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Phylogenomics of *Brucella abortus* isolated from African Buffalo in Kruger National Park: New perspectives on wildlife-cattle disease dynamics

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ABSTRACT

In South Africa, *Brucella abortus* biovar 1 is the primary cause of bovine brucellosis, significantly impacting cattle production and trade. Serological studies have revealed brucellosis in African wildlife, complicating control efforts due to limited epidemiological data. In 1977, *B. abortus* biovar 1 was isolated from an African buffalo fetus in Kruger National Park (KNP), raising speculation that buffalo may serve as reservoir hosts. This study investigated *Brucella* spp. in free-ranging buffalo in KNP using serological, molecular, and bacteriological methods. *Brucella abortus* bv 1 was isolated from lymph nodes and spleens of three sub-adult buffalo in 2022, marking the first documented recurrence in 50 years. Phylogenomic analyses revealed connections between buffalo isolates and cattle strains from South Africa and South America, suggesting spillover and shared origins from Europe. Further genomic and epidemiological surveillance is required to clarify the role of buffalo as reservoir hosts for brucellosis.

1. Introduction

Brucellosis is caused by Gram-negative bacteria belonging to the genus *Brucella*, family *Brucellaceae*. *Brucella* species are facultative intracellular pathogens that target monocytes and macrophages of terrestrial and marine animals, and humans (WOAH, 2024). *Brucella abortus* is known to infect bovids and is historically reported in domestic cattle (*Bos taurus*), water buffalo (*Bubalus bubalis*) (Guarino et al., 2001), African buffalo (*Syncerus caffer*) (Gradwell et al., 1977), elk (*Cervus elaphus*) (Etter and Drew, 2006) and American bison (*Bison bison*) (Williams et al., 1993). *Brucella abortus* is the most disseminated *Brucella* species worldwide and, along with *B. melitensis*, represents the primary cause of brucellosis in both animals and human (Janke et al., 2023).

In South Africa, *B. abortus* bv 1 is predominantly isolated from cattle (90 %) (Matle et al., 2021) and is endemic with varying prevalence in the provinces of this country (Kolo et al., 2021). In wildlife, surveys have revealed up to 13.7 % seropositivity over a 50-year period in African buffalo from the Kruger National Park (KNP) (Simpson et al., 2021). Antibodies against *Brucella* spp. were also detected in 6/51 hippopotamus (*Hippopotamus amphibius*), 1/120 impala (*Aepyceros melampus*) and 1/1 waterbuck (*Kobus ellipsiprymnus*) from KNP by serum agglutination tests (Vos and Van Niekerk, 1969). *Brucella abortus* bv 1 has been isolated from antelope and unspecified wildlife (Matle et al., 2021) including African buffalo in 1977 in KNP (Gradwell et al., 1977), waterbuck and eland (*Taurotragus oryx*) (Kaliner et al., 1973).

The *B. abortus* genome of approximately 3.3 Mb is highly monomorphic, with intraspecies average nucleotide identities (ANI) greater than 99 % (Jumas-Bilak et al., 1998). Variable number of tandem

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repeats (VNTR) are present at various conserved loci in the *Brucella* genome and proved to be an important source of intraspecies polymorphism. The associated multiple locus VNTR analysis (MLVA) method is a valuable tool to cluster *Brucella* isolates from different host species, as well as for epidemiological trace-back studies (Vergnaud et al., 2018). The multi-locus sequence typing (MLST) is an alternative to MLVA relying on sequence analysis of selected genes rather than VNTR copy numbers estimation. It is currently less expensive to sequence the whole genome and to deduce the core genome MLST (cgMLST) or whole genome MLST (wgMLST) *in silico*. The most common methods for comparing whole genomes include the gene-by-gene approach or SNP-based typing (wgSNP) with or without a reference strain sequence (Uelze et al., 2020). Given the limited information and knowledge about *Brucella* species and transmission amongst host species in South Africa, particularly between livestock and wildlife, our aim was to isolate and characterize *Brucella* spp. infections in free-ranging African buffalo in KNP. To achieve this, we employed a comprehensive epidemiological and sub-typing approach using MLVA, leveraging the extensive global database available for *B. abortus* to detect potential transmission between wildlife and livestock. Additionally, we utilized whole genome sequencing of the African buffalo *B. abortus* bv 1 isolates from KNP to perform cgMLST and wgSNP for improved resolution and robust phylogeny, investigating the epidemiology by comparing these data with the global and available South African bovine *B. abortus* genomes.

2. Materials and methods

The study design focused on *Brucella* spp. in free-ranging African buffalo (*Syncerus caffer*) collected from 22 February to 5 September 2022 in KNP using serological, molecular and bacteriological methods. *Brucella* isolated from buffalo were characterized using MLVA with sixteen loci (MLVA-16), cgMLST and wgSNPs analysis. The buffalo *Brucella* MLVA data was compared with *B. abortus* isolated from cattle during 1994–2006 at the Agriculture Research Council -Onderstepoort Veterinary Research (ARC-OVR) and global MLVA data. Similarly, the buffalo *Brucella* cgMLST and wgSNPs data were compared with global WGS data.

2.1. Brucella investigation in African buffalo in Kruger National Park

KNP is located in the Limpopo and Mpumalanga provinces of South Africa. It is regarded as one of the largest and most important National Parks in Africa, hosting 148 wild mammal species in a 19,485 km^2 fenced conservation area. KNP is considered an endemic area for brucellosis, largely due to the persistence of *Brucella* spp. in African buffaloes consisting of a population of 48 000 (www.sanparks.org).

