

1 **Population structure of *Mycobacterium bovis* in Germany: A long-term study**
2 **using Whole Genome Sequencing combined with conventional molecular typing**
3 **methods**

4 Thomas A. Kohl^{1,2}, Katharina Kranzer^{3,4}, Sönke Andres³ Thierry Wirth^{5,6,*}, Stefan
5 Niemann^{1,2,*}, Irmgard Moser^{7,*}

6 ¹ Molecular and Experimental Mycobacteriology, Research Center Borstel, Germany.

7 ² German Center for Infection Research (DZIF), partner site Hamburg-Lübeck-Borstel, Germany.

8 ³ Division of Mycobacteriology (National Tuberculosis Reference Laboratory), Research Center
9 Borstel, Borstel, Germany.

10 ⁴ Clinical Research Department, London School of Hygiene & Tropical Medicine

11 ⁵ Laboratoire Biologie Intégrative des Populations, Evolution Moléculaire, EPHE, PSL University, Paris,
12 France.

13 ⁶ Institut de Systématique, Evolution, Biodiversité, UMR-CNRS 7205, Muséum National d'Histoire
14 Naturelle, Université Pierre et Marie Curie, Université des Antilles, Ecole Pratique des Hautes Etudes,
15 Sorbonne Universités, Paris, France.

16 ⁷ Friedrich-Loeffler-Institut, Federal Institute for Animal Health, Institute of Molecular Pathogenesis,
17 Jena, Germany

18 * shared senior authorship

19

20 Key words: Tuberculosis, *Mycobacterium bovis*, human, animal, transmission,
21 spoligotyping, MIRU-VNTR-typing, Whole Genome Sequencing

22

23 Running title: Population structure of *Mycobacterium bovis* in Germany

24

25 Corresponding author: Irmgard Moser

26

27

28 **Abstract**

29 *Mycobacterium bovis* (Mbov) is the primary cause of bovine tuberculosis (bTB), and
30 also infecting a wide range of domestic animal and wildlife species and humans. In
31 Germany, bTB still emerges sporadically in cattle herds, free-ranging wildlife, diverse
32 captive animal species, and humans. In order to understand the underlying population
33 structure and estimate the population size fluctuation through time, we analyzed 131
34 Mbov strains from animals (n = 38) and humans (n = 93) in Germany from 1999 to
35 2017 by whole genome sequencing (WGS), MIRU-VNTR typing, and spoligotyping.
36 Based on WGS data analysis, 122 out of the 131 Mbov strains were classified into 13
37 major clades, six contained strains from both human and animal cases, and seven only
38 from human cases. Bayesian analyses suggest that the Mbov population went through
39 two sharp anticlimaxes, one in the middle of the 18th century and another one in the
40 1950's. WGS based cluster analysis grouped 46 strains into 13 clusters ranging in size
41 from 2-11 members and involving strains from distinct host types, e.g. only cattle, and
42 also mixed hosts. Animal strains of four clusters were obtained over a nine-year time
43 span, pointing towards autochthonous persistent bTB infection cycles. As expected,
44 WGS had a higher discriminatory power than spoligotyping and MIRU-VNTR typing. In
45 conclusion, our data confirm that WGS and suitable bioinformatics is the method of
46 choice to implement a prospective molecular epidemiological surveillance of Mbov.
47 The population of Mbov in Germany is diverse, with subtle, but existing interactions
48 between different host groups.

49

50

51

52

53 **Introduction**

54 Tuberculosis (TB) is one of the high priority infectious diseases affecting humans and
55 animals worldwide (1, 2), and the leading cause of death by a single infectious agent
56 in humans (2). Causative agents for TB are the members of the *Mycobacterium*
57 *tuberculosis* complex (MTBC), namely *M. tuberculosis*, *M. africanum*, *M. bovis*, *M.*
58 *caprae*, *M. microti*, and *M. pinnipedii*. In addition, *M. canettii*, *M. mungii*, and *M. orygis*
59 have been proposed as separate ecotypes. However, their taxonomic classification is
60 still under debate (3).

61 *M. bovis* (Mbov) is the primary cause of bovine TB (bTB) but also affects a wide range
62 of other domestic animal and wildlife species and even humans (4, 5, 6, 7). After time
63 periods of high prevalence of bTB infection in cattle until the second half of the 20th
64 century, Germany has reached the status of being officially free of bTB. Since July, 1st,
65 1996 (Decision 97/76/EC), 99.9 percent of the cattle herds remained officially free of
66 bTB infection and disease for at least six consecutive years (Article 2(d) of Council
67 Directive 64/432/EEC, 8, 9, 10). However, bTB is still emerging sporadically in cattle
68 herds (11), free-ranging wildlife, captive animal species (12), and humans (13).
69 Confirmed animal bTB cases are notified through an electronic national disease
70 information system (TSN) and published annually (14). From January 1999 to
71 December 2015, a total of 214 bTB outbreaks in cattle herds were notified in Germany,
72 with about half of the cases caused by either *M. bovis* or *M. caprae*. In general, *M.*
73 *caprae* is reported mainly in middle European countries with sporadic cases also in
74 Asia and Peru (15,16), with cattle and wildlife cases in Germany restricted to an area
75 at the German-Austrian border (17,18). *M. caprae* was therefore not included in this
76 study. According to the European Food Safety Authority (EFSA) 2017, from 2013-
77 2017, 43-56 bTB cases in humans were diagnosed annually (13). Notification rates for
78 bTB ranged from 0.05- to 0.07 per 100,000 population. Mbov and the closely related

79 *M. caprae* make up about 1% of all human TB cases (5,486 cases in 2017, more than
80 six per 100.000 population) (13, 19).

81 As disease transmission dynamics of Mbov within and between host groups are only
82 partially understood (20), molecular typing methods could offer insights into
83 transmission routes and inform pathogen surveillance (21, 22, 23). Classical
84 genotyping methods including spoligotyping, restriction fragment length polymorphism
85 (RFLP) and mycobacterial interspersed repetitive unit variable number of tandem
86 repeat (MIRU-VNTR) detection allow analyzing outbreaks, assessing population
87 structures, and performing longitudinal molecular epidemiological studies (24, 25, 26,
88 27, 28, 29, 30, 31).

