Genomic epidemiology of *Mycobacterium bovis* infection in sympatric badger and cattle populations in Northern Ireland.

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Abstract

Background

Bovine tuberculosis (bTB) is a costly epidemiologically complex, multi-host, endemic disease. Lack of understanding of transmission dynamics may undermine eradication efforts. Pathogen whole genome sequencing improves epidemiological inferences, providing a means to determine the relative importance of inter- and intra- species host transmission for disease persistence. We sequenced an exceptional data set of 619 *Mycobacterium bovis* isolates from badgers and cattle in a 100km^2 bTB 'hotspot' in Northern Ireland. Historical molecular subtyping data permitted the targeting of an endemic pathogen lineage, whose long-term persistence provided a unique opportunity to study disease transmission dynamics in unparalleled detail. Additionally, to assess whether badger population genetic structure was associated with the spatial distribution of pathogen genetic diversity, we microsatellite genotyped hair samples from 769 badgers trapped in this area.

Results

Graph transmission tree methods and structured coalescent analyses indicated the majority of bacterial diversity was found in the local cattle population. Results pointed to transmission from cattle to badger being more common than badger to cattle. Furthermore, the presence of significant badger population genetic structure in the landscape was not associated with the spatial distribution of *M. bovis* genetic diversity, suggesting that badger-to-badger transmission may not be a key determinant of disease persistence.

Significance

Our data were consistent with badgers playing a smaller role in the maintenance of *M. bovis* infection in this study site, compared to cattle. Comparison to other areas suggests that *M. bovis* transmission dynamics are likely to be context dependent, and the role of wildlife difficult to generalise.

1. Introduction.

Mycobacterium bovis infection in cattle (*Bos taurus*) and badgers (*Meles meles*) is a persistent and costly problem for the farming industries and governments of the United Kingdom and Republic of Ireland (Allen et al. 2018). In Northern Ireland alone, the bovine tuberculosis (bTB) eradication scheme cost £44 million in 2017/2018 (Northern Ireland Audit Office, 2018). The complex epidemiology of the disease is well recognised, with the role of wildlife in transmitting infection to cattle acknowledged as an impediment to eradication (Godfray et al. 2018). A major knowledge gap for this disease has been, until recently, a detailed understanding of inter-host transmission dynamics and their relative importance (Kao et al. 2016).

Multi-host zoonotic infections of slowly-evolving pathogens, such as the members of the *M. tuberculosis* complex (MTBC), present significant challenges to researchers who wish to use molecular epidemiological methods to understand disease transmission dynamics (Biek et al. 2015). Previously, multi-locus variable number of tandem repeats analysis (MLVA) and spoligotyping were used to characterise spatio-temporal patterns in bTB epidemiology (Kamerbeek et al. 1997; Skuce et al. 2010; Milne et al. 2019; Skuce et al. 2020). These methods have demonstrated how *M. bovis* infections typically present as a series of geographically localised micro-epidemics (Skuce et al. 2010; Skuce et al. 2020). However, MLVA and spoligotype loci, whilst extremely useful in defining the home ranges of endemic infections (Trewby, 2016a; Milne et al. 2018), evolve at rates considerably slower than the inter-host transmission rate, thereby limiting their utility for contemporary disease outbreak investigations (Meehan et al. 2018).

Whole Genome Sequencing (WGS) technologies and associated phylogenetic analytical frameworks, have helped to reveal sources of infection and to improve surveillance and control for various pathogens (Harris et al. 2013; Mellman et al. 2011; Walker et al. 2013). These phylodynamic methods have been most effectively applied to fast-evolving viral pathogens, whose mutation rates can, with dense sampling, permit inference of fine scale disease dynamics, over short time intervals (Volz et al. 2009; Biek et al. 2015). While the latter degree of resolution may be unobtainable for slowly evolving bacterial pathogens, recently it has been shown that provided dense sampling is undertaken across a wide temporal window, much can be revealed about inter-host disease transmission dynamics of bTB (Biek et al. 2012; Crispell et al. 2017; Salvador et al. 2019, Crispell et al. 2019; Rossi et al. 2020a and 2020b).

Biek et al (2012) were the first to apply WGS and Bayesian phylogenetics to the *M. bovis* epi-system, focusing on an emerging endemic strain found in the east of Northern Ireland. In this proof of concept study, they demonstrated ongoing transmission between sympatric cattle and badgers at the farm scale. However, due to the small number of samples and the fact that these samples were not strictly contemporaneous, it was difficult to infer which inter-host transmission rates were most important. Subsequent work in New Zealand, applied to *M. bovis* isolates from cattle and brushtail possums (*Trichorus vulpecula*), revealed that the possum population was likely the major reservoir and the primary driver for infection in cattle (Crispell et al. 2017). However, the study also revealed that biases in sampling structure could affect inferences of the relative importance of transmission routes whenever discrete traits analyses were used. More recently, Salvador et al. (2019) used WGS and Bayesian phylogenetic methods to clarify the roles that cattle, white tailed deer (*Odocoileus virginianus*) and elk (*Cervus canadensis*) play in the epidemiology of an endemic *M. bovis* problem in Michigan, USA. Elk and cattle infections were observed to cluster in space, leading to concerns that the wide-ranging behaviour of elk could be a risk for disease spread across the landscape. However, phylogenetic data clarified that deer were the primary source of inter-species transmission in the

region. In the UK, a recent study from Woodchester Park in Gloucestershire has shown that badger and cattle intra-species transmission accounts for the majority of disease dissemination events (Crispell et al. 2019). However, Bayesian structured coalescent methods show that badger-to-cattle transmission occurred up to nine times more commonly than cattle-to-badger transmission in this dataset, further clarifying the role that wildlife can play in maintaining livestock infections in this epi-system (Crispell et al. 2019). In Ireland, phylogenetic methods were recently used to show that in County Wicklow, local deer populations harbour a diversity of *M. bovis* consistent with them being a source of infection for cattle (Crispell et al. 2020).

Pathogen spread across landscapes is recognised to be an inherently spatial process (Biek and Real, 2010), leading to distinct patterns in pathogen genetic structure. Similarly, free ranging wildlife hosts, exhibit partitioning of their own genetic variation across landscapes (Guerrero et al. 2018), for example, isolation by distance (IBD) (Wright, 1943). An appreciation of how these types of landscape-genetic phenomena intersect can help to inform epidemiological investigations in wildlife populations (Biek and Real, 2010). A key question within localised bTB micro-epidemics is whether significant badger population structure is observed over smaller geographic scales in Ireland, and if so, whether it explains any of the partitioning of *M. bovis* genetic variation in the landscape.

In this study we sought to better understand the interspecies transmission dynamics of *M. bovis* and the effect of badger population structure on the spatial partitioning of pathogen diversity. Compared to some previous studies, we undertook a systematic, sympatric sampling of both cattle and badgers. The study was conducted in a small 100 km^2 area of Northern Ireland (Figure 1A), using data from a recently completed (2014-2018) wildlife intervention. The 'test and vaccinate or remove' (TVR) selective culling protocol applied to the local badger population (Arnold et al. 2021; Menzies et al. 2021) provided an important opportunity to systematically sample sympatric cattle and badger populations for *M. bovis*, and to apply WGS, phylodynamic and population genetic methods to determine the roles that both cattle and badgers play in the local disease dynamics.

2. Methods.

2.1 Sampling of cattle and badgers.

The TVR zone (Figure 1A), was chosen because it has a high prevalence of cattle TB (24% confirmed bTB herd prevalence over two years [2011/2012]), and is embedded within County Down, which also has one of the highest average badger densities (3.88 badgers per km^2) in Northern Ireland – (Reid et al. 2012).

