaw et al., 2021). Based on our findings, there is a plausible indication that the Eu3 complex is expanding within the African region, and it's conceivable that the introduction of these strains into Malawi originated from Zambia (Fig. 3A & B).

We observed a high genetic diversity of *M. bovis* circulating in Malawian cattle (Fig. 2). The drivers are most likely linked to the traditional animal production systems largely employed by local livestock farmers (FAO & NEPAD, 2005), involving unregulated animal movements in search of grazing pastures and watering spots. Such movements permit regular contact between domestic animals and/or wildlife from various areas, resulting in constant infection and the spread of diseases such as bTB. Pathogen genotype clustering can reveal the spatial and temporal patterns of hosts in bTB outbreaks or endemic settings (Egbe et al., 2017). The phylogenetic clustering infers an endemic situation as opposed to an outbreak (Fig. 2). This hypothesis is supported by a low CR (33%), which is also reflected by low RTI (0.231, 0.323, and 0.333) and a low TMI (0.0035, 0.0041, and 0.0054) for the southern, central, and northern regions, respectively.

In the Southern African evolutionary network constructed using the 5-MIRU-VNTR loci, a close genetic relationship was observed among Malawian, Zambian, Mozambican, and South African isolates (Fig. 3A). This pattern found further support and validation through the examination of the 10-MIRU-VNTR analysis (Fig. 3B). Importantly, the results of both analyses remained consistently aligned, pointing towards a common source or origin for these isolates. Pre-and post-colonial livestock trade between these countries might have contributed to the close relationships of the

isolates. Previous reports highlighted that to improve the dairy industry, Malawi imported exotic dairy breeds from South Africa and Zambia (Kapalamula et al., 2021). It is interesting to mention that SB0425, previously identified as an ancestral type (Loiseau et al., 2020), showed no close relationship with similar spoligotype profiles from Tanzania suggesting limited cattle movement between the two countries despite sharing borders. We understand that the number of sampled animals was less than the anticipated, this was due to logistical challenges. Therefore, we acknowledge that this may have a potential sampling bias effect. However, the discovery of ancestral strains from northern region (Malawi) suggests that the pathogen has been in circulation over a long period of time and that bTB is a common occurrence in this region.

While the total number of isolates in the two trees varied (Fig. 3A & B), there were marked differences in the clustering rates between the two MIRU-VNTR loci sets (Table 2). The analysis of 5-loci MIRU-VNTR resulted in a high clustering rate (90.64%), signifying limited discriminatory power and a tendency to group genetically similar isolates together, indicating lower resolution in distinguishing the isolates. On the other hand, the 10-loci MIRU-VNTR analysis yielded a lower clustering rate (68.26%), indicating stronger discriminatory power and a higher resolution in distinguishing genetic differences among isolates, resulting in fewer strains clustering together. These findings underscore the importance of selecting the most suitable loci for specific research objectives and demonstrate the impact of sample size on the observed clustering rates. While the congruity of outcomes between the 5- and 10-MIRU-VNTR loci analyses underscores the robustness of our findings, it is worth noting that a more comprehensive understanding of transmission dynamics could potentially be attained through high-throughput approaches such as whole genome sequencing. This progressive approach holds the potential to furnish a more holistic perspective on the intricate patterns of evolution and transmission.

In conclusion, this study discovered a high genetic diversity of *M. bovis* circulating in Malawian cattle and possible transmission links. Further, Malawian isolates are phylogenetic related to isolates

from neighboring countries except from Tanzania suggesting possible transmission linkages. This calls for alternative approaches to support current mitigating strategies.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical statement

The study obtained ethical approval (Protocol Number 19/09/2398) for sampling cattle from major abattoirs from the National Health Science Research Committee (NHSRC), Ministry of Health, and the Department of Animal Health and Livestock Development (DAHLD), Lilongwe, Malawi.

Authors' contributions

TFK, CN, and YS contributed to the conception and design of the study, TFK, JYC, LB, DS, and HMC contributed to the acquisition of data, TFK, JYC, MLA, JT, PB, SVG, J Thapa, CN, and YS contributed to the analysis and interpretation of data, TFK, JYC, CN, YS contributed to the drafting the article, All authors contributed to the revising it critically for important intellectual content and finally approved the version to be submitted.