89 Spoligotyping (25) is based on the analysis of CRISPR-CAS spacer sequences located
90 in a genomic region prone to convergent evolution (21), possibly leading to uncertainty
91 of strain relatedness. Spoligotyping patterns submitted to international databases
92 receive unique identifiers: SITVIT (32, 33, 34) allowing for MTBC isolates from any
93 host, and mbovis.org accepting MTBC strains from animals only (35). As of October
94 2018, 39,609 MTBC spoligotypes have been collected in the SITVIT database from
95 more than 121 countries (32). At mbovis.org, 2,117 patterns are available (last update
96 April 2020). RFLP is a method with high potential for discrimination for *M. tuberculosis*
97 but not Mbov strains due to the small number of analyzed insertion element copies
98 present in the respective genomes. MIRU-VNTR typing possesses a higher
99 discriminatory power, allowing automated high throughput typing and web-based
100 translation into a digit code identifier (29, 30, 36, 37). The method has high potential to
101 define clusters of related strains, but cannot differentiate between closely related
102 strains within outbreaks (38).

103 Next generation sequencing (NGS) allows for analysis of the nearly-complete genome
104 of a pathogen by whole genome sequencing (WGS), providing deeper insights into the
105 population structure, pathogen evolution, transmission chains, and biology of bacteria
106 (38, 39, 40, 41). WGS analysis facilitates the detection of recent transmission chains
107 and monitoring re-emerging of strains after years of non-detection (42, 43, 44, 45).

108 In this study, we used WGS, spoligotyping and MIRU-VNTR to determine the diversity
109 of Mbov strains isolated from animals and humans in Germany and define possible
110 transmission chains within and between different host populations over a 19-year
111 period (1999-2017). Using Bayesian analyses, we sought insights into the dynamics of
112 strain diversity over the last 800 years in Germany.

113

114 **Materials and Methods**

115

116 Strain selection and DNA extraction

117 In total, 131 Mbov strains were available for WGS including the reference strain Mbov
118 BCG (DSM 43990 / ATCC 27289), with 38 strains from the Friedrich-Loeffler-Institut
119 (FLI), Federal Institute for Animal Health, and 93 strains from the National Reference
120 Center (NRC) for Mycobacteria in Borstel, Germany (supplementary table S1). From
121 January 1999 to December 2015 (the study period), a total of 214 bTB outbreaks in
122 cattle herds were notified in Germany by the electronic system implemented by the FLI
123 to monitor bTB outbreaks, with about half of the cases in cattle caused by *M. bovis*.
124 Mbov strains from ten cattle bTB outbreaks, from five other domestic animal species ,
125 14 zoo animals, and wild boars were analyzed (supplementary table S2), spanning the
126 time period from 1999–2015, and covering different regions of the country, including
127 the known hot spot regions in the north and south. At the NRC in Borstel, all German

128 *M. bovis* strains cultured and archived from 2000 to 2017 were included. The NRC
129 receives samples from all districts in Germany, and while it is not the only laboratory
130 offering specialist mycobacterial diagnostics in Germany, it receives an estimated 50%
131 of all MTBC isolates. At both institutions, strains were cultured according to standard
132 procedures (46, 47, 48, 49), and genomic DNA was extracted using the High Pure PCR
133 Template Preparation kit (Roche Life Science; FLI) and with the
134 cetyltrimethylammonium bromide (CTAB) procedure (NRC), respectively (50).

135

136 Classical genotyping

137 Spoligotyping of animal strains was performed using a microarray format (Alere
138 Technologies, Jena, Germany) (51). Binary codes were automatically compared with
139 data available through SITVIT and mbovis.org to identify concordant species and
140 lineages. For human strains, the conventional spoligotyping method was used (25).
141 MIRU-VNTR-typing of the strains isolated from animals was performed using
142 conventional PCR and agarose gel electrophoresis (27, 29, 52). For human strains,
143 the automated high-throughput method was used (29). VNTR copy numbers were
144 assessed according to allele calling tables (www.miru-vntrplus.org, EU Reference
145 Laboratory for bovine Tuberculosis, www.visavet.es). The discriminatory power of the
146 method was calculated according to Hunter and Gaston (53); (supplementary tables 3
147 and 4).

148

149 Whole genome sequencing and data analysis

150 Libraries for WGS were prepared from genomic DNA with a modified Illumina Nextera
151 protocol (54) and run on the Illumina NextSeq NGS platform (Illumina, San Diego, CA,
152 USA). We employed the MTBseq pipeline with default parameters for variant detection
153 and a joint analysis (55), employing a threshold of 12 SNPs for cluster detection (56).

154 As deduced from the pairwise SNP distances distribution, we used a cutoff of 350
155 SNPs to detect major groups (figure 2). For all sequenced strains, mean coverage
156 depth was at least 50-fold, and at least 95% of the reference genome fulfilled the
157 MTBseq thresholds for variant detection. From the aligned sequences of concatenated
158 SNP positions produced by MTBseq, we calculated a maximum likelihood tree with
159 FastTree (57) with a general time reversible (GTR) substitution model, 1,000
160 resamples and Gamma20 likelihood optimization to account for rate heterogeneity
161 among sites. The consensus tree was rooted with the “midpoint root” option in FigTree
162 (<http://tree.bio.ed.ac.uk/software/figtree>), and nodes were arranged in increasing
163 order. The resulting tree was annotated with the EvolView software (58). Additionally,
164 we built maximum parsimony trees with the software BioNumerics version 7.5 (Applied
165 Maths, Gent, Belgium) with default settings.

166 For the coalescent-based analyses, evolutionary rates and tree topologies were
167 analyzed using the general time-reversible (GTR) and Hasegawa-Kishino-Yano (HKY)
168 substitution models with gamma distributed among-site rate variation with four rate
169 categories (Γ_4). The substitution rate was estimated by plotting a regression line that
170 depicts for the sole WGS clusters, in a pairwise manner, the relationship between the
171 elapsed time and the accumulated number of SNP's. Under this model, the slope
172 corresponds to the mutation rate. We tested both a strict molecular clock (which
173 assumes the same evolutionary rates for all branches in the tree) and a relaxed clock
174 that allows different rates among branches. Constant-size, exponential and Bayesian
175 skyline plot models, based on a general, non-parametric prior that enforces no
176 particular demographic history were used in BEAST v1.10.4 (59). For each model, two
177 independent chains were conducted for 200 million generations and convergence was
178 assessed by checking ESS values for key parameters using TRACER V1.7.1 (60). We
179 used TRACER V1.7.1 to calculate the log₁₀ Bayes factors in order to compare the

180 models after a burn-in of 10% of the chain. Bayes factors represent the ratio of the
181 marginal likelihood of the models being compared. Approximate marginal likelihoods
182 for each coalescent model were calculated via importance sampling (1,000 bootstraps)
183 using the harmonic mean of the sampled likelihoods. A ratio between 3 and 10
184 indicates moderate support that one model better fits the data than another, whereas
185 values greater than 10 indicate strong support. For correlation with known clonal
186 complexes, we selected 33 strains representing the known clades contained in a recent
187 publication (61), and performed a joint analysis as described previously.