2.2. Sample collection

Serum, liver, spleen and lymph nodes samples were opportunistically collected. Animals were harvested for sustainable utilization as part of standard park procedures. An amount of one cm^3 was excised from each organ using sterile blades and collected in specimen containers separately. Tonsils, sub-mandibular, retro-pharyngeal, sub-mammary and inguinal lymph nodes were pooled together in the same specimen bottle. Samples were kept in a cooler box in the field until storage in PBS at −20°C in access-controlled freezers. Frozen organ samples from buffaloes that tested positive in series to Rose Bengal test (Onderstepoort Biological Products, OBP, Pretoria, South Africa) and indirect ELISA (ID-VET, Grabels, France) were eligible for culture. Macroscopic inspection was also conducted and recorded.

2.3. Inoculation of organs on Farrell's media

Brucella selective supplement was obtained from ThermoFisher Scientific (Waltham, Massachusetts, USA) to prepare Farrell's media. The antibiotic mix was prepared according to the manufacturer's instructions. Frozen tissues were thawed slowly at fridge temperature (4 °C) and inoculated on selective media. Plates were incubated at 37 °C and 5 % CO_2 for 7–14 days and examined daily. Suspect colonies were investigated using catalase reaction (degradation of hydrogen peroxide) to identify strict and facultative aerobes (WOAH, 2024), Gram and Stamp's modified Ziehl Nielssen (MZN) staining (WOAH, 2024), and molecular screening using the conventional 16–23S ribosomal DNA internal transcribed spacer region (ITS) PCR (Keid et al., 2007). Colonies confirmed as *Brucella* spp. were further identified using AMOS-PCR (Bricker and Halling, 1994) and Bruce-Ladder PCR (López-Goñi et al., 2008) before sending for biotyping to the ARC-OVR bacteriology laboratory.

2.4. DNA extraction, quantification and quality check

For subculture and PCR screening purposes, DNA was extracted from suspect *Brucella* spp. isolates using a crude-boiling method. Briefly, the colony of interest was dissolved in 100 µl of sterile distilled water and incubated at 95 °C for 15 min and then used as template in the PCR (see Molecular screening).

For molecular subtyping (MLVA and WGS), DNA was extracted from each culture using the PureLink genomic DNA extraction kit (Thermo-Fisher Scientific, Waltham, Massachusetts, USA). Concentration of extracted DNA was assessed with Qubit® 4.0 Fluorometer (Thermo-Fisher Scientific, Waltham, Massachusetts, USA). Quality check was performed with a NanoDrop, ensuring that the 260/280 ratio fell in between a 1.8–2.0 range.

2.5. Molecular screening

Crude-boiled extracts of suspect cultures/subcultures were first screened using *Brucella* genus-specific 16–23S ribosomal DNA ITS-PCR as described by Keid et al (Keid et al., 2007). *Brucella abortus*, *B. melitensis*, *B. ovis* and *B. suis* were identified using a multiplex AMOS PCR assay as previously described (Bricker and Halling, 1994). If any of the species-specific and size-specific amplicon was amplified, the species-specific forward primer was used at 0.4 µM in singleplex PCR with 0.4 µM reverse primer to verify the *Brucella* species amplicon. Bruce-Ladder PCR was finally used to confirm *Brucella* species (López-Goñi et al., 2008).

For all PCR reactions, double distilled water was used as negative control, while the *B. abortus* RB51, *B. melitensis* Rev.1 and *B. ovis* from a clinical sample identified by the bacteriology laboratory at the Faculty of Science, University of Pretoria, South Africa were used as positive controls.

2.6. MLVA

Brucella isolates from African Buffalo in KNP were analyzed using MLVA and compared with 115 *Brucella* strains isolated at ARC-OVR from cattle from 1994 and 2006 in South Africa and other strains retrieved from Liu et al. (2023). (Supplementary material S1) to evaluate their relationships. The *B. abortus* from cattle and buffalo from this study were characterized at ARC-OVR using bacteriological methods and identified using biotyping as indicated previously (WOAH, 2024).

MLVA-11 and MLVA-16 were performed as previously described (Ferreira et al., 2012). Genotype was scored by visual analysis of the gel images. Band size estimates were converted to repeat units following the published allele calling table (Supplementary Table 2 in Vergnaud et al. (2018)). MLVA data were analysed as a character data set within Bio-Numerics software (version 8.1) (Applied Maths, Sint-Martens-Latem, Belgium). Clustering analysis was performed using the Minimum Spanning Tree method or using the Manhattan distance coefficient and the Bio-Neighbor Joining tree algorithm with permutation resampling as implemented in BioNumerics.

2.7. Whole genome sequencing

Isolates were sequenced with the Illumina NextSeq 2000 platform (Illumina, Inc; San Diego, California, US) with 150 bp paired-end chemistry using a P1 flow cell type. The genome libraries were prepared with the Nextera XT Library Prep kit (Illumina, Inc; San Diego, California, US), according to the manufacturer's instructions. Isolates were sequenced with Illumina's recommended dual index barcoding protocol. Raw reads were imported into the Genpat system (https://genpat.izs.it), which is the bioinformatic web application developed by the National Reference Center for Genomic Sequences of Microbial Pathogens at IZSAM. On Genpat, sequences underwent an initial pre-processing analyses including trimming with trimmomatic (Bolger et al., 2014) and quality check with FastQC (https://github.com/s-andrews/FastQC), followed by *de novo* assembly with Shovill (https://github.com/tseemann/shovill), a pipeline for assembly of bacterial isolate genomes from Illumina paired-end reads. Taxonomic identification was done using Kraken (Wood and Salzberg, 2014), which is a sequence classifier that queries the database of k-mers for exact-matches. Sequences were deposited in GenBank under BioProject accession number PRJNA1139423.