An initial survey was conducted in 2014 to establish sett locations (DAERA, 2018). Overnight cage trapping was used across all years between the months of May and October 2014-2018. Trapped badgers were anaesthetised, trap-side, hair sampled and tested for *M. bovis* infection using the dual path platform (DPP) serology test based on the StatPak method (Chambers et al. 2009). Tracheal aspirate was taken from all trapped badgers, whether DPP-positive or -negative and sent for bacteriological culture as described below. From 2015-2018, DPP positive badgers were humanely euthanized and DPP-negative badgers were vaccinated using injectable Bacillus Calmette Guerin (BCG), and released (Menzies et al. 2021). Culled badgers underwent post-mortem examination according to a standardised protocol (Courcier et al. 2020) and specified tissues were submitted for bacteriological culture.

All cattle in Northern Ireland are TB tested annually using the standardised (European Council, 1964) single intradermal comparable cervical tuberculin (SICCT) test (Abernethy et al. 2006). Specified tissue

from all SICCT positive bTB reactor cattle in the TVR region from 2014-2017 were harvested at the time of slaughter and submitted for bacteriological culture.

To provide extra temporal depth, a selection of historical isolates (n=243) from both hosts in the TVR zone were re-cultured from the AFBI *M. bovis* Northern Ireland wide, strain archive. The temporal window for all isolates runs from 1986 to 2017. All historical badger isolates were derived from road traffic accident (RTA) post-mortems in a surveillance scheme run by the Department of Agriculture, Environment and Rural Affairs in Northern Ireland (DAERA-NI) (Courcier et al. 2017). In addition, an area directly neighbouring the study zone has a distinct lineage of *M. bovis* present, four isolates of which were detected in the study zone. A random sample of historical isolates from this neighbouring area was collected to provide additional phylogenetic context.

2.2 *M. bovis* culture and genomic DNA extraction.

M. bovis isolates were initially cultured in the liquid BD BACTEC MGIT system, solid Stonebrinks and Löwenstein-Jensen media and single colonies were selected for sub-culture (Skuce et al. 2005). Isolates were heat-killed in a water bath at 80°C for 30 min. DNA was extracted using standard high salt/cationic detergent cetyl hexadeycl trimethyl ammonium bromide (CTAB) and solvent extraction protocols (Parish and Stoker, 2001; van Soolingen et al. 2002).

2.3 Spoligotyping and MLVA analysis.

M. bovis isolates were genotyped by spoligotyping (Kamerbeek et al., 1997) and 8 locus MLVA using previously described methods (Skuce et al., 2010). Authoritative names for spoligotype patterns were obtained from www.mbovis.org (Smith and Upton, 2012). MLVA profiles were named using a laboratory nomenclature (Skuce et al. 2005).

2.4 Genome sequencing and bioinformatic analyses.

Sequencing libraries were prepared using the Illumina Nextera XT method to produce inserts of approximately 500-600bp. One hundred samples were sequenced at AFBI using an Illumina MiSeq platform with Illumina V2 chemistry, producing paired-end reads of 250 bp. A further 100 samples were sequenced at the Glasgow Polyomics facility using an Illumina Miseq producing 2×300 bp paired end reads. All remaining samples were sequenced by Eurofins Scientific using an Illumina HiSeq producing 2 x250 bp paired end reads. For quality assurance (QA) purposes, comparison of sequencing performance across all three sites was undertaken at AFBI. Specifically, we re-sequenced 15 randomly selected isolates from those sent to other institutes and compared these duplicates to the initial sequencing data.

Reads for each sample were mapped to the recently updated/annotated (Malone et al. 2017) reference genome for *M. bovis* strain AF2122/97 (GenBank accession LT708304.1) using the mapping-based phylogenomics pipeline, RedDog V1beta.10.3 (Edwards et al. 2016) to identify SNP variants across all isolates. Alignment and mapping were carried out in Bowtie2 v2.2.9 (Langmead et al. 2012) using the sensitive local mapping setting. SNP calling was undertaken using SAMtools and BCFtools (Li et al. 2009) using the consensus caller setting. A minimum depth of 10x was set for SNP calling. The average coverage failure filter, average depth filter and average mapping failure filters were set at 98%, 10x, and 75% respectively. Transposable elements, repeat regions and the PE/PPE regions as defined in the Genbank annotation, were excluded from SNP calling using the parseSNP tool in the RedDog pipeline (Edwards et al. 2016).

Some isolates were sequenced by Glasgow Polyomics, others were sequenced by Eurofins laboratories. 15 randomly selected isolates were re-sequenced by AFBI. No SNP distance was observed between duplicates and the previously sequenced isolates.

2.5 Badger microsatellite genotyping.

Nuclear DNA extracted from all badger hair samples was genotyped at 14 microsatellite loci using methods previously described (Guerrero et al. 2018). We re-profiled 5% of DNA samples as a quality assurance measure.

2.6 Testing for IBD in badger population.

Using the package 'PopGenReport' (Adamack and Gruber, 2014) in R (R Development Core Team, 2020), we constructed a microsatellite distance matrix (Smouse and Peakall method) for all unique badgers captured in the study zone, and for the subset of badgers that produced *M. bovis* cultures from the endemic lineage (see below), to ensure we had sufficient power to detect IBD in sub-populations. For both datasets, we then constructed inter-animal Euclidean distance matrices using the R package, 'Geosphere' (Hijmans et al. 2019). We then performed a Mantel test, with 10,000 repetitions, for IBD using the package 'ade4' (Dray and Dufour, 2007) in R. For the larger meta-population, Mantel tests and linear regressions were carried out for each capture year, with an analysis of covariance carried out to compare the slopes of each genetic distance vs euclidean distance relationship.

2.7 Preliminary phylogenetic analyses.

The most appropriate nucleotide substitution model for our phylogenetic analyses of the FASTA alignments of informative SNPs was assessed using the 'modelTest' function of the package 'Phangorn' (Schliep, 2011) in R. Specifically, the fit of the General Time Reversible (GTR), Jukes Cantor, and Hasegawa Kishino Yano (HKY) models to the data were assessed. The nucleotide substitution model with the lowest AIC (GTR – AIC: 26860.39) was used to build a maximum likelihood phylogeny using RAxML v8 (Stamatkis, 2014). The rapid bootstrapping search method in RAxML was selected and stopped after 400 replicates. The phylogeny was visualized and assessed in FigTree v1.4.4 (Rambaut, 2018) and the ape package (Paradis and Schliep, 2018) in R. Final figures of maximum likelihood trees were produced using ggtree (Yu, 2020). The presence/absence of a temporal signal in the phylogenetic data of the endemic clade was assessed using the program TempEst v1.5.1 (Rambaut *et al.* 2016). After selecting the reference strain (AF2122/97) as the best-fitting, outgroup root isolate, which maximises the temporal signal, the root to tip divergence model was fitted using the residual mean squared method.

To test the significance of the temporal signal in our dataset, we randomised the tip dates for the 302 chosen isolates from the endemic clade, in ten replicate analyses, as *per* Firth et al (2010). Tip dates were randomised using the 'Tipdatingbeast' package (Rieux and Khatchikian, 2017) in R. The original dataset and each replicate were subjected to a simplified BEAST 2 (Bayesian Evolutionary Analysis by Sampling Trees - Bouckaert et al. 2014), constant population, unstructured coalescent analysis, using a relaxed clock model, the GTR nucleotide substitution model and a chain length of 200,000,000 MCMC steps, of which 10% was discarded as burn-in. After checking convergence in Tracer 1.7.1 – (ESS > 200 for all parameters), the median substitution rate for each replicate was compared to those of the non-randomised data set. In addition, we also compared the substitution rates to those inferred from the BASTA and MASCOT analyses (see below) to verify that not taking population structure into account did not result in biased clock rate estimates.