188

189 Data availability

190 All WGS data was submitted to the EMBL-EBI ENA SRA archive (supplementary table
191 S1).

192

193 **Ethics statement**

194 Ethical approval was not sought, as no patient data was used.

195

196 **Results**

197 In total, 131 Mbov strains, 93 of human and 38 of animal origin (supplementary table
198 S1) isolated in Germany from 1999–2017, including one *M. bovis* BCG reference
199 strain, were investigated by spoligotyping, MIRU-VNTR-typing, and WGS. WGS data
200 analysis revealed 12,726 variable SNP positions among the genomes analyzed that
201 were used for the calculation of a phylogenetic tree (figure 1). Interestingly, the strain
202 mbov-49 was clearly separated from the rest of the study collection. This strain has

203 been isolated at the FLI in 2000 from a Nilgau antelope (*Boselaphus tragocamelus*),
204 which died in a German zoo, and found to be not intrinsically pyrazinamide resistant
205 (62).

206 Overall, the median pairwise distance in distinct SNP positions of the 131 strains was
207 516 SNPs, and distinct peaks emerged in the frequency distribution between 0-30, 70-
208 350, 370-620, and 780-840 distinct SNPs, agreeing with the groups of related strains
209 found by cluster detection with a threshold of 12, 30, and 350 distinct SNPs (d12, d30,
210 d350) between nearest group members (figure 1, figure 2). Using the d350 threshold
211 to group strains, we found 13 cladistic groups containing 122/131 strains ranging in
212 size from 2-35 members, with on average eight years (2-18) between the earliest and
213 latest year of isolation.

214 Six of the d350 groups contained both human and animal cases, and seven only
215 human cases. When comparing d350 groups with the known clonal complexes African
216 1 and 2 (Af1, Af2), European 1 and 2 (Eu1, Eu2), as well as newly determined Unknown
217 1-8 (61), we could correlate clonal complexes Af1, Eu1, Eu2, and Unknown2 with d350
218 groups 08, 07, 06, and 13 (supplementary figure S1, supplementary table S6). For
219 clonal complexes Af2, Unknown1, and Unknown7, we found only one corresponding
220 strain in our collection (mbov-118, mbov-49, mbov-119). Interestingly, three d350
221 groups (10, 11, 12) were attributed to clonal complex Unknown3, and four d350 groups
222 (01, 02, 03, 04) to clonal complex Unknown4. We found no representatives of
223 complexes Unknown5 and Unknown6 in our study, as well as correlates of d350
224 groups 05 and 09 among the collection of known clonal complexes.

225

226 Putative transmission clusters

227 We used a threshold of at most 12 distinct SNP positions to the nearest group member
228 as indication for possible recent transmission (54), which yielded 13 d12 clusters of
229 altogether 46 strains (figure 1, figure 3, table 1). The d12 clusters ranged in size from
230 2-11 members, spanned up to 15 years and involved distinct host types, with d12
231 clusters 5 and 12 only comprising cattle hosts, clusters 4, 7, 11, and 13 only human
232 hosts, and the rest mixed hosts (table 1). In total, 32 of the 38 animal strains (the pair
233 of Mbov BCG in d12 cluster 13 not counted) were grouped into WGS d12 clusters. In
234 four of these clusters, animal strains were recovered more than nine years apart,
235 pointing towards autochthonous persistent bTB infection cycles. In contrast, only 12
236 out of the 93 human strains were grouped into d12 clusters, with nine human strains
237 forming four WGS d12 clusters of two and three members, respectively (table 1). The
238 members of these groups were isolated within at most two years from each other.
239 Overall, we found one cluster (cluster 8) with a putative transmission from cattle to
240 humans with respective strains separated by two SNPs, and one cluster (cluster 6) of
241 raccoon and human strains separated by 12 SNPs.

242 As the frequency distribution of pairwise SNP distances featured a peak between 0-30
243 SNPs (figure 2), we also clustered strains with a threshold of 30 SNPs. This yielded
244 two new clusters of related strains with two members each, an additional member of
245 d12 cluster 13, and d12 clusters 2 and 8 were joined together (figure 1).

246

247 Comparison with classical genotyping

248 The 131 strains were differentiated into 45 known spoligotypes and 11 spoligotypes
249 not contained in the established databases (supplementary tables S1 and S5). Five or
250 more strains each fell into four known spoligotypes: SB 120/IT0482 (35 strains), SB

251 121/IT0481 (13 strains), and SB 989/IT1118 (12 strains), SB 288/IT685 (5 strains). Of
252 these, SB 120 and SB 121 have been reported as predominant spoligotypes circulating
253 among animals around the world (63). Strains of these spoligotypes were present in
254 different branches of the constructed phylogenetic tree and in different MIRU-VNTR
255 and d12 clusters (figure 1).

256 Comparing the composition of the d350 groups in terms of the respective spoligotypes
257 (figure 1), we found correlations with the well-established clonal complexes 1 and 2 and
258 Af 1 and 2, as well as with the newly determined complexes named unknown 1 – 8(61;
259 supplementary table S7). For example, SB0120 found in d350 groups 01, 02, 04, 05,
260 10, and 13 was detected in complexes Unknown 2–5. This spoligotype has been
261 reported as predominant circulating among animals around the world (63). Seven
262 spoligotypes present in d350 groups 01, 02, 03, and 04 were reported for complex
263 Unknown4 (61). The 15 spoligotypes found for d350 group 06 corresponded to those
264 for complex Eu2, and the nine spoligotypes present in d350 groups 10, 11 and 12 were
265 found in clade Unknown3 (61). The spoligotype SB0989 found in d350 group 09 was
266 reported for singletons not contained in a complex (61).

267 MIRU-VNTR analysis yielded 92 distinct patterns with 21 strain clusters ranging from
268 two to seven members comprising altogether 62 strains. Using 121 supposedly
269 unrelated strains, the discriminatory power index (HGDI; 51) of each of the 24+1-locus
270 MIRU-VNTR loci was determined finding allelic heterogeneity mainly restricted to 2-4
271 repeat copies (supplementary table S3). Allele heterogeneity of > 0.5 was found for the
272 loci VNTR 2163a, 2163b, 2165, 2461 and 4052 (supplementary table S4). Overall,
273 MIRU-VNTR types correlated well with both the phylogenetic tree and the d12 clusters.
274 However, 21 strains grouped by MIRU-VNTR were not clustered by d12 analysis, and

275 four d12 clusters encompassed strains with different MIRU-VNTR patterns, with four
276 distinct loci in one, and one distinct locus in three of these cases (figure 1, figure 3).