2.8. cgMLST

MLST profiles were identified for the *Brucella* isolates using the MLST typing tool in the Microbial Genomics Module of CLC genomics workbench version 23.0.5 (Qiagen Digital Insights, Redwood City, California, USA) and compared with the latest MLST scheme publicly available at PubMLST (https://pubmlst.org/bigsdb?db=pubmlst_brucella_seqdef&page=schemeInfo&scheme_id=3). A total of 532 *B. abortus* global isolates were included in the phylogenetic analysis (Supplementary material S2). The allelic profiles were compared with KNP buffalo isolates, and the resultant pairwise distances were used to generate a minimum spanning tree (MST) as part of the MLST typing tool.

2.9. wgSNPs

wgSNP analysis was run independently by two participants as a consistency check. The genome-wide SNPs on Genpat were predicted without reference genome using the kSNP (v.3) tool (Gardner et al., 2015) with k-mer value of 21. Based on the distributions of wgSNPs among 472 genomes listed in Supplementary material S3, a phylogenetic tree was constructed using maximum likelihood method under kSNP3 tool. The phylogenetic tree was visualized and edited using MegaX software (version 10.2.6).

3. Results

Pure *Brucella* spp. cultures were obtained from the tissues of three sub-adult female buffaloes that tested seropositive using RBT and iELISA. Tissues of origin were lymph nodes (SAN63 *alias* 2023.TE.26531.1.1 and SAN94 *alias* 2023.TE.26531.1.3) and spleen (SAN68 *alias* 2023.TE.26531.1.2). All buffaloes were clinically healthy and no lesions compatible with *Brucella* spp. were detected during post-mortem examination. Isolated cultures were catalase positive, Gram negative and acid-fast coccobacilli 0.4 × 0.8 μm in size (Supplementary figure S1). Samples were first confirmed as *Brucella* spp. with *Brucella* specific ITS-PCR (Fig. 1A), then *B. abortus* with AMOS-PCR (Fig. 1B) and Bruce-ladder PCR (Fig. 1C), and finally characterized as *B. abortus* biovar 1 by ARC-OVI.

The three African buffalo *B. abortus* biovar 1 isolates shared the same MLVA-11 allelic profile. SAN63 and SAN68 had identical MLVA-16 genotype while SAN94 differed from them on one VNTR namely the highly variable bruce07 locus (five repeat units in SAN94 vs four in SAN63 and SAN68). The MLVA-16 genotypes of SAN63 and SAN68 from the African buffalo were identical or similar (differing at one VNTR) to BRUa0044, 45, 46, 48 and 52 from cattle in Brazil (Minharro et al., 2013), Bru0819–0820, 0835, 0833, 0837, 0839, 0872 and 0879 from cattle in South Africa (this study), Zimbabwean strain ZW053 and strains from India, Brazil and Portugal (Supplementary material S1) (Ledwaba et al., 2021). SAN94 MLVA-16 genotype was similar to South African cattle isolates (Bru0834, 0874, 0875 ad 0896) from Brazil (BRUa0121–122, a0128) and Portugal isolates (BRUa0861, a0866 (Fig. 2).

High-throughput short-read sequencing yielded an average of 3,486,911 raw reads per isolate (min 1664,496, max 5360,388), leading to a mean coverage of 137X (min 65, max 211). The African buffaloes *B. abortus* biovar 1 strains sequenced in the present study were all classified as *B. abortus* by the Kraken taxonomic classifier.

Core genome MLST analysis revealed new cgSTs for the three SA buffalo isolates (Fig. S2). SAN94 differed more consistently (*i.e.* by 18 allelic differences) from the other two (Fig. 3). Buffalo isolates clustered close to Argentinian (SAMN01047740 to SAMN01047742) and Portuguese (SAMN00839699) isolates. In particular, Argentinian human isolate SAMN01047740 is the closest sequence type with 27 allelic differences from SAN68 (SAMN42831030), 31 from SAN63 (SAMN42831029) and 45 from SAN94 (SAMN42831031). Conversely, ten *B. abortus* South African cattle isolates identified by Ledwaba et al. (2021). clustered separately from buffalo strains, with at least 311 allelic differences, and showed close genetic similarity to strains from Zimbabwe (SAMN00811173), Mozambique (SAMN00811169), India (SAMN09273432, SAMN09273438, SAMN15438790) and UK (including Northern Ireland) isolates (Fig. 3).

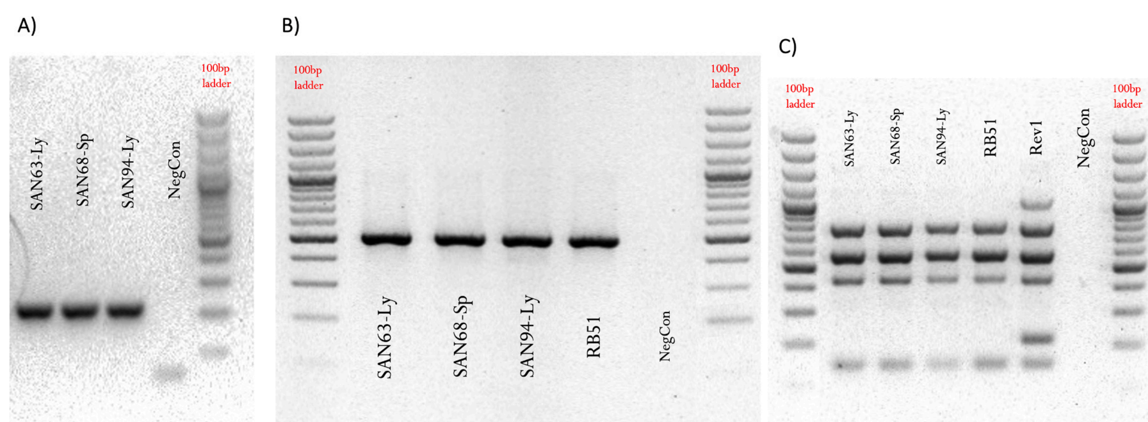

Fig. 1. Molecular confirmation of *Brucella abortus* cultures isolated from African buffalo from Kruger National Park tissues with A) 16–23S ribosomal DNA ITS-PCR of 218 bp *Brucella* genus-specific amplicon, B) Singleplex *B. abortus* AMOS-PCR of 498 bp amplicon and C) Bruce-Ladder PCR with *B. abortus* profile consisting of 450, 587 and 794 bp products with *B. melitensis* rev 1 profile consisting of 218, 450, 587, 794 and 1071 bp.