2.8 Structured coalescent analyses to determine inter-species *M. bovis* transition rates.

To facilitate the goal of determining the transmission dynamics of bTB in the TVR zone, existing spoligotyping and MLVA data were used to rationally target the historically endemic strain family in this region (Skuce et al. 2010; Skuce et al. 2020). Specifically, we applied two Bayesian structured coalescent methods, using BEAST2 (Bouckaert et al. 2014; Barrido-Sottani et al. 2017). Specifically, we used BASTA (DeMaio et al. 2015) (Bayesian Structured Coalescent Approximation - De Maio et al. 2015) and MASCOT (Marginal Approximation of the Structured Coalescent - Müller et al. 2018)) which can estimate both ancestral population structure and transition rates in the presence of biased sampling. To ensure valid comparisons with previously investigated bTB epi-systems in the UK, we used BASTA as *per* Crispell et al (2019) and Rossi et al (2020a and 2020b). In addition, we used MASCOT to estimate the effective population size / diversity of the *M. bovis* population in both cattle and badger hosts.

Our study population for estimation of transition rates, was a subset of 302 isolates from the endemic lineage defined below. We randomly selected 248 cattle isolates (one isolate per annum per herd) and 54 isolates from unique badgers. All 302 isolates were collected in the period 1986-2017. We implemented BASTA and MASCOT analyses in BEAST2 to determine inter-species transition rates. We used uncorrelated lognormally distributed relaxed clock models, as these have been observed to best describe the rates of molecular evolution in *M. tuberculosis* and *M. bovis* in previous studies (Crispell et al. 2017; Crispell et al. 2019; Menardo et al. 2019). Each BASTA and MASCOT analysis was run with three replicates. For each BASTA analysis, a chain length of 300,000,000 MCMC steps was set, sampling every 15,000 steps. For each MASCOT analysis, a chain length of 500,000,000 MCMC steps was set, sampling every 50,000 steps. Full details of analyses are supplied in the xml files in Supplementary files. Following the removal of a 10% burn-in, chains were combined using LogCombiner v2.6 (Bouckaert et al. 2014) and analyses were compared based upon the log likelihood scores, model convergence and posterior support of parameters in Tracer v1.7.1 (Rambaut et al. 2018). Maximum Clade Credibility (MCC) trees were constructed for combined chains using TreeAnnotator v2.6 (Bouckaert et al. 2014)) using the median ancestor heights criterion. BASTA log and tree output files were used to estimate inter-species transition rates and inter- and intra-species transition counts, using custom R and Java scripts which analysed 10,000 trees from each of the model runs as per the method of Crispell et al (2019) – these scripts are provided as Supplementary files.

2.9 Graph methods to assess inter-species transitions.

To reconstruct the transmission tree of the set of 302 *M. bovis* isolates collected from badgers and cattle between 1986 and 2017, we used the graph method *SeqTrack* (Jombart et al., 2011). *SeqTrack* infers the most likely genealogy from genomic and temporal data. The matrix of pairwise genetic distances between *M. bovis* isolates, the isolates' date of collection and associated host information, an estimated *M. bovis* substitution rate, and the sequences total (in number of SNPs) were given as input. *SeqTrack* produced a *M. bovis* genealogy tree, with the number of SNP differences associated to the branch between each ancestor and descendant. The output files were used to compute the distribution of *M. bovis* genetic distances between ancestors and descendants, as well as the percentage of intra and inter species transmission events. To check the effect of *M. bovis* substitution rates on the ancestral history, we ran the analysis with values from previous *M. bovis* genomic studies in NI (0.15 and 0.20 substitutions per genome per year from Biek et al. 2012 and Trewby et al. 2016b,

respectively, and 0.35 from this work (Section 3.5). *SeqTrack* was implemented in R using the adegenet package (Jombart and Ahmed, 2011) (scripts provided as Supplementary files).

2.10 Assessing effect of badger population structure on *M. bovis* spatial partitioning.

We sought to determine if *M. bovis* inter-isolate SNP distance is associated with pairwise Euclidean distance between trapped badger locations, pairwise difference in time of *M. bovis* isolation and pairwise host genetic distance.For *M. bovis* genome sequences derived from badgers infected with the endemic lineage, we constructed two inter-isolate distance matrices: (i) SNP distance using the R package 'ape' (Paradis and Schliep, 2018); (ii) time of isolation difference using the 'dist' function in R.

Using these *M. bovis* distance matrices and the two already produced to assess IBD in the infected badgers (see above), we performed a multiple regression on distance matrices (MRM) analysis using the R package 'ecodist' (Goslee and Urban, 2007) with 10000 repetitions.

3. Results

3.1 Sampling of cattle and badgers.

A total of 642 *M. bovis* isolates were used in this study, of which 611 were collected from badgers and cattle in the TVR zone, and 31 from a neighbouring region. Of the 642 isolates, 15 were sequencing QA duplicate controls as discussed in section 2.4. In addition, we re-sequenced the *M. bovis* reference sample, AF2122/97 as an internal control. Of the 642 survey isolates, 399 (282 cattle; 117 badger) were sampled contemporaneously in the TVR zone during the project (2014-2017). A further 242 historical isolates (232 cattle; 10 badger=10) from 1986-2013 were available from archived cultures from the zone and its environs. Cattle isolates across all years were single isolates per animal from 185 herds. Multiple isolates (n=86) were cultured from 24 individual badgers, with single isolates derived from a further 36 badgers. In total, between 1986 and 2017, we collected *M. bovis* isolates from 60 unique badgers and 483 unique cattle (Figure 1B). Full details of sample locations, year of isolation and species of origin are given in Supplementary Data 1.

3.2 Spoligotyping and MLVA analysis.

22 MLVA types and 6 spoligotypes were observed in the 642 isolates. From prior analyses (Skuce et al. 2010), the spoligotype and MLVA genotypes could be grouped into eight related 'strain families'. Each is dominated by a probable founder genotype (source of the family name) with related daughter strains varying by spoligotype and / or MLVA polymorphism. Numbers of isolates per strain family are shown in Table 1A. The MLVA 6, spoligotype 263 family (6.263) was considered to be endemic in the region and accounted for most of the observed isolates (Skuce et al. 2010; Skuce et al. 2019). The remaining seven strain families (1.140, 2.142, 3.140, 4.140, 5.140, 19.140 and 20.131) were not likely to be endemic in the TVR cull zone as each has a home range elsewhere in Northern Ireland (Skuce et al. 2010; Trewby, 2016a). Of the 36 isolates from strain family 20.131, 32 were collected from a region neighbouring the TVR zone, 4 badger isolates were found within the zone. Full details of isolate MLVA genotypes and spoligotypes are supplied in Supplementary Data 1.

3.3 Sequencing, bioinformatic analyses and Quality Assurance.

The RedDog pipeline was used to process the isolates. 24 (22 cattle and 2 badgers) failed the sequencing QA filters (98% coverage filter for the reference genome) and were excluded. The remaining 618 survey isolates plus AF2122/97 control passed all QA filters. Detailed QA meta-data for all 619 isolates are given in Supplementary Data 2. Forward and reverse reads for all QA passing

isolates are deposited at the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA), bio-project XXXXXXXX. Accession numbers for all reads are given in Supplementary Data 2.

From the 619 isolates with good quality sequence reads, 1562 SNPs passed QA calling rules and were used to conduct phylogenetic analyses. Details of all SNPs passing QA, and their location in the reference sequence are given in Supplementary Data 3.