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Insight into the genetic diversity of *Mycobacterium bovis* isolated from cattle in Malawi

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Abstract

We describe the genetic diversity and phylogenetic relationships of *Mycobacterium bovis*, isolated from cattle in Malawi. Deletion analysis, spoligotyping, and MIRU-VNTR typing were used to genotype the isolates. Combined with a larger dataset from neighboring countries, the overall *M. bovis* diversity in Southern Africa was contextualized. From the southern and northern regions of Malawi, 24 isolates were confirmed as *M. bovis*. We pooled data for the central region (60 isolates) from our recent publication to conceptualize the genetic and phylogenetic relationships of *M. bovis* in Malawi. European 1 was the dominant *M. bovis* clonal complex, with 10 unique spoligotype patterns, and SB0131 was ubiquitous. High genetic diversity, a low clustering rate, and many singletons, coupled with a low mutation transmission index, infer a low level of recent transmission, and suggest an endemic status of bovine tuberculosis (bTB) in Malawi. *M. bovis* isolates from Zambia, Mozambique, and South Africa were genetically related to Malawian isolates, whereas Tanzanian isolates were distantly related. The diversity and phylogenetic analysis suggest earlier introductions and maintenance of *M. bovis* by constant reinfection from reservoir animals. These findings are fundamental to understanding the source and route of infection in order to establish alternative management strategies for bTB.

Keywords

Mycobacterium bovis; bovine tuberculosis; genetic diversity; deletion analysis; spoligotyping; MIRU-VNTR

Introduction

Mycobacterium bovis, a member of the *Mycobacterium tuberculosis* complex (MTBC), is the primary cause of bovine tuberculosis (bTB) in cattle (Smith et al., 2006). Furthermore, *M. bovis* potentially infects other mammalian hosts, including wildlife and humans (OIE, 2021). The implications of bTB extend far beyond the rural livelihood socioeconomic and animal health direct impacts to issues encompassing wildlife health and management and public health, diagnosis, and treatment efforts (OIE, 2021; Zinsstag et al., 2006). Even though bTB is found world-wide, Africa suffers the greatest burden (OIE, 2021). Unfortunately, there is a lack of monitoring and reporting for bTB, leading to an underestimate of the disease's real impact. In industrialized countries, bTB has almost been eliminated in cattle by testing and slaughtering of the reactors (FAO, 2015). However, due to financial constraints, this method cannot be used in developing countries like Malawi. In such situations, spatial patterns, flows, and pathogen diversity become crucial information for determining the source and route of infection and developing alternative disease management techniques such as animal movement control (Egbe et al., 2017).

Previously, we reported highly diversified and clonally expanding *M. bovis* strains circulating in cattle from the central regions of Malawi (Kapalamula et al., 2021). These isolates showed genetic similarities to those reported in neighboring countries, suggesting possible transmission links, wide spread of the disease, and a lapse in bTB mitigating efforts (Kapalamula et al., 2021). However, these findings may not be generalizable to the entire country but rather shed more light on what might be going on in terms of bTB molecular epidemiology and transmission dynamics in Malawi.

We therefore collected granulomatous bTB-like lesions from cattle slaughtered at major abattoirs in the northern and southern regions of Malawi and genotyped the isolates. Additionally, we combined the dataset from the central region (Kapalamula et al., 2021) (based on deletion analysis, spoligotyping patterns, and MIRU-VNTRs) that allowed us to conceptualize the *M. bovis* genetic diversity in Malawi. Furthermore, we combined Malawian isolates with a larger dataset from neighboring countries to contextualize *M. bovis* phylogenetic networks in Southern Africa.