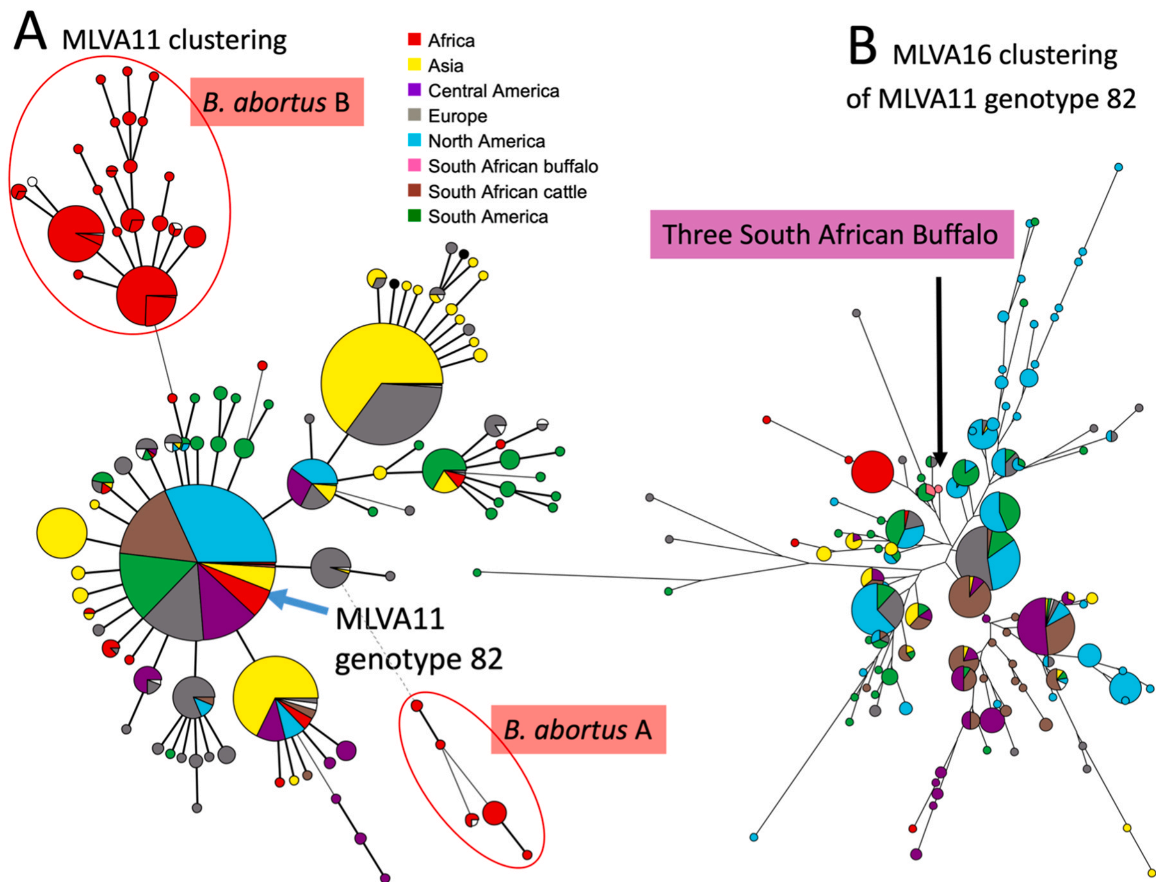

Fig. 2. Clustering analysis of MLVA data. Nodes are colored according to geographic origin (continent) as indicated in the legend. Node size is proportional to the number of strains sharing an identical genotype. Part A: Minimum spanning tree (MST) showing MLVA11 clustering of 2195 *B. abortus* isolates. The three strains recovered from South African buffaloes belong to the most represented MLVA11 genotype (arrowed, MLVA11 genotype 82 in MLVAbank), present worldwide. Part B: clustering based on MLVA16 data of the 564 strains belonging to MLVA11 genotype 82. Clustering is based on the Manhattan distance coefficient and the BioNeighbor-Joining algorithm. The position of the three strains isolated from South African buffaloes is indicated. Closest neighbors are strains from South America and Europe (Portugal, Italy). Italian strain 8979 was isolated from a buffalo (Garofolo et al., 2013). Strains LNIV-441Ba1–07 and LNIV-442-Ba1–07 were isolated from cattle in the St Miguel Island, Azores (Portugal). Strains 42, 43, 44, 46, 47 were isolated from cattle in Governador Valadares, Minas Gerais, Brazil (Minharro et al., 2013).

Based on the phylogenetic tree built with wgSNPs of 490 *B. abortus* isolates (Fig. 4), four major lineages could be distinguished as described by Janke et al (Janke et al., 2023).: clades A and B containing African isolates and defining the deepest nodes, clade C with Euro-Asiatic isolates, and clade D. The branches defining the deepest nodes within clade D are constituted by a few strains isolated in Western Europe (Poland, Italy, France, Spain). The rest of clade D in Fig. 4 is dominated by American (from North, Central, South Americas) as well as UK isolates. In this study, clade D is designated as comprising the "New World" isolates, based on the hypothesis that *B. abortus* originated in the Middle East following the emergence of multi-species livestock economies (L'Hôte et al., 2024) or possibly Africa as argued by Janke et al., (Janke et al., 2023) (based on the geographic distribution of clades A and B), spread into Europe (clade D), and eventually reached the Americas ("New World" clade D) through global trade.