The AF2122/97 control exhibited a 3 SNP distance from the reference sequence (Genbank LT708304.1) likely due to accrual of mutations after culture passages at AFBI.

3.4 Badger microsatellite genotyping and IBD analyses.

769 unique badgers were captured, location recorded, sampled and successfully genotyped between 2014 and 2018. Random 5% re-genotyping for QA purposes produced identical allele calls. Microsatellite profiles, capture locations and date of capture can be found in Supplementary Data 4. Summary population genetic statistics for all animals, per year are collated in Supplementary Table S1.

Samples from 45 badgers produced positive *M. bovis* cultures. Spoligotyping and MLVA placed them in the major endemic lineage in the study zone. Capture locations for the 45 endemic strain positive badgers are illustrated in Supplementary Figure S1A.

Across all years, the badger population exhibited consistent levels of IBD, as indicated by significant Mantel tests (r=0.11-0.17, $p<0.05$) (Table 2). The slopes of the relationships between genetic distance and Euclidean distance were very similar. Small, significant differences were observed in the ANCOVA (Supplementary Figure S2 and Table 2), mainly due to the increased slope of the IBD relationships years 2015, 2016, and 2018, consistent with badger genetic differentiation being observed over shorter distances.

The 45 *M. bovis* culture positive badgers also exhibited significant IBD (Table 2) similar to that of the larger study population.

3.5 Preliminary phylogenetic analyses.

3.5.1. All isolates.

The Maximum Likelihood tree constructed in RAxML for all 619 sequenced isolates is shown in Figure 2. The phylogeny was rooted using the 20.131 strain family as an out-group, as these isolates are known to derive from an older common ancestor than other extant strains (Allen et al. 2013). Eight major lineages, each with high bootstrap support, were observed in the phylogeny. The eight strain families defined previously by MLVA and spoligotyping were monophyletic and in perfect concordance with the SNP based tree topology. We determined the within-lineage diversity, as defined by total number of SNPs recorded, for each of the eight major lineages observed (Table 1B). Additionally, from distance matrices generated during phylogenetic analyses, pairwise, inter-isolate SNP distance, summary statistics were computed within all eight major lineages (Table 1C).

3.5.2 Endemic lineage – 6.263.

The endemic major lineage of 6.263 presented the best opportunity to investigate *M. bovis* transmission dynamics among cattle and badgers in the TVR zone. 6.263 has been consistently associated with the study area for over two decades in local cattle and badgers, unlike lineages whose home range is elsewhere in Northern Ireland (Skuce et al. 2020). A higher resolution SNP phylogeny of 6.263 is shown in Figure 3, using the subset of 302 isolates described above. Cattle and badger

isolates were observed in all sub-lineages of the endemic lineage, with no sub-lineages made up of isolates exclusively from a single host species. Major sub-lineages had good bootstrap support (>90). A bar-plot showing frequency of isolates per host species taken from the endemic clade over the period 1986-2017, is presented in Supplementary Figure S3. A smaller maximum likelihood phylogeny of the 45 endemic clade badgers is presented in Supplementary Figure S1B.

Badger isolates were predominantly found in the endemic 6.263 lineage. No badger isolates were found in the 1.140, 2.142, 3.140, 5.140 and 19.140 lineages. Four isolates, from two unique badgers, sampled in the TVR zone, were found in the 20.131 lineage, whilst five isolates from four unique badgers were found in the 4.140 lineage (Supplementary Figures S4 and S5). A full breakdown of number of isolates from each species in each lineage is shown in Table 1A.

The n=302 endemic lineage phylogeny analysed in TempEst, rooted against the AF2122/97 reference genome, exhibited a positive correlation between genetic divergence (root to tip distance) and sampling time, with moderate evidence of temporal signal (R^2=0.25; p<0.001), and a conservative clock rate of approximately 0.22 substitutions per genome, per year - see Supplementary Figure S6A. All ten tip date randomised replicate datasets run using the simple unstructured coalescent model, exhibited similar substitution rates, all of which were considerably lower than the substitution rate inferred on the non-randomised data set (0.35 substitutions per genome, per year 95% HPD: 0.28-0.43) and exhibited no overlap in 95% highest posterior density (HPD) (Supplementary figure S6B). Furthermore, the BASTA and MASCOT analyses showed similar substitution rate estimates (see below) to the non-randomised data set under a simple unstructured coalescent model. This confirmation of the presence of a temporal signal permitted further analyses using structured coalescent methods within BEAST2 (Firth et al. 2010).

3.6 Structured coalescent analyses – BASTA and MASCOT.

The three replicate BASTA and MASCOT chains converged to similar intra-method values across all parameters. Log-combined Data for derived parameters are shown in Table 3. Substitution rates are presented in Supplementary Figure S6B.

BASTA indicated a median substitution rate of 0.38 substitutions per genome per year (95% HPD interval 0.27-0.49). MASCOT indicated a similar rate of 0.34 substitutions per genome per year (95% HPD interval: 0.28-0.42).

BASTA and MASCOT estimates for median time to the most recent common ancestor (tMRCA) of the endemic lineage were 47.93 years (95% HPD 32.6-85.3) and 37.3 years (95% HPD 31.3-49.4) before the last date of sampling (in 2017), respectively - suggesting an emergence for endemic lineage 6.263 in the late 1960s to early 1980s.

BASTA estimated the median badger-to-cattle interspecies transition rate to be 0.03 transitions per lineage per year (95% HPD interval: 0.00002-0.2039). In contrast, the cattle-to-badger median rate was approximately three times larger, at 0.098 transitions per lineage per year (95% HPD interval: 0.0006-0.154) – see Figure 4A. MASCOT estimated badger-to-cattle transitions to be 0.86 transitions per lineage per year (95% HPD interval: 0.55-1.24), and cattle-to-badger median rate to be approximately three times larger, at 2.73 transitions per lineage per year (95% HPD interval: 0.53-5.83) – see Figure 4B.

Time stamped maximum clade credibility (MCC) trees for the BASTA and MASCOT analyses are presented in Supplementary Figures S7 and S8.

MASCOT-derived estimates of the *M. bovis* effective population size (N_e) were also different between hosts, suggesting that the vast majority of pathogen diversity was found in cattle (median N_e = 465.76; 95% HPD: 118.13-1173.25) compared to badgers (median N_e = 1.98: 95% HPD: 0.25-7.52) – see Figure 4C.

BASTA-derived counts of intra- and inter-species transitions are discussed in the Supplementary Material and presented in Supplementary Figure S9.

3.7 Graph methods – inter-species transitions.

The *SeqTrack* analysis inferred the ancestry of the 302 *M. bovis* isolates by distinguishing (based on date of collection and genetic distances), ancestors from descendants (Figure 5). It identified 68 ancestors, of which 56 were associated with cattle and 12 with badgers. The number of cattle descendants was more than twice the number of badger ones (μ=4.41, σ=8.44 and μ=1.93, σ=1.89, respectively). The inferred ancestry displayed very few mutations between ancestors and descendants (maximum of 11 mutations with μ=1.66, σ=1.95) suggesting direct transmission links between isolates (Supplementary Fig S10). From these, 71.8% were within cattle, 13.6% were from cattle to badgers, 10.3% were from badgers to cattle, and 4.3 % were within badgers (Supplementary Fig S11). Running the analysis with different substitution rate values did not influence these results (results not shown).