277

278 Mutation rate estimation and demographic inference

279 The geographically widespread and phylogenetically diverse nature of our strain
280 collection did not allow implementing a Bayesian tip-dating approach. We therefore
281 focused on the 13 d12 clusters where the measurably evolving dimension of Mbov
282 could be captured to infer a realistic estimation of the mutation rate. A positive
283 correlation ($r^2 = 0.682$) was found between the time elapsed between two strains and
284 the number of accumulated SNPs (figure 4). The slope was close to 1, corresponding
285 to the acquisition of one SNP every year between two strains and translating to a
286 mutation rate of 1.14×10^{-7} substitutions/nucleotide/year.

287 To estimate the effective population size fluctuation through time, three demographic
288 models were compared and the best fitting evolutionary model was obtained under the
289 Bayesian skyline model with a relaxed clock (figure 4). The relaxed clock model
290 outperforms the constant clock model (BF = 40) and the Bayesian skyline was favored
291 to its closest model, constant size (BF = 14). The TMRCA (TIME to Most Recent
292 Common Ancestor) corresponding to our Mbov strain collection dated back some 950
293 years ago (95% HPD [highest posterior density] interval, 836-1062). According to the
294 coalescent-based demographic reconstructions, the German Mbov population went
295 through three successive expansions, a first twentyfold increase in the late middle age,
296 followed by two mild expansions in the middle of 18th century and the early 20th century
297 (figure 4).

298

300 **Discussion**

301 This investigation provides insights into population structure, persistence and
302 population size fluctuation of Mbov strains in Germany over time and the complex
303 interrelations in a multi-host pathogen system. In the context of a country declared
304 officially free of bTB for more than two decades, special consideration was given to
305 strain persistence attempting to understand recurrent outbreaks and possible links to
306 human cases, while other publications have mainly concentrated on microevolution of
307 strains in the context of geospatial spreading and transmission dynamics between
308 animal reservoirs (64, 65).

309

310 The main limitation of our study is that, due to practical limitations related to access to
311 strains, we were not able to collect a fully comprehensive set of Mbov strains from
312 human and animal cases in Germany. Additionally, due to the restrictions set by data
313 protection regulations, the available metadata for the strains was limited to year and
314 host of isolation. Regrettably, this does not allow an epidemiological analysis of the
315 WGS d12 and d30 clusters. Still, our collection covers a time span from 1999-2017
316 and diverse host species. While we took care to identify and remove duplicate strains
317 from the same host, we cannot fully exclude this possibility for human strains.

318

319 We successfully performed WGS for a collection of 93 human and 38 animal Mbov
320 strains, isolated in Germany from 1999–2017. The pairwise distance distribution and
321 the reconstructed phylogenetic tree indicate the presence of 13 d350 groups within the
322 study population. These encompassed the majority of strains (122/131) and represents
323 a snapshot of Mbov sublineages historically spreading in Germany. Correlating our

324 phylogeny and detected groups with described clonal complexes revealed that our
325 collection contains representatives of the well-known Mbov complexes Af1, Af2, Eu1,
326 and Eu2, as well as of additional groups defined recently (61). Interestingly, there are
327 at most two strains of complexes Af1, Af2, and Eu1 in our study, and we found no
328 representatives of complexes Unknown5 and Unknown6, or correlating complexes for
329 d350 groups 05 and 09. This might indicate a geographically uneven distribution of
330 subgroups and that the Mbov phylogeny needs to be refined by WGS-based studies
331 with larger, geographically diverse collections.

332

333 Using a threshold of 12 distinct SNP positions to identify strains possibly involved in
334 recent transmission events (56), we found 32 out of the 38 animal strains and 12 out
335 of the 93 human strains grouped into 13 d12 clusters. In four of these clusters, animal
336 strains were recovered more than nine years apart, pointing towards autochthonous
337 persistent bTB infection cycles. This is further supported by the combination of d12
338 clusters 2 and 8 into a joint group when clustering with a threshold of 30 SNPs, with
339 the phylogenetic analysis and the number of distinct SNP positions suggesting a
340 relatively recent common source for both clusters. Human strains within clusters were
341 isolated within at most one-year difference and with one sole exception had at most
342 one SNP distance, possibly indicating direct transmission.

343 Despite the imbalance of Mbov strains included from humans and animals, there seem
344 to be distinct infection dynamics for animals and humans. For cattle and other animals,
345 the majority of strains were found within d12 clusters and several strains were
346 persistently spreading over up to 15 years, pointing towards potential reservoirs of
347 these strains, for example in the German wildlife population. The mostly un-clustered
348 human cases might represent progression to active disease from latently infected
349 individuals as indicated previously (17). In general, human mobility is also higher

350 compared to cattle and wild animals. Here, patients having contacts to sources of
351 infection outside Germany may contribute to the detected high diversity of strains
352 isolated from human patients. As reported in 2003 (17), the majority of patients with
353 Mbov disease in Germany, was over 60 years of age suggesting that they might have
354 acquired the infection at a young age when the prevalence of bTB in cattle in Germany
355 was much higher than today. Unfortunately, Mbov strains isolated from cattle before
356 1999 were not available.

357

358 Two of the d12 clusters (6 and 8) contained both animal and human strains, indicating
359 possible recent transmission between humans and animals. The detection of only one
360 human strain contained in a d12 cluster with cattle strains may indicate that the overall
361 risk of human infection with Mbov is low with respect to consumption of food (milk,
362 meat) or direct contact to indigenous cattle, while transmission can happen in
363 outbreaks settings.

364 The study results show that WGS is superior in unequivocally detecting genetic
365 relationship between strains and clarify transmission routes compared to spoligotyping
366 and MIRU-VNTR. While spoligotyping provides some information of strain relatedness,
367 our results demonstrate that it cannot reliably establish clusters of related strains.
368 MIRU-VNTR typing results correlated well with WGS data. However, MIRU-VNTR
369 cannot accurately trace gradual evolution within a transmission cluster. Twenty-one
370 strains clustered by MIRU-VNTR were not clustered by d12 analysis, and four d12
371 clusters encompassed strains with distinct MIRU-VNTR patterns.