Fig. S3 shows a focus on "New World" clade D. In particular, the majority of strains were isolated in the USA. As is the rule, this view is to some extent distorted by sampling biases. However, some remarkable features of the phylogeny can be noticed. The majority of European strains assigned to "New World" clade D originate from the UK. In addition, UK strains are dispersed within the phylogeny. Another remarkable feature of the phylogeny is that Central (Costa Rica) and South American lineages appear to result from a limited number of recent introductions (19th-20th century). Whereas the Costa Rica lineages appear embedded within North American branches, the Brazilian and Argentinian clade D lineages are associated with branches from the UK and Portugal. Dating points compiled from different reports (Janke et al., 2023) are indicated, suggesting that Brazilian-Argentinian clade D lineages were introduced in the second part of the nineteenth century or early 20th, with UK as the currently most likely source.

The ten and the three South African strains isolated from cattle and African buffalo from KNP respectively belong to two clearly distinct lineages (Fig. S2 and S3).

The African buffalo strains define one branch within a polytomy of four branches (Fig. 5). The other three branches are each defined by a unique strain isolated in Argentina. More ancient nodes in the immediate vicinity are defined by one strain from Portugal, then strains from UK and Egypt, then UK-Iran and Brazil-Argentina. Interestingly, although the Brazilian isolates were collected all over Brazil and represent currently circulating strains, all known clade D strains from Brazil are located in this particular sublineage.

The ten *B. abortus* strains isolated from South African cattle (Ledwaba et al., 2021) and nearest neighbors constitute a three-branches polytomy (Fig. 6). One of the branches is defined by strains from India. A second one comprises five of the South African strains and one strain from Zimbabwe. The third branch contains the other five South African strains plus one strain from La Réunion (France) and one strain from Mozambique. The polytomy including the South African cattle strains is embedded within branches defined mostly by UK strains.

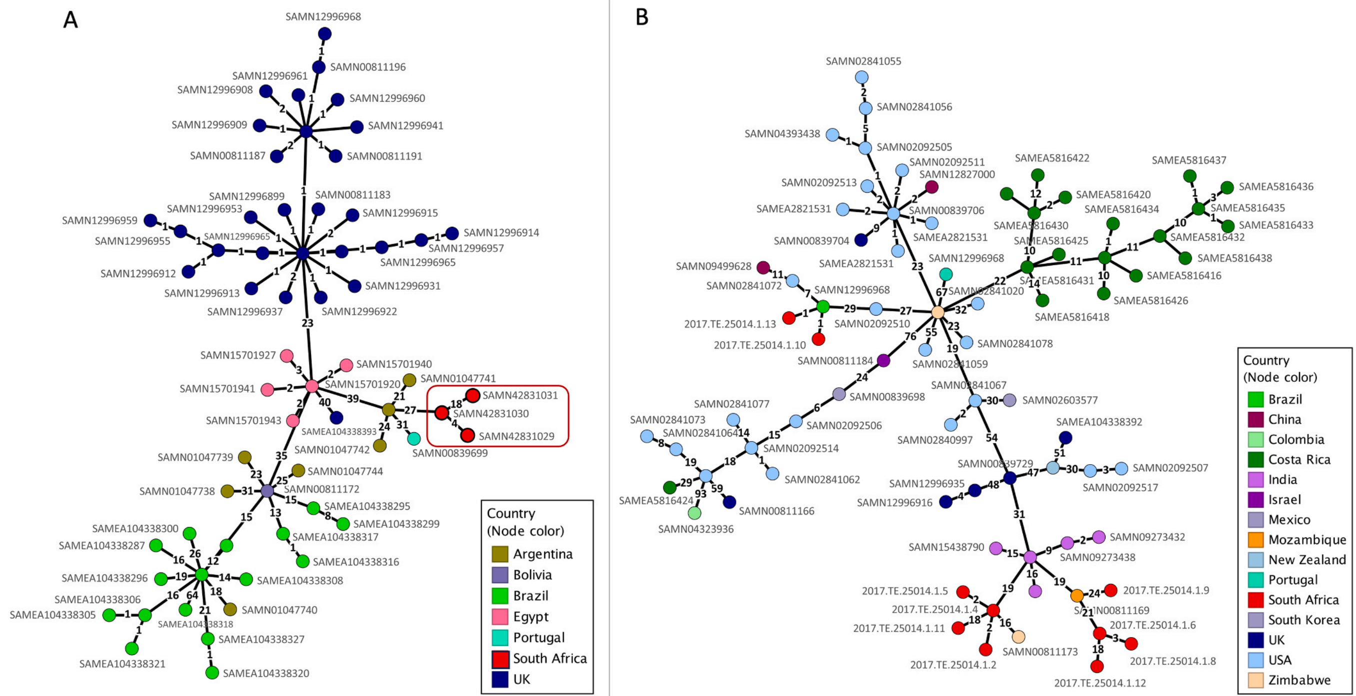

Fig. 3. Minimum spanning tree representing cgMLST results of: A) Kruger National Park (KNP) buffalo isolates (highlighted in red) and corresponding clade composed by South American (Argentinian, Brazilian and Bolivian), Portuguese, Egyptian and UK isolates; B) South African cattle isolates and closest strains from Zimbabwe, Mozambique, India, UK and US. Numbers on branches length indicate allelic distance. Colour legend provides stratification per country of isolation. Biosample accession numbers are displayed for each isolate/node. Complete cgMLST phylogeny is reported in Supplementary Material S2.