3.8 Effect of badger population structure on *M. bovis* spatial partitioning.

From the full model in the MRM analysis, modeling *M. bovis* genetic distance (SNP-based) as a function of inter badger genetic distance, inter badger Euclidean distance and inter *M. bovis* time of isolation difference, we observed that only inter-badger Euclidean distance was significantly associated with *M. bovis* genetic distance (p = 0.04). However, the overall fit of the model was non-significant (F-test p >0.05). Badger microsatellite derived genetic relatedness was therefore not associated with *M. bovis* SNP-derived genetic differentiation. A full summary of MRM findings is presented in Supplementary Table S2.

4. Discussion

We applied Bayesian phylogenetic methods to investigate transmission dynamics in a systematically sampled, multi-host, endemic disease. Our data shed further light on the intra- and interspecies dynamics of *M. bovis* transmission in an endemic area. They are also useful for informing control policies and comparison to epi-systems in different regions. From the latter, novel insights into drivers of disease transmission dynamics may come to light.

Our structured coalescent analyses suggest that the endemic 6.263 lineage has been present in the TVR zone since the 1960s-1980s. The rate of molecular evolution of 0.34 to 0.38 substitutions per genome per year is consistent with previous *M. bovis* phylodynamic studies – see Supplementary Table S3.

The presence of bacterial isolates from both cattle and badgers throughout all sub-lineages of the endemic 6.263 lineage indicates likely bi-directional transmission between both species. The structured coalescent BASTA and MASCOT models both suggest that infection in this region is driven primarily by cattle. Point estimates for transition rates from both analyses, and raw transition counts from BASTA do exhibit considerable uncertainty, however, when taken alongside additional data on N_e, seqtrack outputs and badger genetic structure (see below), they are consistent with a reduced role

for badgers in this epi-system. While BASTA and MASCOT models had similar magnitudes of difference in inter-species transition rates and point to their same relative importance, point estimates of the transition rates were considerably different. This is perhaps not surprising, since while both are approximations of the structured coalescent, they make considerably different assumptions about how the coalescent process affects the shape of the phylogenetic tree from which transition rates are calculated (Müller et al. 2017). Estimates of raw transition counts both between- and within-hosts (see Supplementary material) are broadly congruent and point towards the study area being a primarily cattle-driven, epi-system. An important caveat when using Bayesian structured coalescent methodologies, is that transmission events and transitions are subtly different variables (Crispell et al. 2019) – see the Supplementary Materials for some discussion on this and sampling representativeness.

Estimates of the effective population size from MASCOT models consistently indicated that most of the pathogen diversity is in cattle. The graph methods were largely in agreement with the reconstructed phylogenies, suggesting that the majority of bacterial ancestors were found in cattle hosts and that after within-cattle transmission events, transmissions from cattle to badgers were the main contributors to this epi-system. The graph methods complemented the phylogenetic analyses by their ability to infer correct genealogies of isolates in densely sampled areas such as this one. This method considers that ancestries can be inferred between sampled isolates within the given dataset following three assumptions: 1) each isolate can only have one ancestor; 2) ancestors must have an earlier collection date than their descendants; and 3) the likelihood of the ancestries can be indicated by the genetic differences among isolates. If any of these assumptions are violated, the method could provide wrong results, however, if this bias exists, it would also affect the graph-based and the phylogenetically analyses. The maximum number of SNP differences between isolates was 11, with a mean number of 1.66, suggesting that most (if not all) transmission events were direct. This result is not surprising since this study was performed in a small area in which an endemic lineage is present. However, it also highlights the quality of data sampling during the study period.

These independent lines of evidence suggest that the *M. bovis* strains found in badgers in the area represent a limited sample of the diversity found in the cattle *M. bovis* population, and likely result from recent cattle-to-badger transmission. Indeed, the raw transition count data (Supplementary Table S4 & Supplementary Figure S9) suggest that the number of cattle-to-badger transitions is approximately double that of badger-to-badger transitions, suggesting that spill-over events from cattle are having a greater impact on badger TB status than within-species transmission events.

Badger genetic population structure, whilst remaining stable over the intervention period, had no association with how *M. bovis* genetic diversity was spatially distributed. It is possible that this lack of association is due to factors other than reduced badger to badger intraspecies transmission dynamics. The endemic lineage, if it were a relatively recent incursion, may have had little time to establish foci of persistent infection in badgers, and diffuse across the landscape through philopatric contact networks. This lineage has, however, been present in the region for ~40-50 years, providing ample time for establishment to occur. Alternatively, perturbation of the badger population, and associated dispersal arising through the application of culling, even at a small-scale (Bielby et al. 2014), may have served to obscure any association between pathogen and host population structures. The relative stability of the IBD relationship we observe, and the fact that during the culling period of 2015-2018, genetic differentiation occurs over shorter distances than the survey-only year of 2014, are consistent with no perturbation signal. GPS collar data from badgers from this same region have also suggested badger ranging did not increase significantly in cull years compared to survey (O'Hagan et al 2021). Given these observations, and the results of the structured coalescent and graph methods, we

postulate that our badger genetic data further support the hypothesis of a relatively reduced role for badgers in bTB transmission and persistence in this region, compared to cattle.

Our data contrast starkly with the findings of Crispell et al (2019) from the Woodchester Park region of Gloucestershire, GB, that found badger-to-cattle transitions were up to nine times more common than cattle-to-badgers. Without employing detailed, comparative methods, it is difficult to definitively understand why transmission dynamics between the two regions are so divergent. However, it may be due to differences in host density and geography. The Woodchester Park badger population is one of the densest in Europe, with an average of 30-40 badgers per km^2 during the period covered by the recent phylodynamic study (Delahay et al. 2013; Crispell et al. 2019). The TVR region's badger population is approximately eight to ten times less dense than this - 3.88 badgers per km^2 from a County Down wide survey (Reid et al. 2012), and ~5.6 badgers per km^2 as assessed in the TVR study (Menzies et al. 2021), and more comparable to the average badger density observed in the UK Randomised Badger Culling Trial (RBCT - 3.92 badgers per km^2 (ISG, 2007)). Conversely, the cattle density in the immediate vicinity of Woodchester Park, in Gloucestershire, is quite variable with estimates ranging from 25-100 cattle per km^2 (APHA, 2017). Northern Ireland is recognised as having some of the highest cattle densities in western Europe with recent estimates suggesting an average of 112 cattle per km^2 (Allen et al. 2018). The landscapes are also geographically different, with the Irish landscape made up largely of pastureland interspersed with hedgerows that are the primary location for badger setts (Reid et al. 2012), while Woodchester Park consists of prime badger woodland habitat (Delahay et al. 2013).

Interestingly, the transmission dynamics we describe here are very similar to those reported by Rossi et al (2020b) in a recently introduced incursion of bTB into the low-risk area of Cumbria in northern GB. Specifically, they observed an initial phase of primarily cattle-to-cattle transmission, spilling over into local badgers. Whilst exact estimates of GB wide badger density are not available (Rossi et al. 2020b), it is likely that density in Cumbria is considerably lower than that observed in Woodchester Park, and comparable to that observed in our study area - perhaps in the order of 0.3 to 2.5 badgers per km^2 (Judge et al. 2017). It is interesting that the transition counts between hosts are very similar given that the Cumbria outbreak has not persisted as long as the endemic situation in the TVR area.

Host density is a major driver of *M. bovis* persistence (Allen et al. 2018) and it is conceivable that the relative densities of cattle and badgers in different regions, alongside other factors, may affect transmission dynamics. The observed disparity between epi-systems suggests that there may be no simple bTB transmission paradigm on which to base all interventions. Such heterogeneity in regional disease epidemiology may well call for a more heterogeneous approach in the application of disease eradication schemes.