Materials and methods

Study design: A cross-sectional study and basic purposive sampling method were utilized to collect granulomatous bTB-like lesioned tissues during routine abattoir cattle examination at major regional abattoirs in Blantyre (Southern region) and Mzuzu (Northern region) between August 2019 and February 2020. These abattoirs are supplied by animals from districts within the respective region as observed on the accompanied livestock movement permits. Sample size was estimated at ~1000 based on a 50% prevalence assumption at 95% confidence interval and 5% absolute precision since there were no previous studies on bTB epidemiology in the area. Sample collection, preparation and culturing have been described in detail in our previous publication (Kapalamula et al., 2021). Genomic DNA was extracted by the boiling method involving heating at 95^{o}C for 15 minutes and immediate cooling at -20^{o}C for 30 minutes.

Mycobacterial identification and confirmation: Isolates were identified as MTBC, by a specific PCR targeting the insertion sequence 6110 (*IS6110*) with slight modifications (Kapalamula et al., 2021). The PCR products were run on 1.5% agarose gel stained with gel red (Biotium, Inc., CA, USA) for 18 min at 100V with an expected band size of 123bp. A multiplex PCR targeting the region of difference 4 (RD4) was performed to identify *M. bovis* as described previously (Bakshi et al., 2005). The expected PCR products were 168 bp for *M. bovis* and 337 bp for *M. tuberculosis*.

Genotyping of *M. bovis* isolates: DNA of all confirmed *M. bovis* isolates was used for genotyping experiments. The isolates were characterized by (a) deletion typing based on a PCR targeting chromosomal deletions of RDAf1 (African 1 complex), RDAf2 (African 2 complex) and RDEu1 (European 1 complex) (Kapalamula et al., 2021), (b) spoligotyping (Kamerbeek et al., 1997) and (c) MIRU-VNTR typing based on 26 loci (Kapalamula et al., 2021). *M. bovis* spoligotype database was used for referencing (https//: www.mbovis.org).

Data management and statistical analysis: Data was saved in Excel spreadsheet and imported to Bionumerics software package version 7.6 (Applied Maths, Sint-Martens-Latem, 2016) and MIRU-VNTR plus online software (https://www.miru-vntrplus.org/).

Phylogenetic and genetic diversity analyses: An unweighted pair group average linkage algorithm (UPGMA) and Neighbor Joining (NJ) trees were used to analyze isolates' genetic relationships based on distance matrices, with minimum phylogenetic distances set at a single locus variation (SLV). Spoligo- and MIRU-VNTR typing discriminatory power was estimated by the Hunter Gaston Discriminatory Index (HGDI) (Hunter & Gaston, 1988). Additionally, HGDI was used to estimate *M. bovis* genetic diversity for each region. In this study, a cluster was defined as comprising two or more isolates that share similar spoligotype or MIRU-VNTR patterns and are grouped within the same branch of a phylogenetic tree.

Transmission estimates: To reflect on the extent of *M. bovis* transmission, two transmission indices were computed and compared among the regions. The RTI was computed using the formula (Tanaka & Francis, 2005),

RTI = (sample size-total number of unique genotypes)/(the sample size-1) (1)

The TMI, described as the measure of the number of mutations caused by a transmission event was computed by

$$TMI = \tilde{\mu}\left(\frac{n-g+v_1}{v_1}\right) \quad (2)$$

where $\tilde{\mu}$ is an independent estimate of the mutation rate of the genetic marker (Tanaka & Francis, 2005). A composite mutation rate for MIRU-VNTR (0.0027) per loci per year was used as calculated in previous studies (Egbe et al., 2017). And *g* is the number of unique genotypes, whereas v_1 is the number of single-step mutation events inferred from the data. These indices range from 0 (the least recent transmission) to 1(maximum genotypes transmission).

M. bovis clustering rate (CR) was estimated using the following formula:

CR= (number of clustered isolates-number of clusters) / total number of isolates) (3)

The diversity and phylogenetic relationships of *M. bovis* in Malawi: The phylogenetic networks of *M. bovis* were determined by integrating spoligotyping and MIRU-VNTR typing data using the UPGMA tree. Data from this study was combined with recent published (60 isolates) (Kapalamula

et al., 2021) to compute the diversity and phylogenetic relationships of *M. bovis* in Malawi. Isolates were classified to *M. bovis* clonal complexes based on the criteria published previously (Berg et al., 2011; Müller et al., 2009; Smith et al., 2011).