372 We estimated a mutation rate of 1.14×10^{-7} substitutions/nucleotide/year for Mbov. A
373 recent publication on the molecular clock with over 6,000 samples representing the
374 global diversity and covering different epidemiological settings estimated a clock rate

375 between 1×10^{-8} and 5×10^{-7} , while stating that sampling times below 15-20 years could
376 be insufficient to calibrate a clock rate (67). In another study dealing explicitly with
377 globally distributed Mbov strains, the clock rate was estimated between 6.66×10^{-8} and
378 1.26×10^{-7} (61). Our collection of 131 samples of German Mbov strains spans a time
379 period of 19 years, maybe limiting our ability to estimate the clock rate. However, the
380 rate we inferred is in full agreement with estimates published for *M. tuberculosis*
381 outbreaks in Germany (37) and Eurasia (66). Estimates of the effective population size
382 fluctuation through time according to coalescent-based demographic reconstructions
383 suggested that, the German Mbov population went through three successive
384 expansions, a first twentyfold increase in the late middle age, followed by two mild
385 expansions in the mid 18th century and the early 20th century (figure 4). These
386 expansions might be due to increasing growth and movement of human and cattle
387 populations as well as increasing growth of human communities and of intensive
388 animal husbandry with time. The population size sharply declined after the 1970's,
389 underlining the absence of ongoing epidemics in Germany and confirming the bTB free
390 status of the country. Indirectly supporting the data, the Bayesian skyline detected an
391 anticlimax in the 1740 to 1760 period. This observation coincides with the cattle plague
392 outbreak (RPV virus) that severely impacted the European stocks during that period
393 (68).

394 In conclusion, in this study for the first time the persistence of infectious cycles of Mbov
395 in the officially bTB free country of Germany over more than ten years has been clearly
396 demonstrated pointing towards the challenges controlling this pathogen. As
397 exemplified here, WGS is definitively the method of choice for establishment of an
398 integrated molecular surveillance of Mbov as well as for outbreak investigations.

399

400 **Acknowledgements**

401 We thank V. Mohr, F. Boysen, T. Ubben, A. Lüdemann, U. Brommer and G. Kauth for
402 excellent technical assistance. Parts of the work have been funded by grants from
403 German Center for Infection Research, Federal Ministry of Education and Research,
404 Germany, from Deutsche Forschungsgemeinschaft (DFG, German Research
405 Foundation) under Germany's Excellence Strategy – EXC 22167-390884018, and
406 grants from the Leibniz Science Campus EvoLUNG.

407 All authors provided substantial scientific contributions, have read and approved the
408 final manuscript and agreed to the submission. Furthermore, all authors disclose any
409 conflicts of interest relevant to this study.

410

411

412

413

414 **References**

- 415 1. UN general assembly high-level meeting on ending TB. 26 September 2018, New
416 York. www.who.int/tb/features_archive/UNGA_HLM_ending_TB/en/.
- 417 2. World Health Organization. Global tuberculosis report 2019. [released on 17
418 October 2019]. www.who.int/tb/publications/global_report/en/ last accessed
419 1.1.2020.
- 420 3. Riojas MA, McGough KJ, Rider-Riojas CJ, Rastogi N, Hazbón MH. Phylogenomic
421 analysis of the species of the *Mycobacterium tuberculosis* complex demonstrates
422 that *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium caprae*,
423 *Mycobacterium microti* and *Mycobacterium pinnipedii* are later heterotypic

- 424 synonyms of *Mycobacterium tuberculosis*. 2018. *Int J Syst Evol Microbiol*.
425 68:324-332.
- 426 4. O'Reilly LM, Daborn CJ. 1995. The epidemiology of *Mycobacterium bovis*
427 infections in animals and man: a review. 1995. *Tuber Lung Dis*. 76 (Suppl 1):S1-
428 46.
- 429 5. Palmer MV, Thacker TC, Waters WR, Gortázar C, Corner LA. 2012.
430 *Mycobacterium bovis*: A model pathogen at the interface of livestock, wildlife, and
431 humans. *Vet Med Int*: 236205. doi: 10.1155/2012/236205. Epub 2012 Jun 10.
- 432 6. Müller B, Dürr S, Alonso S, Hattendorf J, Laisse CJ, Parsons SD, van Helden PD,
433 Zinsstag J. 2013. Zoonotic *Mycobacterium bovis*-induced tuberculosis in
434 humans. *Emerg Infect Dis* 19: 899-908.
- 435 7. Barasona JA, Vicente J, Díez-Delgado I, Aznar J, Gortázar C, Torres MJ. 2017.
436 Environmental presence of *Mycobacterium tuberculosis* complex in aggregation
437 points at the wildlife/livestock interface. *Transbound Emerg Dis* 64:1148-1158.
- 438 8. Council Directive 64/432/EEC of 26 June 1964 on animal health problems
439 affecting intra-Community trade in bovine animals and swine. [https://eur-
440 lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A31964L0432](https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A31964L0432)
- 441 9. European Commission Health & Consumer Protection Directorate-General,
442 Veterinary and International Affairs, Unit G5 –Veterinary Programmes. Working
443 Document on Eradication of Bovine Tuberculosis in the EU Accepted by the
444 Bovine tuberculosis subgroup of the Task Force on monitoring animal disease
445 eradication.
446 SANCO/10067/2013.[https://ec.europa.eu/food/sites/food/files/animals/docs/
447 diseases_erad_tb_workingdoc2006_en.pdf](https://ec.europa.eu/food/sites/food/files/animals/docs/diseases_erad_tb_workingdoc2006_en.pdf)
- 448 10. Meyn A. 1961. Die Fortschritte der Rindertuberkulosebekämpfung in der
449 Bundesrepublik. *Monatsh f Tierheilkunde* 14: 71-78.

- 450 11. Probst C, Freuling C, Moser I, Geue L, Köhler H, Conraths FJ, , Kramer M. 2011.
451 Bovine tuberculosis: making a case for effective surveillance. *Epidemiol Infect*
452 139:105-112.
- 453 12. Kohl TA, Utpatel C, Niemann S, Moser I. *Mycobacterium bovis* persistence in two
454 different captive wild animal populations in Germany: a longitudinal molecular
455 epidemiological study revealing pathogen transmission by whole-genome
456 sequencing. 2018. *J Clin Microbiol*. 56. pii: e00302-18. doi: 10.1128/JCM.00302-
457 18. Print 2018 Sep.
- 458 13. European Food Safety Authority and European Centre for Disease Prevention
459 and Control (EFSA and ECDC). The European Union summary report on trends
460 and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017
461 EFSA Journal 2018; 16 (12):5500. Approved: 19 November 2018. Amended: 18
462 February 2019.
463 www.efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2018.5500
- 464 14. Annual Animal Health Reports. www.fli.de/en/publication
- 465 15. Prodinger WM, Brandstätter A, Naumann L, Pacciarini M, Kubica T, Boschioli
466 ML, Aranaz A, Nagy G, Cvetnic Z, Ocepek M, Skrypyk A, Erler W, Niemann S,
467 Pavlik I, Moser I. 2005. Characterization of *Mycobacterium caprae* isolates from
468 Europe by mycobacterial interspersed repetitive unit genotyping. *J Clin Microbiol*
469 43:4984-492.
- 470 16. Loiseau C, Menardo F, Aseffa A, Hailu E, Gumi B, Ameni G, Berg S, Rigouts L,
471 Robbe-Austerman S, Zinsstag J, Gagneux S, Brites D. 2020. An African origin
472 for *Mycobacterium bovis*. *Evol Med Public Health*. 2020:49-59. doi:
473 10.1093/emph/eoaa005. eCollection 2020.