An important consideration from this study is that while our data are supportive of badgers playing a lesser role in intra-species bTB transmission dynamics in this region, this may not be capturing the full impact of badger-to-cattle transmission, which seeds new infection into herds. Subsequent within herd 'amplification' by cattle-to-cattle transmission may mean the initial seeding event has greater impact than that standalone event, results in an outsized contribution to disease spread, as has been postulated before from RBCT data. While the badger-to-cattle contribution was estimated at 5.7%, this was modelled to amplify to ~52% (bootstrap 95% CI: 9.1 – 100%), although confidence intervals were very wide (Donnelly and Nouvellet, 2013). Owing to the low rate of molecular evolution observed in *M. bovis*, the phylogenetic methods we employ lack the resolution over shorter outbreak time scales to identify such amplification events and inform on their impact.

Any study of a multi-host infectious disease system in the field will have biases that compromise the representativeness of the sample set assembled. However, we believe the systematic sampling undertaken here, and analytical approaches taken, largely mitigate these issues. See Supplementary materials for a full discussion.

A separate issue with our findings is that they come from a badger population undergoing selective culling and vaccination, both interventions that are likely to affect interspecies disease transmission dynamics (Buddle et al. 2018; ISG, 2007). However, any study seeking to harvest systematically sampled, culturable *M. bovis* from wildlife, would have involved disturbance and culling of badgers for post-mortem and pathogen isolation. The application of vaccination is admittedly a different matter however, and without the necessary non vaccinated control population in which to study transmission dynamics, we are unable to determine the likely impact of the 5 years of vaccination.

Conclusions

We describe how in a small region of Northern Ireland, cattle-associated transmission appears to drive bTB disease dynamics. Of the inter-species transitions estimated, cattle-to-badger transitions were three times more common than badger-to-cattle. We contrast this to the recently published work of Crispell et al. (2019) from Woodchester Park, in which starkly different transmission dynamics were observed. There may be regional heterogeneity in the epidemiology of bTB. Further work in other regions of the UK and Ireland is required to assess just how heterogeneous disease dynamics may be. If substantial heterogeneity is observed, it may be advisable for different regions to adopt bespoke eradication schemes tailored to the prevailing host dynamics in their areas, leading to superior control outcomes.

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Research Ethics

All badger field work was carried out under licences issued by the Northern Ireland Environment Agency. All scientific procedures performed on badgers were conducted according to the guidelines of the Animals Scientific Procedures Act (ASPA - Licence 2767) overseen by the Department of Health for Northern Ireland.

Conflict of interest

The authors declare no conflict of interest in the production of this work.

Supplementary Files

R scripts and BEAST xml run files used in the performance of this work are curated at the following GitHub repository:

https://github.com/AdrianAllen1977/Genome-epidemiology-of-Mycobacterium-bovis-infection-in-contemporaneous-sympatric-badger-and-cattle

Figures

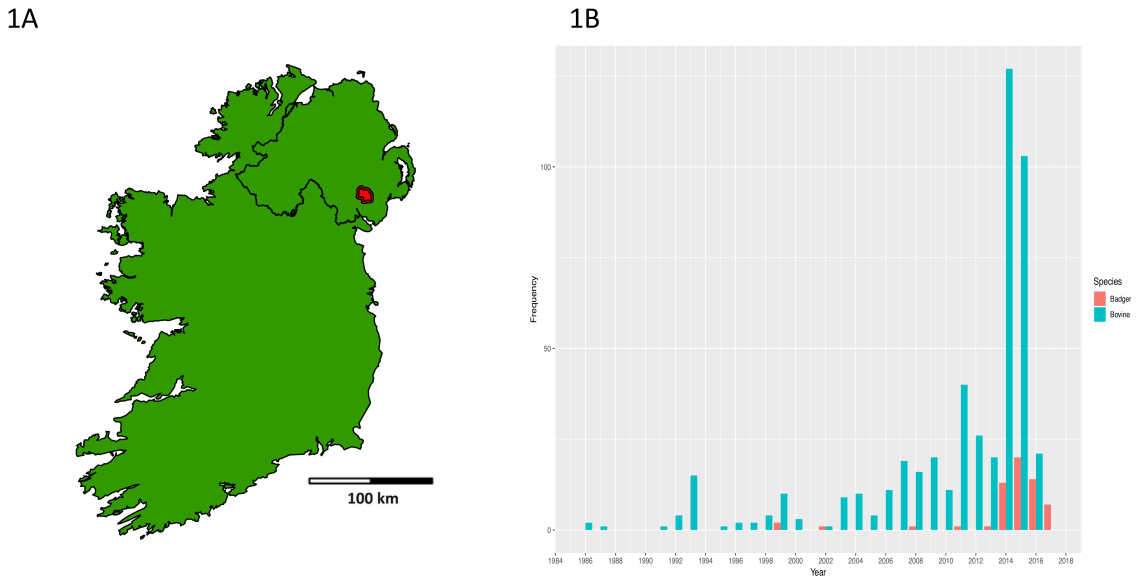

Figure 1 – A: Map of Ireland with location of 100 km^2 TVR cull zone highlighted in red. B: Frequency bar-plot of *Mycobacterium bovis* isolates sampled annually, from unique badgers and cattle, in the TVR zone prior to (1986-2013) and during the intervention (2014-2017).

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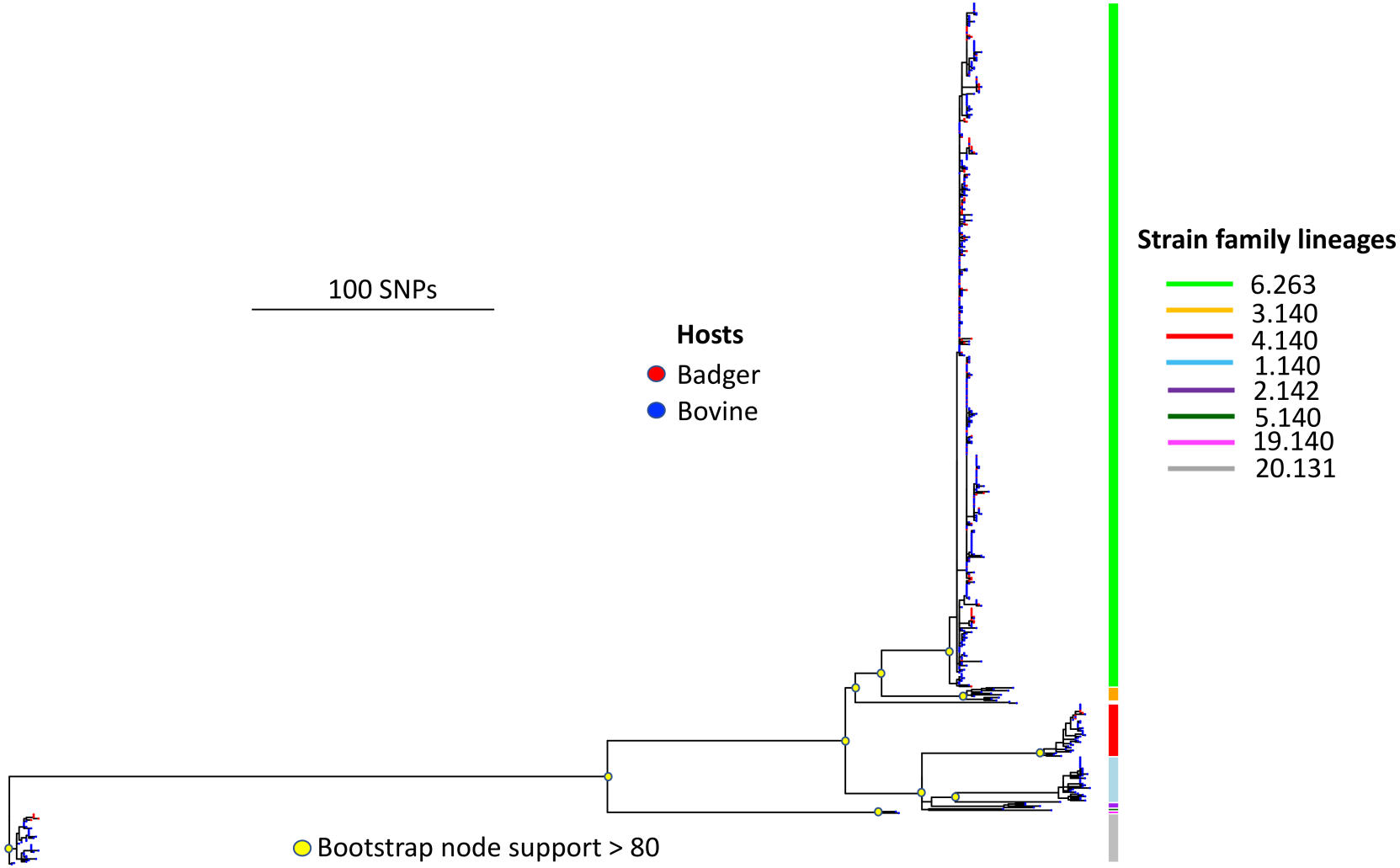