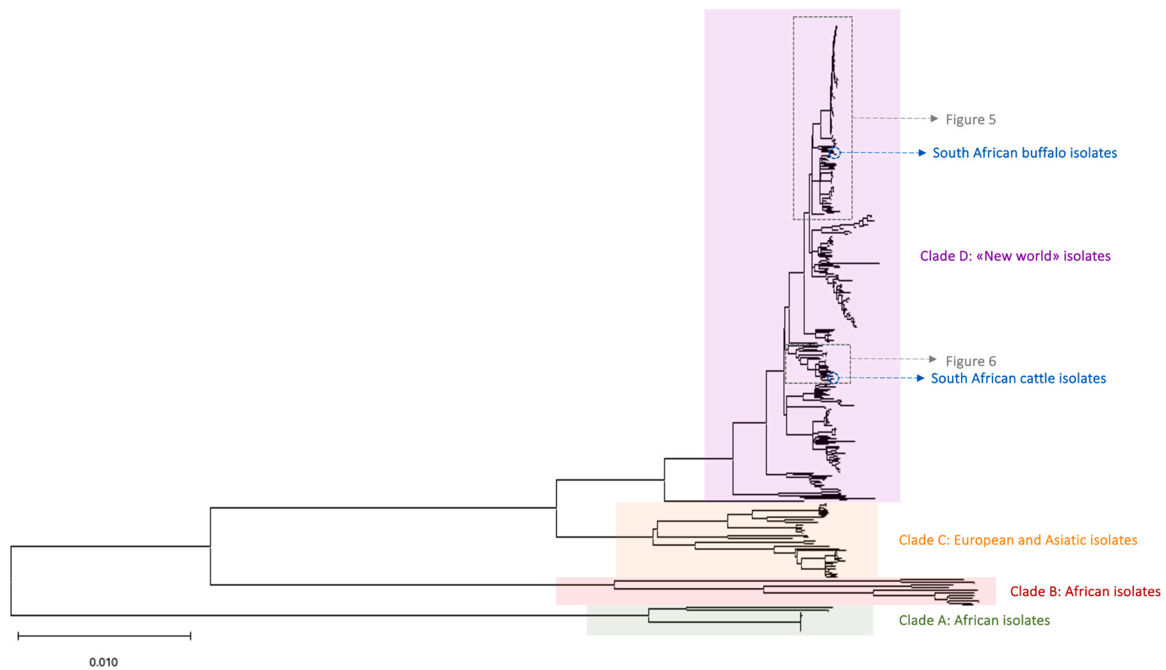

Fig. 4. Maximum Likelihood phylogenetic tree displaying evolutionary relationships of three Kruger National Park buffalo isolates with 472 *Brucella abortus* genomes deposited in Genbank. The four major clades (A, B, C, D) can be clearly observed. Branches lengths are scaled on nucleotide substitutions per site. Dashed grey lines indicate subtrees of interest. Dashed blue lines indicate the placement of South African isolates. Tree was rooted using *B. melitensis* biovar 1 strain 16 M (GCF_000740415) as outgroup.

4. Discussion

In this study, *B. abortus* biovar 1 was isolated from free-ranging African buffalo in KNP, South Africa. This finding, coupled with the historical isolation of *B. abortus* biovar 1 in the region (Gradwell et al., 1977), suggests the possibility that this biovar has been circulating in this species for more than 50 years. Unfortunately, since the historical strain was lost, it is not possible to evaluate the long-term persistence of