Contextualizing the diversity of *M. bovis* isolates between Malawian isolates and those from neighbouring countries (Southern Africa): We constructed a phylogenetic network using *M. bovis* isolates reported in neighboring countries to contextualize the diversity of *M. bovis* in Southern Africa. Using literature, a large data set (481isolates) were used; South Africa (193 isolates (Musoke et al., 2015 (n=31); Hlokwe et al., 2013 (n=131); Hlowe., et al 2011 (n=31)), Zambia (120 isolates) (Squarre et al., 2021 (n=42); Malama et al., 2014 (n=25); Hang'ombe et al., 2012 (n=53)), Tanzania (27 isolates (Katale et al., 2017 (n=5); Berg et al., 2011 (n=8); Müller et al., 2009 (n=14)), Mozambique (57 isolates) (Machado et al., 2018) and Malawi (Kapalamula et al.,2021 (60 isolates)) and (24 isolates) this study). Phylogenetic network was constructed based on spoligotyping data and 5 VNTR loci (ETR A, B, C, D and E) found common among the studies. Furthermore, an extended analysis was conducted, encompassing a broader spectrum of loci with a direct comparison to the previous dataset comprising only 5 MIRU-VNTR loci. The primary objective of this expanded analysis was to augment the study's resolution, thereby enabling the identification of potential hidden relationships considering that using 5 MIRU-VNTR loci may have limitations of masking the diversity. In the pursuit of this goal, our inclusion criteria were structured around two key parameters: (1) the utilization of datasets specifically from within southern African countries, and (2) the incorporation of isolates featuring no more than 3 missing loci data points.

Results

Animal characteristics summary: A total of 587 cattle were examined during the study period as follows: 313 from the southern region and 274 from the northern region. Out of these, 32/313 (southern region) and 18/274 (northern region) had bTB-like lesions in various tissues and samples were collected. A total of 31 isolates were identified as MTBC of which 24 were confirmed as *M.*

bovis. It is noteworthy to mention that the remaining seven isolates (identified as MTBC) were not further investigated to other MTBC species because it was out of scope of the present study.

Geographical distribution and diversity of *M. bovis* spoligotypes: Out of 24 isolates, 21 (88%) had RDEu1 deletion hence belonged to the European 1 (Eu1) clonal complex. We did not find isolates that belonged to African 1 or African 2 clonal complexes. Three isolates (12%) were categorized as having 'BCG-like' spoligotype pattern for lacking additional spacers in addition to the absence of *M. bovis* spacers makers 3, 9, 16, and 39–43. Spoligotyping for the northern and southern regions revealed a total of six types SB0131, SB0130, SB0273, SB0425, SB0961 and SB2733. Compared to the global database (http://www.mbovis.org), only one had not been previously reported and was assigned spoligotype number SB2733 which was characterized by the absence of spacers 2, and 35-36. Combining with data from the central region (Kapalamula et al., 2021), ten spoligotypes were revealed and SB0131, SB0273 and SB0961 were ubiquitous. (Fig 1). Interestingly, SB0425 was only found in the northern and the central regions. SB0274, SB1031 and SB2466 were exclusively found in central region whereas SB0130 and SB2733 were found in Southern region (Fig 1). The distribution of spoligotypes in each region is summarized (Supplementary table 1).

Fig. 1 | Spatial distribution of spoligotype diversity and dominance in the three regions of Malawi. Ten spoligotypes were revealed and spoligotype SB0131 was dominant in Malawi.

Genotype diversity of *M. bovis* in Malawi: The unique spoligotypes recovered from the northern and southern regions were further differentiated into 16 distinct 'genotypes' (8 singletons and 8 clusters) by a 26 loci MIRU-VNTR typing. Combined with data from the central region (Kapalamula et al., 2021), high genetic diversity was observed in the central region (0.980) followed by the southern region (0.971) and the northern region (0.933). Using the combined analysis of Spoligo- and MIRU-VNTR typing, the 84 isolates (60 from a previous study (Kapalamula et al., 2021) and 24 from this study) revealed 56 genotypes (39 singletons and 17 clusters) with a

discrimination index of 0.985 and clustering rate of 33% (Fig. 2). The largest cluster comprised of seven isolates, followed by four isolates, three isolates and two isolates (Fig. 2).