634

635 **Figure 2** – 1562 SNP Maximum Likelihood phylogeny of all 619 isolates that passed sequencing QA.

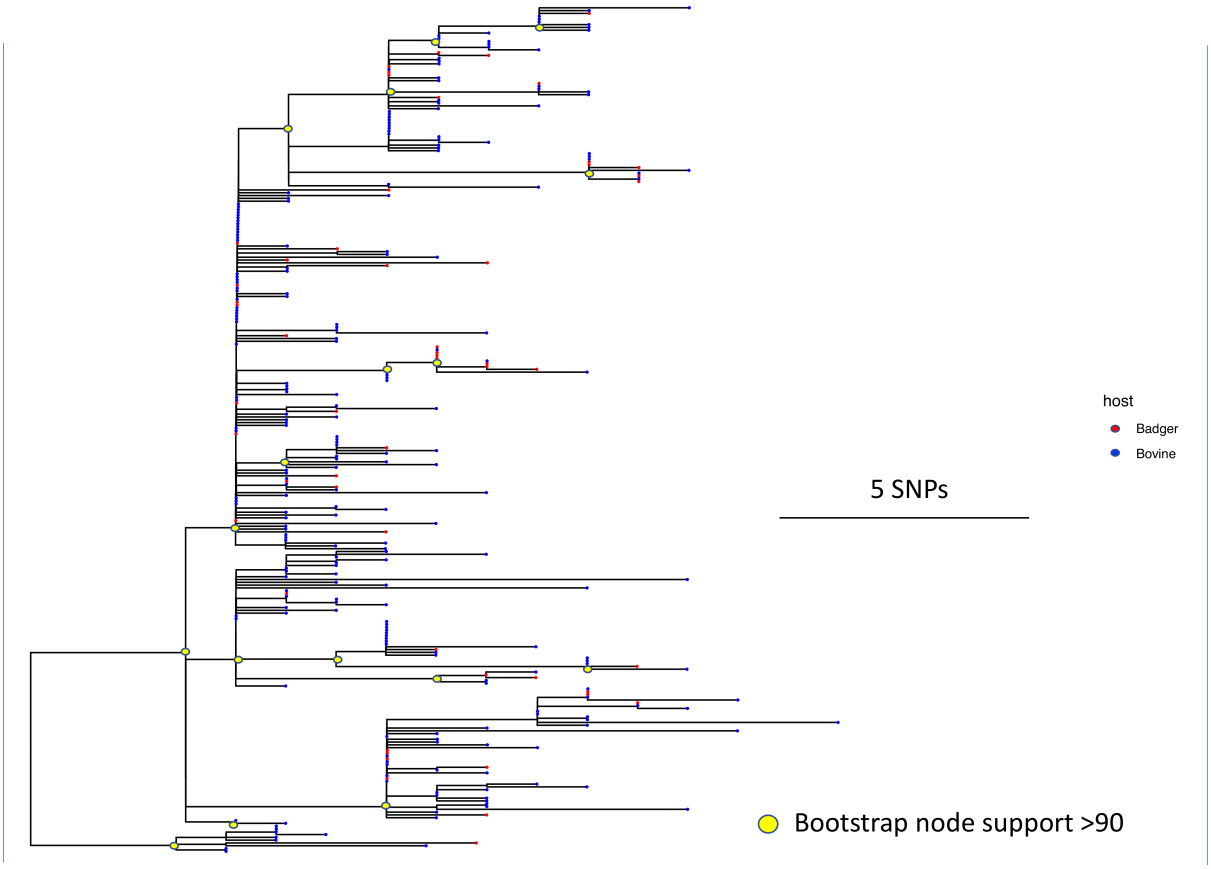

Figure 3 – 377 SNP Maximum Likelihood phylogeny of 302 isolates from the 6.263 endemic lineage in the TVR zone. Tree rooted to AF2122/97 reference, but reference removed for aid of visualisation.