- 474 17. Kubica T, Rüscher-Gerdes S, Niemann S. 2003. *Mycobacterium bovis*_subsp.
475 *caprae* caused one-third of human *M. bovis*-associated tuberculosis cases
476 reported in Germany between 1999 and 2001. J Clin Microbiol 41:3070-3077.
- 477 18. Domogalla J, Prodinger WM, Blum H, Krebs S, Gellert S, Müller M, Neuendorf E,
478 Sedlmaier F, Büttner M. Region of difference 4 in alpine *Mycobacterium caprae*
479 isolates indicates three variants.2013. J Clin Microbiol 51:1381-1388.
- 480 19. Epidemiologisches Bulletin 11/12 2019 - RKI
481 www.rki.de › Content › Infekt › EpidBull › Archiv › 2019.
- 482 20. Brooks-Pollock E, Roberts GO, Keeling MJ. 2014. A dynamic model of bovine
483 tuberculosis spread and control in Great Britain. Nature 511:228-231.
- 484 21. Comas I, Homolka S, Niemann S, Gagneux S. Genotyping of genetically
485 monomorphic bacteria. 2009. DNA sequencing in *Mycobacterium tuberculosis*
486 highlights the limitations of current methodologies. PLoS One 4:e7815. doi:
487 10.1371/journal.pone.0007815.
- 488 22. Schürch AC, van Soolingen D. DNA fingerprinting of *Mycobacterium tuberculosis*:
489 from phage typing to whole-genome sequencing. 2012. Infect Genet Evol.
490 12:602-609.
- 491 23. Merker M, Kohl TA, Niemann S, Supply P. 2017. The evolution of strain typing in
492 the *Mycobacterium tuberculosis* Complex. Adv Exp Med Biol 1019:43-78.
- 493 24. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B.
494 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting:
495 recommendations for a standardized methodology. J Clin Microbiol 31:406-409.
- 496 25. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S,
497 Bunschoten A, Molhuizen H, Shaw R, Goyal M, van Embden J.1997.
498 Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis*
499 for diagnosis and epidemiology. J Clin Microbiol 35: 907-1014.

- 500 26. Cousins DV, Skuce RA, Kazwala RR, van Embden JD. 1998. Towards a
501 standardized approach to DNA fingerprinting of *Mycobacterium bovis*.
502 International Union against Tuberculosis and Lung Disease, Tuberculosis in
503 Animals Subsection. Int J Tuberc Lung Dis 2 471-478.
- 504 27. Frothingham R, Meeker-O'Connell WA. 1998. Genetic diversity in the
505 *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA
506 repeats. Microbiology 144:1189-1196.
- 507 28. Skuce RA, Neill SD. 2001. Molecular epidemiology of *Mycobacterium bovis*:
508 exploiting molecular data. Tuberculosis (Edinb.) 81: 169-175.
- 509 29. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsç-Gerdes S, Willery E,
510 Savine E, de Haas P, van Deutekom H, Roring S, Bifani P, Kurepina N, Kreiswirth
511 B, Sola C, Rastogi N, Vatin V, Gutierrez MC, Fauville M, Niemann S, Skuce R,
512 Kremer K, Locht C, van Soolingen D. 2006. Proposal for standardization of
513 optimized mycobacterial interspersed repetitive unit-variable-number tandem
514 repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 44: 4498-4510.
- 515 30. Oelemann MC, Diel R, Vatin V, Haas W, Rüsç-Gerdes S, Locht C, Niemann S,
516 Supply P. 2007. Assessment of an optimized mycobacterial interspersed
517 repetitive- unit-variable-number tandem-repeat typing system combined with
518 spoligotyping for population-based molecular epidemiology studies of
519 tuberculosis. J Clin Microbiol 45:691-697.
- 520 31. Wirth T, Hildebrand F, Allix-Béguet C, Wölbeling F, Kubica T, Kremer K, van
521 Soolingen D, Rüsç-Gerdes S, Locht C, Brisse S, Meyer A, Supply P, Niemann
522 S. 2008. Origin, spread and demography of the *Mycobacterium tuberculosis*
523 complex. PLoS Pathog 4(9):e1000160. doi: 10.1371/journal.ppat.10001
- 524 32. Web Page of the SITVIT database,
525 www.pasteurguadeloupe.fr:8081/SITVIT_ONLINE

- 526 33. SITVIT2. www.pasteur-guadeloupe.fr:8081/SITVIT2/
- 527 34. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, Allix C,
528 Aristimuño L, Arora J, Baumanis V, Binder L, Cafrune P, Cataldi A, Cheong S,
529 Diel R, Ellermeier C, Evans JT, Fauville-Dufaux M, Ferdinand S, Garcia de
530 Viedma D, Garzelli C, Gazzola L, Gomes HM, Gutierrez MC, Hawkey PM, van
531 Helden PD, Kadival GV, Kreiswirth BN, Kremer K, Kubin M, Kulkarni SP, Liens
532 B, Lillebaek T, Ho ML, Martin C, Martin C, Mokrousov I, Narvskaiä O, Ngeow YF,
533 Naumann L, Niemann S, Parwati I, Rahim Z, Rasolofo-Razanamparany V,
534 Rasolonavalona T, Rossetti ML, Rüsç-Gerdes S, Sajduda A, Samper S,
535 Shemyakin IG, Singh UB, Somoskovi A, Skuce RA, van Soolingen D, Streicher
536 EM, Suffys PN, Tortoli E, Tracevska T, Vincent V, Victor TC, Warren RM, Yap
537 SF, Zaman K, Portaels F, Rastogi N, Sola C. 2006. *Mycobacterium tuberculosis*
538 complex genetic diversity: mining the fourth international spoligotyping database
539 (SpolDB4) for classification, population genetics and epidemiology. BMC
540 Microbiol 6:23.
- 541 35. The *Mycobacterium bovis* Spoligotype Database. www.mbovis.org
- 542 36. Allix-Béguec C, Harmsen D, Weniger T, Supply P, Niemann S. 2008. Evaluation
543 and strategy for use of MIRU-VNTRplus, a multifunctional database for online
544 analysis of genotyping data and phylogenetic identification of *Mycobacterium*
545 *tuberculosis* complex isolates. J Clin Microbiol 46:2692-2699.
- 546 37. Weniger T, Krawczyk J, Supply P, Niemann S, Harmsen D. 2010. MIRU-
547 VNTRplus: a web tool for polyphasic genotyping of *Mycobacterium tuberculosis*
548 complex bacteria. Nucleic Acids Res 38 (Web Server issue):W326-31.
- 549 38. Roetzer A, Diel R, Kohl TA, Rückert C, Nübel U, Blom J, Wirth T, Jaenicke S,
550 Schuback S, Rüsç-Gerdes S, Supply P, Kalinowski J, Niemann S. 2013. Whole
551 genome sequencing versus traditional genotyping for investigation of a