Fig. 2: A UPGMA phylogenetic tree showing the relationships of spoligotypes from cattle slaughtered in major regions of Malawi. The tree is constructed based on a combined spoligotyping and a 26 loci MIRU-VNTR analysis.

The transmission rates of *M. bovis* within the three regions of Malawi were estimated using the parameters from empirical molecular data (Table 1) using formulae 1 & 2. We observed low RTI and TMI in all regions (Table 1)

Table 1: Attributes used for the comparative analysis for the calculation of recent transmission index and transmission mutation index of *M. bovis* within the three regions in Malawi

	Region		
Attributes	**Southern**	**Central**	**Northern**
Number of isolates (n)	14	63	10
Number of unique genotypes (g)	11	43	7
Number of clusters and size	3 (2,2,2)	11(2,2,2,2,2,2,2,3,3,4,7)	3 (2,2,2)
Single locus variation genotypes (v_1)	10	38	3
RTI	0.231	0.3226	0.3333
TMI	0.0035	0.0041	0.0054

Contextualizing the diversity of *M. bovis* isolates between Malawian isolates and those from neighboring countries (Southern Africa)

Using 5-MIRU VNTR loci, we constructed phylogenetic median networks based on a comprehensive dataset of 481 isolates from the literature. This analysis aimed to elucidate hypothetical linkages and evolutionary relationships among isolates in Southern Africa. The results indicate a significant genetic relatedness among isolates from the Southern African region (Fig. 3A). Specifically, Malawian isolates appear to share a closer genetic affinity with Zambian and

Mozambican isolates, as evidenced by their common spoligotypes SB0131 and SB0961 (Fig. 3A). The clustering rate for this analysis was determined to be 90.64% (Table 2).

Furthermore, an additional analysis was conducted to evaluate as many loci as possible, aiming to enhance the study's resolution and facilitate the detection of hidden relationships. It was discovered that a ten-loci approach was prevalent among the southern African isolates, leading to the establishment of a sample population of 259 isolates. Phylogenetic median networks were constructed to elucidate the hypothetical linkages and evolutionary relationships among these isolates, and these results were compared with the findings from the 5-MIRU-VNTR loci analysis. Malawian isolates were closely related to Zambian and Mozambican isolates sharing SB0131 spoligotype (Fig. 3B). The 10-MIRU VNTR loci showed high allelic diversity with the clustering rate was 68.26% (Table 2). Furthermore, eight loci (QUB11a, ETR-B, MIRU16, MIRU26, QUB11b, ETR-A, MIRU10, ETR-C) seem suitable for the Southern African context offering the potential to optimize costs in future research endeavors (Table 2).

Fig 3: Phylogenic trees showing strains from different countries constructed using unweighted pair group method with arithmetic mean (UPGMA) based on (A) 5 MIRU-VNTR loci and (B) 10 MIRU-VNTR loci. The key shows the SB numbers and country where the strains were isolated from illustrated by different colors. The trees were constructed by MIRU VNTR Plus online software (https://www.miru-vntrplus.org/) and visualized with R-studio (https://posit.co/products/open-source/rstudio/)

Table 2: Allelic diversity of the 10 and 5 MIRU-VNTR loci analysis on the regional strains and their clustering rate.

	Allelic Diversity	
Loci	**10 MIRU-VNTR loci**	**5 MIRU-VNTR loci**
MIRU10	0.90	
ETR-B	0.83	0.75
MIRU26	0.79	
MIRU16	0.78	
ETR-A	0.76	0.64
QUB11b	0.74	
QUB11a	0.74	
ETR-C	0.63	0.70
MIRU04 (ETR-D)	0.49	0.47
MIRU31 (ETR-E)	0.47	0.41
Clustering rate (%)	**68.26**	**90.64**

Discussion

Since the major control and/or eradication technique of bTB in cattle, i.e., testing and slaughter of bTB-positive cattle, is financially constrained in most developing countries, e.g., Malawi, the application of population-based molecular markers of bTB is critical in developing alternative mitigating strategies. Such techniques leverage various host population-driven combinations of pathogen genotypes, clustering, diversity, and geographic distribution to predict contact activity, potential transmission events, and, most importantly, disease hotspots (Egbe et al., 2017). We report the genetic diversity and phylogenetic relationships of *M. bovis* isolated from cattle slaughtered at major regional abattoirs in Malawi.