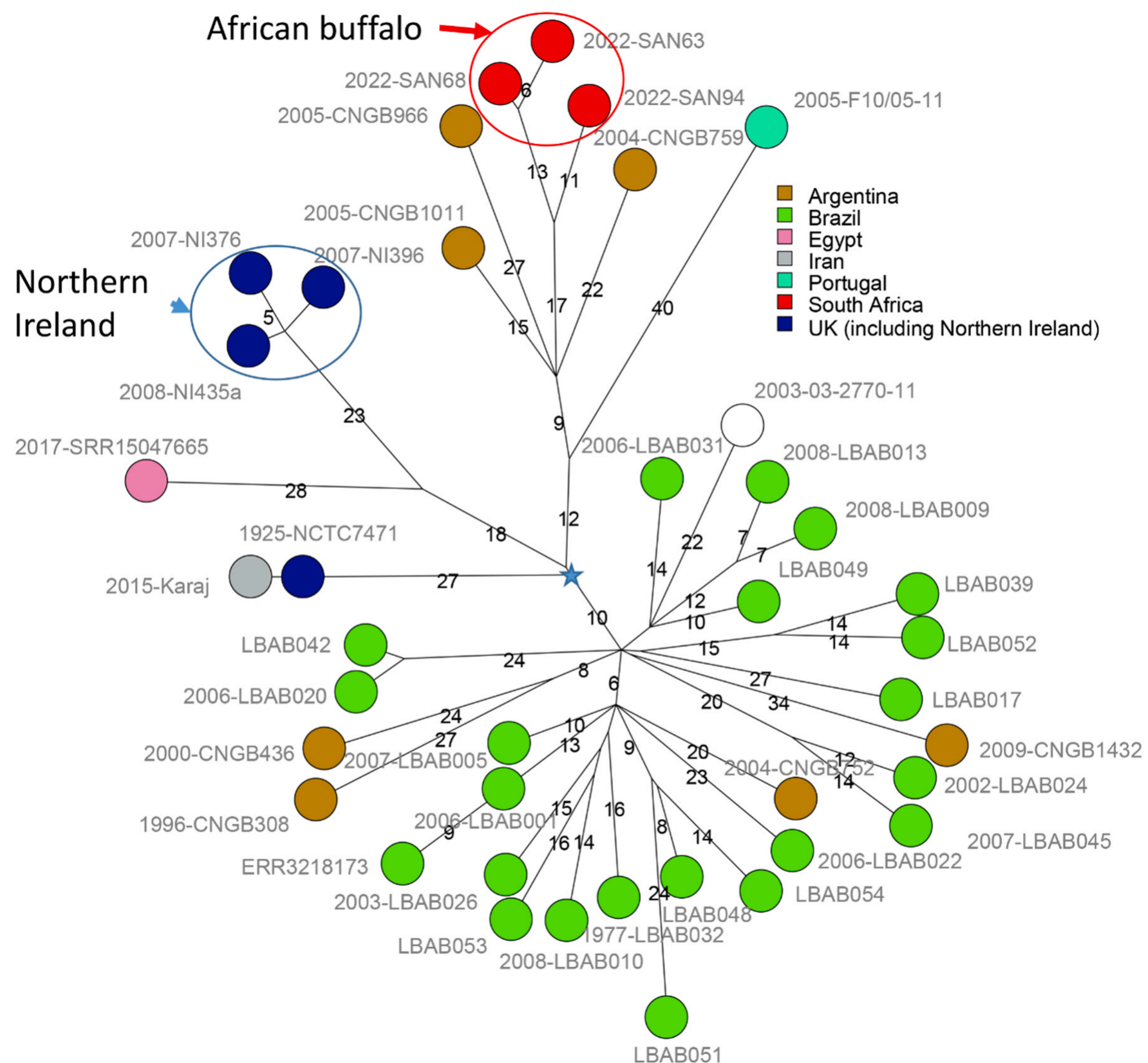

Fig. 5. Phylogeny of the three KNP buffalo *B. abortus* strains from South Africa and their closest neighbors. Maximum parsimony tree based on 816 SNPs called by mapping on *B. abortus* strain 9–941 genome accession GCA_000008145. The tree has no homoplasy. Nodes are colored according to country of origin and labelled with year of isolation when available and strain id. Branch lengths above four are indicated. The blue star indicates the position of the MRCA.

B. abortus biovar 1 within the KNP ecosystem, potentially maintained by African buffalo and possibly other animal hosts (Simpson et al., 2021).

Molecular sub-typing techniques (MLVA, cgMLST and kSNP) consistently revealed that *B. abortus* strains circulating in KNP buffalo are not closely related to the "African" clades A and B which define the most ancestral nodes within the *B. abortus* phylogeny. Unexpectedly, they clustered together with South American strains, within the relatively monomorphic clade D (Figs. 4 and 5). Clade D is considered the most recently evolved lineage, containing the largest number of *B. abortus* genomes from six continents but with little genetic diversity (Janke et al., 2023). The historical events that likely lie at the root of clade D are the global translocations of infected livestock facilitated by European colonization and global trade, which allowed isolates to spread between distant locations (Janke et al., 2023, Minharro et al., 2013, Ledwaba et al., 2021). According to Sluyter (2023), cattle (Criollo breed, *Bos taurus*) were introduced in Brazil and Argentina in the 16th century from Portugal, the Cape Verde Islands, and mainland Africa. More specifically, modern Criollo breeds show a significant contribution of the bovine mitochondrial subhaplogroup T1a, a matriline that is mostly found in Africa (Delsol et al., 2023). The end of the colonial period during the 19th century led to a significant expansion of cattle ranching and the closure of the open range, which allowed for the import and maintenance of European breeds. Starting in the mid-19th century, the introduction of British breeds such as Shorthorn, Aberdeen Angus, and Hereford began to dominate the Pampas, thriving and gradually replacing the native Criollo cattle (Sluyter, 2023). Interestingly, these breeds were also extensively imported also in South Africa, contributing to the creation of the Bonsmara, a South African breed developed in the 1960s using Afrikaner, Hereford, and Shorthorn genetics) (Gouws, 2023).

We thereby postulate that *B. abortus* spillover might have occurred in South American and, in parallel, South African cattle through import of infected cattle from UK, eventually infecting African buffaloes near cattle farms. Another plausible hypothesis is the direct introduction of infected cattle from Brazil or Argentina into South Africa. However, this scenario is not well-documented in the literature, which predominantly discusses the importation of frozen beef from these regions (DAFF, 2024), a practice that poses little to no risk for the transmission of *B. abortus*.

On a separate note, ten *B. abortus* whole genomes obtained from South African cattle fall within clade D, but they cluster with a few strains from African countries (Mozambique and Zimbabwe), as well as strains from Eurasia (India and UK) (Ledwaba et al., 2021), with relevant genomic distance from South African buffalo isolates (Figs. 3 and