- 552 *Mycobacterium tuberculosis* outbreak: a longitudinal molecular epidemiological
553 study. PLoS Med 10:e1001387. doi: 10.1371/journal.pmed.1001387.
- 554 39. Eyre DW, Cule ML, Wilson DJ, Griffiths D, Vaughan A, O'Connor L, Ip CLC,
555 Golubchik T, Batty EM, Finney JM, Wyllie DH, Didelot X, Piazza P, Bowden R,
556 Dingle KE, Harding RM, Crook DW, Wilcox MH, Peto TEA, Walker AS. 2013.
557 Diverse sources of *C. difficile* infection identified on whole-genome sequencing.
558 N Engl J Med 369: 1195-1205.
- 559 40. Harrison EM, Paterson GK, Holden MT, Larsen J, Stegger M, Larsen AR,
560 Petersen A, Skov RL, Christensen JM, Bak Zeuthen A, Heltberg O, Harris SR,
561 Zadoks RN, Parkhill J, Peacock SJ, Holmes MA. 2013. Whole genome
562 sequencing identifies zoonotic transmission of MRSA isolates with the novel
563 *mecA* homologue *mecC*. EMBO Mol. Med 5:509-515.
- 564 41. Niemann S, Merker M, Kohl T, Supply P. 2016. Impact of genetic diversity on the
565 biology of *Mycobacterium tuberculosis* complex strains. Microbiol Spectr 4(6).
566 doi: 10.1128/microbiolspec.TB2-0022-2016.
- 567 42. Biek R, Pybus OG, Lloyd-Smith JO, Didelot X. 2015. Measurably evolving
568 pathogens in the genomic era. Trends Ecol. Evol 30:306-313.
- 569 43. Kao RR, Price-Carter M, Robbe-Austerman S. 2016. Use of genomics to track
570 bovine tuberculosis transmission. Rev Sci Tech 35:241-258.
- 571 44. Bjorn-Mortensen K, Soborg B, Koch A, Ladefoged K, Merker M, Lillebaek T,
572 Andersen AB, Niemann S, Kohl TA. 2016. Tracing *Mycobacterium tuberculosis*
573 transmission by whole genome sequencing in a high incidence setting: a
574 retrospective population-based study in East Greenland. Sci Rep 6:33180.
- 575 45. Bjorn-Mortensen K, Lillebaek T, Koch A, Soborg B, Ladefoged K, Sørensen HC,
576 Kohl TA, Niemann S, Andersen AB. 2017. Extent of transmission captured by

- 577 contact tracing in a tuberculosis high endemic setting. Eur Respir J 49(3). pii:
578 1601851. doi: 10.1183/13993003.01851-2016.
- 579 46. DIN Deutsches Institut für Normung e. V. Medical microbiology – Diagnosis of
580 tuberculosis – Part 3: Detection of mycobacteria by culture methods; Text in
581 German and English; DIN 58943-3: 2011-03. In (Ed.). DIN-Taschenbuch 222 -
582 Medizinische Mikrobiologie und Immunologie - Diagnostische Verfahren“. Beuth
583 Verlag Berlin; 2011.pp 338 – 356.
- 584 47. Rodriguez JG, Mejia GA, Del Portillo P, Patarroyo ME, Murillo LA. 1995. Species-
585 specific identification of *Mycobacterium bovis* by PCR. Microbiology141:2131-
586 2138.
- 587 48. Moser I, Prodingler WM, Hotzel H, Greenwald R, Lyashchenko KP, Bakker D,
588 Gomis D, Seidler T, Ellenberger C, Hetzel U, Wuennemann K, Moisson P. 2008.
589 *Mycobacterium pinnipedii*: transmission from South American sea lion (*Otaria*
590 *byronia*) to Bactrian camel (*Camelus bactrianus bactrianus*) and Malayan tapirs
591 (*Tapirus indicus*). Vet Microbiol 127: 399-406.
- 592 49. Berg S, Firdessa R, Habtamu M, Gadisa E, Mengistu A, Yamuah L, Ameni G,
593 Vordermeier M, Robertson BD, Smith NH, Engers H, Young D, Hewinson RG,
594 Aseffa A, Gordon SV. 2009. The burden of mycobacterial disease in Ethiopian
595 cattle: implications for public health. PLoS One 4:e5068. doi:
596 10.1371/journal.pone.0005068.
- 597 50. van Soolingen D, Hermans PW, de Haas PE, Soll DR, van Embden JD. 1991.
598 Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis*
599 complex strains: evaluation of an insertion sequence dependent DNA
600 polymorphism as a tool in the epidemiology of tuberculosis. J Clin Microbiol
601 29:2578–2586.

- 602 51. Ruettger A, Nieter J, Skrypnyk A, Engelmann I, Ziegler A, Moser I, Monecke S,
603 Ehricht R, Sachse K. 2012. Rapid spoligotyping of *Mycobacterium tuberculosis*
604 complex bacteria by use of a microarray system with automatic data processing
605 and assignment. J Clin Microbiol 50:2492-2495.
- 606 52. Skuce RA, McCorry TP, McCarroll JF, Roring SM, Scott AN, Brittain D, Hughes
607 SL, Hewinson RG, Neill SD. 2002. Discrimination of *Mycobacterium tuberculosis*
608 complex bacteria using novel VNTR-PCR targets. Microbiology 148:519-528.
- 609 53. Hunter PR, Gaston MA. 1988. Numerical index of the discriminatory ability of
610 typing systems: an application of Simpson's index of diversity. Clin Microbiol
611 26:2465-2466
- 612 54. Baym M, Kryazhimskiy S, Lieberman TD, Chung H, Desai MM, Kishony R. 2015.
613 Inexpensive multiplexed library preparation for megabase-sized genomes. PLoS
614 One 10:e0128036.
- 615 55. Kohl TA, Utpatel C, Schleusener V, De Filippo MR, Beckert P, Cirillo DM,
616 Niemann S1. 2018. MTBseq: a comprehensive pipeline for whole genome
617 sequence analysis of *Mycobacterium tuberculosis* complex isolates. PeerJ.
618 13;6:e5895. doi: 10.7717/peerj.5895. eCollection.
- 619 56. Walker TM, Ip CL, Harrell RH, Evans JT, Kapatai G, Dediccoat MJ, Eyre DW,
620 Wilson DJ, Hawkey PM, Crook DW, Parkhill J, Harris D, Walker AS, Bowden R,
621 Monk P, Smith EG, Peto TE. 2013. Whole-genome sequencing to delineate
622 *Mycobacterium tuberculosis* outbreaks: a retrospective observational study.
623 Lancet Infect Dis 13:137–146.
- 624 57. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2--approximately maximum-
625 likelihood trees for large alignments. PLoS One 5: e9490 DOI:
626 10.1371/journal.pone.0009490.