The European 1 (Eu1) clonal complex was detected in the vast majority among the *M. bovis* isolates from Malawi. Eu1 clonal complex has been reported in Mozambican (Machado et al., 2018) and South African cattle (Michel et al., 2008) and identified the latter as the Eu1 clonal complex's transit source to Africa. The presence of the Eu1 complex in Malawian cattle suggests that these strains

have spread throughout the Southern African region. We discovered a small proportion of "BCG-like" spoligotype pattern strains with an intact spacer 11 and an absence of spacer 2 (Fig. 2). These strains appear to have evolved from the spoligotype SB0120 through the loss of spacer 3, in addition to spacers 3, 9, 16, and 39–43 (Ghebremariam et al., 2018). Particularly, spoligotype SB0120 demonstrates dominance among Zambian cattle and wildlife (Hang'ombe et al., 2012). Its prevalence extends across various regions in Africa and Europe (Ghebremariam et al., 2018) and has previously been categorized as European 3 (Eu3) complex (Almaw et al., 2021). Based on our findings, there is a plausible indication that the Eu3 complex is expanding within the African region, and it's conceivable that the introduction of these strains into Malawi originated from Zambia (Fig. 3A & B).

We observed a high genetic diversity of *M. bovis* circulating in Malawian cattle (Fig. 2). The drivers are most likely linked to the traditional animal production systems largely employed by local livestock farmers (FAO & NEPAD, 2005), involving unregulated animal movements in search of grazing pastures and watering spots. Such movements permit regular contact between domestic animals and/or wildlife from various areas, resulting in constant infection and the spread of diseases such as bTB. Pathogen genotype clustering can reveal the spatial and temporal patterns of hosts in bTB outbreaks or endemic settings (Egbe et al., 2017). The phylogenetic clustering infers an endemic situation as opposed to an outbreak (Fig. 2). This hypothesis is supported by a low CR (33%), which is also reflected by low RTI (0.231, 0.323, and 0.333) and a low TMI (0.0035, 0.0041, and 0.0054) for the southern, central, and northern regions, respectively.

In the Southern African evolutionary network constructed using the 5-MIRU-VNTR loci, a close genetic relationship was observed among Malawian, Zambian, Mozambican, and South African isolates (Fig. 3A). This pattern found further support and validation through the examination of the 10-MIRU-VNTR analysis (Fig. 3B). Importantly, the results of both analyses remained consistently aligned, pointing towards a common source or origin for these isolates. Pre-and post-colonial livestock trade between these countries might have contributed to the close relationships of the

isolates. Previous reports highlighted that to improve the dairy industry, Malawi imported exotic dairy breeds from South Africa and Zambia (Kapalamula et al., 2021). It is interesting to mention that SB0425, previously identified as an ancestral type (Loiseau et al., 2020), showed no close relationship with similar spoligotype profiles from Tanzania suggesting limited cattle movement between the two countries despite sharing borders. We understand that the number of sampled animals was less than the anticipated, this was due to logistical challenges. Therefore, we acknowledge that this may have a potential sampling bias effect. However, the discovery of ancestral strains from northern region (Malawi) suggests that the pathogen has been in circulation over a long period of time and that bTB is a common occurrence in this region.

While the total number of isolates in the two trees varied (Fig. 3A & B), there were marked differences in the clustering rates between the two MIRU-VNTR loci sets (Table 2). The analysis of 5-loci MIRU-VNTR resulted in a high clustering rate (90.64%), signifying limited discriminatory power and a tendency to group genetically similar isolates together, indicating lower resolution in distinguishing the isolates. On the other hand, the 10-loci MIRU-VNTR analysis yielded a lower clustering rate (68.26%), indicating stronger discriminatory power and a higher resolution in distinguishing genetic differences among isolates, resulting in fewer strains clustering together. These findings underscore the importance of selecting the most suitable loci for specific research objectives and demonstrate the impact of sample size on the observed clustering rates. While the congruity of outcomes between the 5- and 10-MIRU-VNTR loci analyses underscores the robustness of our findings, it is worth noting that a more comprehensive understanding of transmission dynamics could potentially be attained through high-throughput approaches such as whole genome sequencing. This progressive approach holds the potential to furnish a more holistic perspective on the intricate patterns of evolution and transmission.