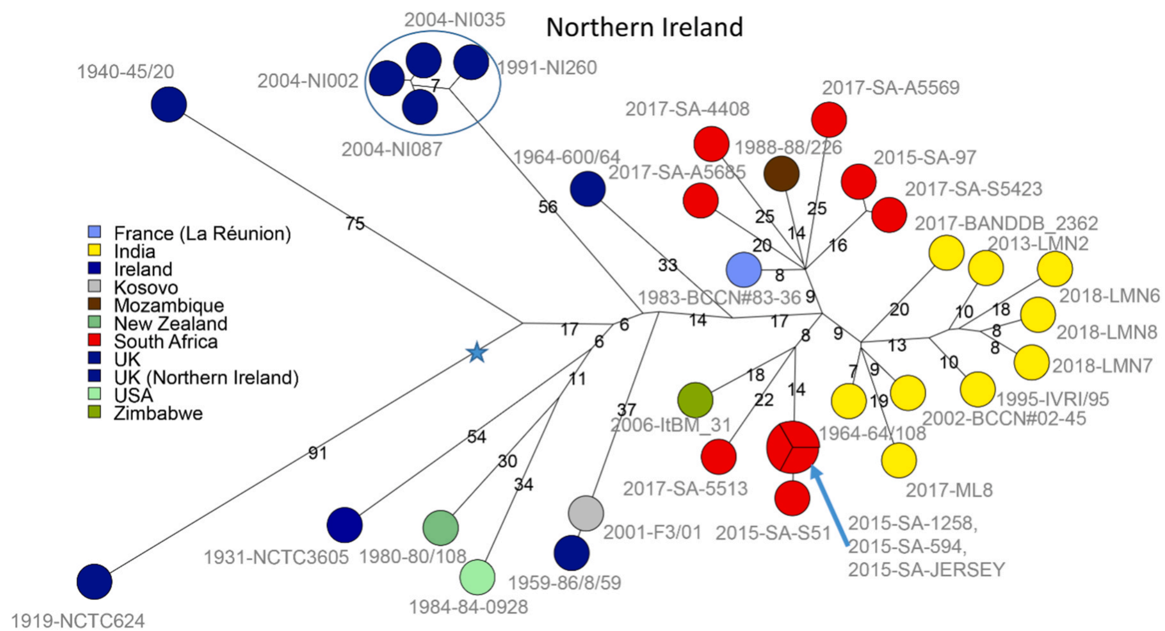

Fig. 6. wgSNP subtree displaying details of the clade composed by ten strains isolated from South African cattle and 24 closest neighbors. Maximum parsimony tree based on 818 SNPs called by mapping on *B. abortus* strain 9–941 genome accession GCA_000008145. The tree size is 825 (homoplasia 0.86 %). Nodes are colored according to country of origin and labelled with year of isolation and strain id. Branch lengths above four are indicated. The blue star indicates the position of the MRCA.

4). This suggests the possibility of an additional brucellosis transmission route across continents, facilitated by trade routes such as those established by the Dutch East India Company (Vereenigde Oost-Indische Compagnie) in the 17th century. The company used Cape Town in South Africa as a refreshment outpost, potentially allowing for the spread of the disease between Europe and India (Dye and La Croix, 2018). Additionally, South Africa's history as a British colony and the subsequent importation of European cattle breeds following the Anglo-Boer war and Rinderpest outbreaks may have further contributed to the introduction of different *B. abortus* strains (Niemand, 2013). European colonization and trade throughout Africa, coupled with migration patterns, could explain the diverse subclades observed within clade D in this study. The increasing global movement of people, animals, and goods, hence the greater global connectivity allows for widespread movement of contagious pathogens, as recently well-reminded by the COVID-19 and Monkeypox Public Health Emergencies of International Concern.

The long-term persistence of *B. abortus* biovar 1 in KNP buffaloes (Gradwell et al., 1977) together with the foreign ancestry revealed by WGS analysis of newly isolated strains from the same region and host (this study), and the presence of similar MLVA16 profiles with some South African cattle strains (Fig. 2), corroborates the hypothesis that *B. abortus* was introduced from livestock to KNP buffalo. This finding emphasizes that wildlife may not always be the origin of pathogen emergence or re-emergence, but it may also serve as the ultimate destination in the chain of infection thus constituting the susceptible host. As a consequence, wildlife disease monitoring is a valuable conservation tool for reserve managers, as it helps identify hidden conditions whose causes originate outside of park borders and may worsen the conservation status of already endangered wildlife species (Mazzamuto et al., 2022). Wildlife epidemiology and pathogen surveillance can also assist governments, veterinarians and stakeholders in directing decisions towards the true origin of disease burden or components of the reservoir. Instead of attempting to control *B. abortus* in wildlife, we suggest enhancing surveillance and control at the wildlife-livestock interface (Gortazar et al., 2015).

In this study, all three buffaloes appeared healthy and showed no visible lesions, consistent with a latent infection. This underscores the challenge of controlling brucellosis in wildlife. The role of other animal hosts in maintaining *Brucella* spp. remains understudied but should not be underestimated (Simpson et al., 2021). Wild ungulates such as kudu (*Tragelaphus strepsiceros*), wildebeest (*Connachaetes taurinus*) and warthog (*Phacocoerus africanus*) can evade the fences further complicating the containment of the disease (Hargreaves et al., 2004). Rather than focusing on controlling *B. abortus* in wildlife, we recommend prioritizing surveillance and control efforts in domestic animals at the wildlife-livestock interface.

Ethics statement

Study protocol was approved by Research and Animal Ethics at the University of Pretoria under reference number REC054–21. Section 20 of the Animal Disease Act, 1984 (ACT NO. 35 of 1984) to test and isolate *Brucella* in KNP was granted with reference 12/11/1/1955 (HP).

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CRediT authorship contribution statement

Massis Fabrizio De: Writing – review & editing, Validation. **Janowicz Anna:** Writing – review & editing, Validation, Formal analysis. **Ledwaba Maphuti Betty:** Writing – review & editing. **Wentzel Jeanette:** Writing – review & editing, Validation, Investigation. **De Klerk Lin-Mari:** Writing – review & editing, Investigation. **Sabeta Claude:** Writing – review & editing. **Vergnaud Gilles:** Writing – review & editing, Visualization, Validation, Software, Formal analysis, Data curation. **Godfroid Jacques:** Writing – review & editing, Validation, Investigation. **Cossu Carlo Andrea:** Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **van Heerden Henriette:** Writing – review & editing, Visualization, Validation,

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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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2025.110493.

Data Availability

Whole genome sequences were deposited in GenBank under BioProject accession number: PRJNA1139423.

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