- 627 58. He Z, Zhang H, Gao S, Lercher MJ, Chen WH, Hu S. 2016. Evolview v2: an online
628 visualization and management tool for customized and annotated phylogenetic
629 trees. *Nucleic Acids Research* 44: W236-241.
- 630 59. Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A. 2018.
631 Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10.
632 *Virus Evol* 4(1):vey016. doi: 10.1093/ve/vey016. eCollection 2018 Jan.
- 633 60. Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018. Posterior
634 summarization in Bayesian phylogenetics using Tracer 1.7. *Syst Biol.* 67:901-
635 904.
- 636 61. Loiseau C, Menardo F, Aseffa A, Hailu E, Gumi B, Ameni G, Berg S, Rigouts L,
637 Robbe-Austerman S, Zinsstag J, Gagneux S, Brites D. 2020. An African origin
638 for *Mycobacterium bovis*. *Evol Med Public Health* 2020:49-59.
- 639 62. Loiseau C, Brites D, Moser I, Coll F, Pourcel C, Robbe-Austerman S, Escuyer V,
640 Musser KA, Peacock SJ, Feuerriegel S, Kohl TA, Niemann S, Gagneux S, Köser
641 CU. 2019. Revised interpretation of the Hain Lifescience GenoType MTBC to
642 differentiate *Mycobacterium canettii* and members of the *M. tuberculosis*
643 complex. *Antimicrob Agents Chemother.* 24;63. pii: e00159-19. doi: 10.1
644 128/AAC.00159-19.
- 645 63. Ghavidel M, Mansury D, Nourian K, Ghazvini K. 2018. The most common
646 spoligotype of *Mycobacterium bovis* isolated in the world and the recommended
647 loci for VNTR typing; A systematic review. . *Microb Pathog* 118: 310-315.
- 648 64. Trewby H, Wright D, Breadon EL, Lycett SJ, Mallon TR, McCormick C, Johnson
649 P, Orton RJ, Allen AR, Galbraith J, Herzyk P, Skuce RA, Biek R, Kao RR. 2016.
650 Use of bacterial whole-genome sequencing to investigate local persistence and
651 spread in bovine tuberculosis. *Epidemics.* 14:26-35.

- 652 65. Biek R, O'Hare A, Wright D, Mallon T, McCormick C, Orton RJ, McDowell S,
653 Trewby H, Skuce RA, Kao RR. 2012. Whole genome sequencing reveals local
654 transmission patterns of *Mycobacterium bovis* in sympatric cattle and badger
655 populations. PLoS Pathog. 8(11):e1003008. doi: 10.1371/journal.ppat. 1003008.
- 656 66. Merker M, Blin C, Mona S, Duforet-Frebourg N, Lecher S, Willery E, Blum MG,
657 Rüscher-Gerdes S, Mokrousov I, Aleksic E, Allix-Béguec C, Antierens A,
658 Augustynowicz-Kopeć E, Ballif M, Barletta F, Beck HP, Barry CE 3rd, Bonnet M,
659 Borroni E, Campos-Herrero I, Cirillo D, Cox H, Crowe S, Crudu V, Diel R,
660 Drobniewski F, Fauville-Dufaux M, Gagneux S, Ghebremichael S, Hanekom M,
661 Hoffner S, Jiao WW, Kalon S, Kohl TA, Kontsevaya I, Lillebæk T, Maeda S,
662 Nikolayevskyy V, Rasmussen M, Rastogi N, Samper S, Sanchez-Padilla E, Savic
663 B, Shamputa IC, Shen A, Sng LH, Stakenas P, Toit K, Varaine F, Vukovic D,
664 Wahl C, Warren R, Supply P, Niemann S, Wirth T. 2015. Evolutionary history and
665 global spread of the *Mycobacterium tuberculosis* Beijing lineage. Nat Genet.
666 47:242-249.
- 667 67. Menardo F, Duchêne S, Brites D, Gagneux S. 2019. The molecular clock of
668 *Mycobacterium tuberculosis*. PLoS Pathog. 2019 Sep 12;15(9):e1008067.
- 669 68. Broad J. 1983. Cattle plague in eighteenth-century England. Agric. Hist. Rev
670 31:104-15.

671

672 **Figures**

673

674 **Figure 1:** Maximum likelihood tree of 131 Mbov strains built from 12,726 SNP
675 positions, annotated with host organism, isolation year, WGS cluster, MIRU-VNTR
676 types, and spoligotypes from the SITVIT (IT) and mbovis.org (SB) databases. Scale

677 bar indicates the likelihood of per-site substitution and therefore reflects a distance of
678 127 SNPs barring reverse mutations. Circles on nodes indicate resampling support of
679 at least 90% (green circles) or at least 70% (black circles).

680

681 **Figure 2:** Pairwise distance distribution of SNP distances between all sequenced
682 strains (blue) and within WGS d350 groups (red), d30 clusters (purple), and d12
683 clusters (yellow), with the color indicator for the respective lower thresholds
684 superimposed. The y-axis indicates the total number of pairwise distances and x-axis
685 the number of distinct SNPs.

686

687 **Figure 3: A** Maximum parsimony trees for the 13 WGS clusters, annotated with host
688 of isolation. Numbers on branches indicate number of distinct SNPs, distances of 1 are
689 not indicated. **B** Maximum parsimony trees for the 13 WGS clusters, annotated with
690 MIRU-VNTR types. Numbers on branches indicate number of distinct SNPs, distances
691 of 1 are not indicated.

692

693 **Figure 4:** Bayesian skyline plot showing the effective population size of the German
694 Mbov sample through time, estimated from the SNP matrix. According to the
695 coalescent-based approach, the Mbov population went through three successive
696 expansions followed by a final decline. **Plot-in-plot** Root-to-tip genetic distances
697 plotted against sampling dates based on 13 WGS clusters. The figure illustrates a
698 positive correlation ($r^2 = 0.682$) of divergence with sampling date and confirms that
699 Mbov is a measurably evolving population (MEP).

700

701

702

703 **Tables**

704 **Table 1:** Synopsis of the 13 d12 clusters as deduced from the maximum likelihood tree
705 built from 131 Mbov strains. To the clusters, the number of strains, the years of
706 isolation, spanning time, the maximum distance as indicated by the number of SNPs
707 and the host organisms are annotated.

708

709