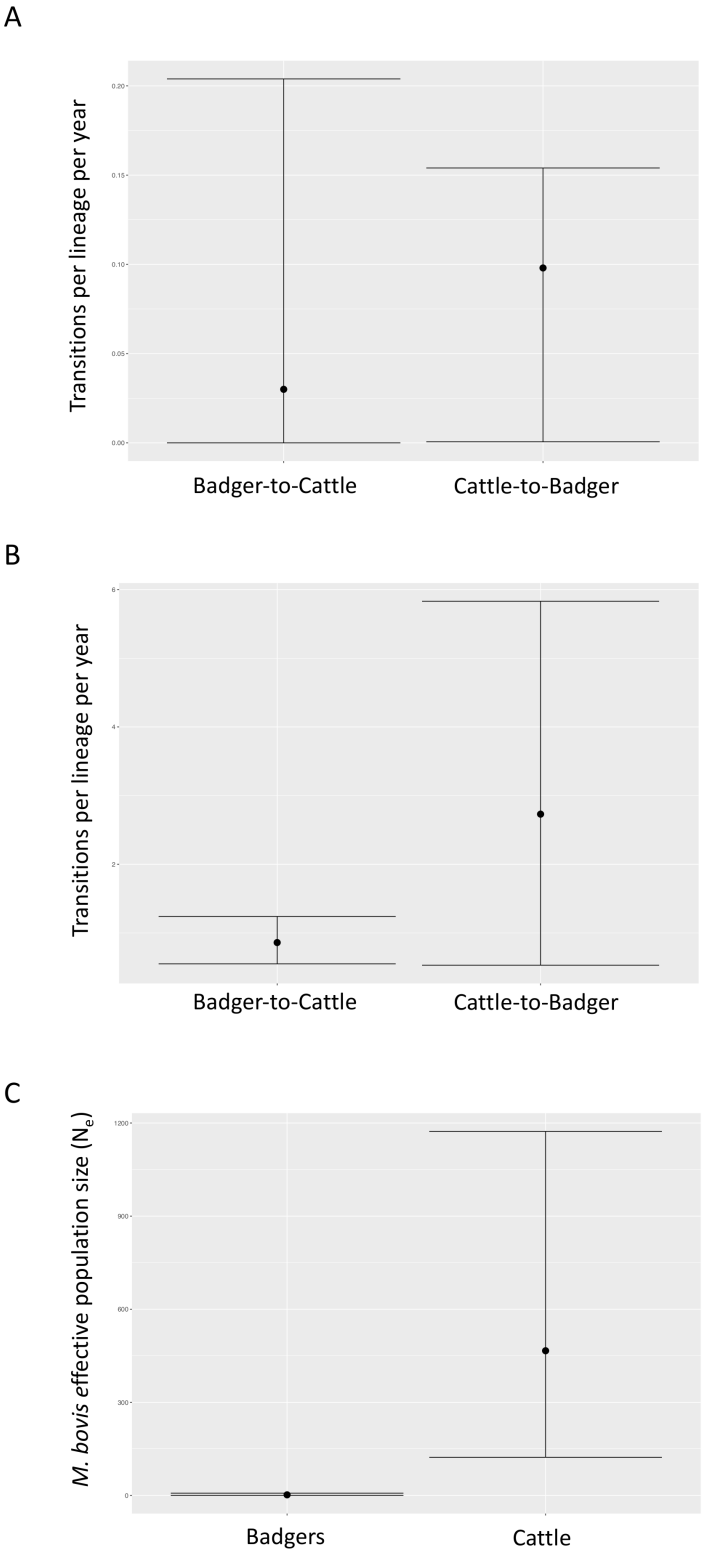

Figure 4 – Logcombined interspecies pathogen transition rates as assessed by A: BASTA and B: MASCOT structured coalescent models. Transition rates are expressed as median transitions per lineage per year and 95% highest posterior density (HPD). C: Logcombined MASCOT inferred *M. bovis* effective population sizes in badgers and cattle and 95% highest posterior density (HPD).

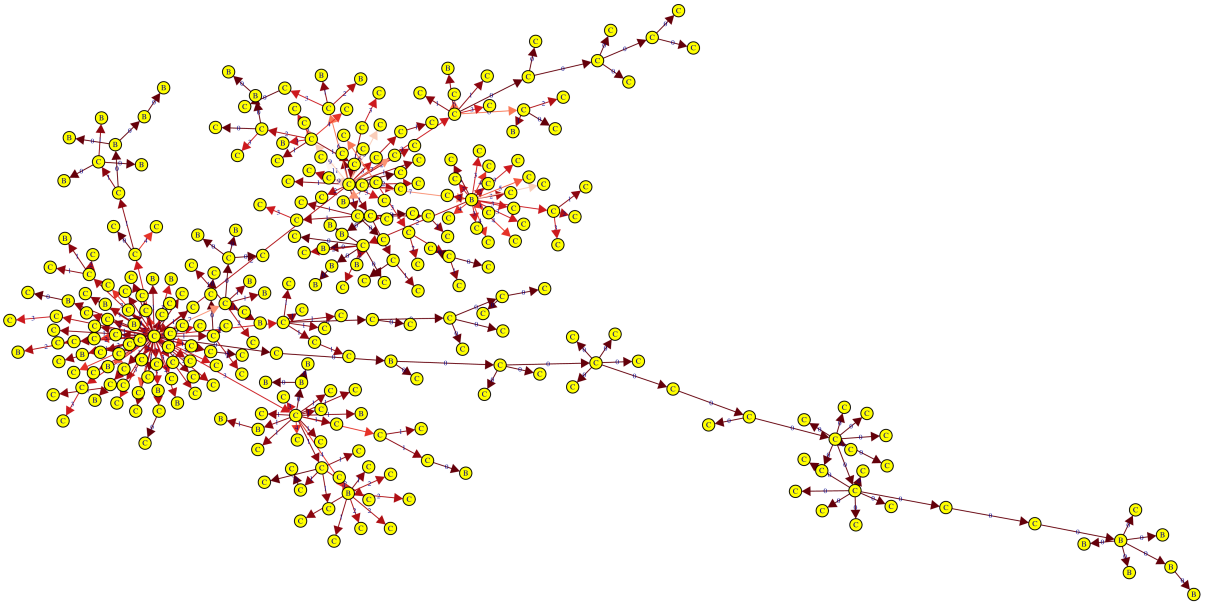

Figure 5 - SeqTrack transmission tree of 302 *M. bovis* isolates collected from cattle and badgers. Nodes shown in yellow labelled as hosts (B for badgers, C for cattle), arrows delineate transmission direction (dark red = no/few mutations, light red = more mutations) with numbers of mutations between isolates shown in blue.

Tables

	MLVA Strain families	1.140	2.142	3.140	4.140	5.140	6.263	19.140	20.131
A	**No. of isolates**	33	4	10	38	2	478	2	36
	No. cattle isolates	33	4	10	33	2	367	2	32
	No. badger isolates	0	0	0	5	0	111	0	4
B	**No. SNPs in clade**	186	38	84	92	82	377	13	53
C	**Min**	0.0	8.0	8.0	0.0	N/A	0.0	N/A	0.0
	1st Quartile	10.0	2.0	16.0	8.0	N/A	5.0	N/A	6.0
	Median	15.0	20.5	21.0	12.0	N/A	7.0	N/A	8.0
	Mean	17.6	19.5	21.6	11.7	N/A	7.6	N/A	8.0
	3rd Quartile	19.0	21.0	27.0	15.0	N/A	10.0	N/A	11.0
	Max	96.0	27.0	34.0	28.0	79.0	25.0	13.0	18.0
	St. Dev	20.0	6.2	7.3	6.3	N/A	4.0	N/A	3.8

Table 1 – A. number of isolates per strain family sampled in the study area with breakdown of number of isolates per host species. B. number of SNPs detected in strain family clades, and C. pairwise SNP distance statistics for each of the eight major lineages of *M. bovis* found in the TVR zone.

Cohort genotyped	No. animals	Mantel test Pearson coefficient r	Mantel p value	Linear model beta	Linear model p value	R^2	1 unit differentiation per x km distance
2014	273	0.11	0.0001	$1.6x10^{-4}$	$<2x10^{-16}$	0.012	6.25 km
2015	152	0.16	0.0001	$2.1x10^{-4}$	$<2x10^{-16*}$	0.024	4.74 km
2016	97	0.17	0.0001	$2.6x10^{-4}$	$<2x10^{-16**}$	0.030	3.84 km
2017	113	0.11	0.0001	$1.5x10^{-4}$	$<2x10^{-16}$	0.011	6.67 km
2018	134	0.13	0.0004	$1.7x10^{-4}$	$<2x10^{-16***}$	0.017	5.88 km
TB +ve	45	0.16	0.0024	$3.1x10^{-4}$	$1.1x10^{-7}$	0.030	3.22 km

Table 2 – Badger meta population isolation by distance (IBD) relationship for all sampling years. * =2015 significantly difference than slopes for years 2014, 2016 and 2017. ** = 2016 slope significantly different than slopes for years 2014 and 2015. ***=2018 slope significantly different than slopes for years 2014 and 2017.

	Substitution rate	tMRCA	Badger *M. bovis* Ne	Cattle *M. bovis* Ne	Cattle to badger transition rate	Badger to cattle transition rate
BASTA	0.38 (0.27-0.49)	47.93 (32.64-85.34)	N/A	N/A	0.098 (0.0006-0.154)	0.030 (0.000002-0.2039)
MASCOT	0.34 (0.28-0.42)	37.30 (31.28-49.39)	1.97 (0.26-7.39)	466.19 (122.94-1172.92)	2.73 (0.53-5.83)	0.86 (0.55-1.24)

Table 3 – Summary statistics for log-combined triplicate BASTA and MASCOT models. All estimates are median and 95% highest posterior density (HPD). Substitution rate is expressed as sites per genome, per year. Transition rates are expressed as transitions per lineage per year. Time to most recent common ancestor (tMRCA) for the endemic 6.263 clade is expressed in years prior to the last sampling date, 2017.

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