In conclusion, this study discovered a high genetic diversity of *M. bovis* circulating in Malawian cattle and possible transmission links. Further, Malawian isolates are phylogenetic related to isolates

from neighboring countries except from Tanzania suggesting possible transmission linkages. This calls for alternative approaches to support current mitigating strategies.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical statement

The study obtained ethical approval (Protocol Number 19/09/2398) for sampling cattle from major abattoirs from the National Health Science Research Committee (NHSRC), Ministry of Health, and the Department of Animal Health and Livestock Development (DAHLD), Lilongwe, Malawi.

Authors' contributions

TFK, CN, and YS contributed to the conception and design of the study, TFK, JYC, LB, DS, and HMC contributed to the acquisition of data, TFK, JYC, MLA, JT, PB, SVG, J Thapa, CN, and YS contributed to the analysis and interpretation of data, TFK, JYC, CN, YS contributed to the drafting the article, All authors contributed to the revising it critically for important intellectual content and finally approved the version to be submitted.

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Declaration of Conflict of Interest:

All authors have no conflict of interest.

Point-to-point responses to Reviewer #1's comments

Comment 1: I would like to thank the authors for taking into account my comments and going back to the data to further analyse the samples with the 10 MIRU VNTR approach. I will add comments to the responses given by the authors in the following lines:

Line 253 and 254: "Furthermore, this hypothesis gained support and validation through the examination of the 10-MIRU-VNTR analysis (Fig. 4)."

I find that the direct comparison between both analyses is very difficult given that the color codes and tree structures are completely different. I think the readers would appreciate a little more cohesion between the two figures, maybe even including as two subfigures within the same figure. I would also like to suggest that the authors use the same type of tree and the same color codes. I think that a cladogram structure as in figure 4 would be better suited, as the genetic relationships are resolved much easier than in figure 3. In addition, I would like to suggest that the authors give a little more information regarding the similarities and differences between trees and combinations of alleles, not only a brief overview.

Response: We have revised the manuscript as suggested, we have changed the Fig 3 and combined with Fig 4 to form Fig 3A & B with revised figure legends. Further we have added information regarding the similarities and differences between trees and combinations of alleles (Lines 196-202, 210-213 and 277-290). Please find them with read letters. Further, we have added a column for allelic diversity for 5 MIRU-VNTR loci (see Table 2 with red letters)

Comment 2: Line 245: Particularly, the strain SB0120 demonstrates dominance among Zambian cattle and wildlife. I believe that the term strain is not adequate here, since there may be multiple strains within the same spoligotype. The same applies for SB0425 (line 278). Please correct.

Response: We have corrected accordingly. Please find them at lines 245 and 270 with red letters, respectively

Comment 3: What criteria was used to establish the genetic clades?

Response: The criteria was based on clustering of isolates.

Comment 4: What was the method used for the definition of cluster? This should be indicated in the text.

Response: We added this L112-114. Please find it with read letters.

Supplementary table 1: Distribution of spoligotypes of *M. bovis* recovered from cattle slaughtered in the three regions in Malawi

	Spoligotype number										
	SB0130	SB0131	SB0273	SB0274	SB0425	SB0961	SB1031	SB1056	SB2466	SB2733	Total
Southern region	1	6	5	0	0	1	0	0	0	1	14
Central region*	0	35	9	1	8	3	3	2	2	0	63
Northen region	0	2	2	0	5	1	0	0	0	0	10
Total	1	43	16	1	13	5	3	2	2	1	87
Frequency	1.149	49.425	18.391	1.149	14.943	5.747	3.448	2.299	2.299	1.149	100

*Data from our previous study (Kapalamula et al 2021)