Comparative Genomic Analysis of *Salmonella enterica* serovar Typhimurium from Passerines Reveals Two Lineages Circulating in Europe, New Zealand, and the United States

Running Title: Genomic Analysis of *S.* Typhimurium from Passerines

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ABSTRACT *Salmonella enterica* serovar Typhimurium from passerines have caused wild bird mortality and human salmonellosis outbreaks in Europe, Oceania, and North America. Here, we performed comparative genomic analysis to explore the emergence, genetic relationship, and evolution of geographically dispersed passerine isolates. We found that passerine isolates from Europe and the United States clustered to form two lineages (EU and US passerine lineages), which were distinct from major *S.* Typhimurium lineages circulating in other diverse hosts (*e.g.*, humans, cattle, pigs, chicken, other avian hosts such as pigeons and ducks). Further, passerine isolates from New Zealand clustered to form a sublineage (NZ passerine lineage) of the US passerine lineage. We inferred that the passerine isolates mutated at a rate of 3.2×10^{-7} substitutions/site/year, and the US, EU, and NZ passerine lineages emerged in *ca.* 1952, 1970, and 1996, respectively. Isolates from the three lineages presented genetic similarity such as lack of antimicrobial resistance genes and accumulation of same virulence pseudogenes. In addition, genetic diversity due to microevolution existed in the three passerine lineages. Specifically, pseudogenization in type 1 fimbrial gene *fimC* (deletion of G at position 87) was only detected in the US and NZ passerine isolates, while a single-base deletion in type 3 secretion system effector genes (*i.e.*, *gogB*, *sseJ*, and *sseK2*) solely concurred in the EU passerine isolates. These findings provide insights into evolution, host adaptation, and epidemiology of *S.* Typhimurium in passerines.

Keywords: *Salmonella enterica* serovar Typhimurium, passerine, outbreak, comparative genomic analysis, host adaptation

IMPORTANCE Passerine-associated *S.* Typhimurium have been linked to human salmonellosis outbreaks in recent years. Here we investigated the phylogenetic relationship of globally distributed passerine isolates and profiled their genomic similarity and diversity. Our study reveals two passerine-associated *S.* Typhimurium lineages circulating in Europe, Oceania, and North America. Isolates from the two lineages presented phylogenetic and genetic signatures that were distinct from isolates of other hosts. The findings shed light on host adaptation of *S.* Typhimurium in passerines and are important for source attribution of *S.* Typhimurium to avian hosts. Further, we found *S.* Typhimurium definitive phage type (DT) 160 from passerines that caused decade-long human salmonellosis outbreaks in New Zealand and Australia formed a sublineage of the US passerine lineage, suggesting that DT160 may have originated from passerines outside Oceania. Our study demonstrates the importance of whole-genome sequencing and genomic analysis of historical microbial collections to modern day epidemiologic surveillance.

INTRODUCTION

Salmonella enterica serovar Typhimurium is a leading cause of salmonellosis worldwide. Generally, *S.* Typhimurium can colonize and infect a broad range of hosts such as humans, livestock, poultry, and wild animals. Examples of broad-host-range *S.* Typhimurium variants circulating worldwide include *S.* Typhimurium definitive phage type (DT) 104 (1) and monophasic *S.* Typhimurium (*S.* 4,[5],12:i:-) sequence type (ST) 34 (2). However, some variants of *S.* Typhimurium are subjected to continuous evolution within specific hosts, thus exhibiting host preference or adaptation. These variants are primarily found in wild birds, which include *S.* Typhimurium DT2 and DT99 from feral pigeons (3), DT8 linked to ducks (4), and DT40 and DT56(v) associated with passerine birds (*i.e.*, any birds in the order *Passeriformes* such as sparrows, siskins, finches) (5, 6). Although the above-mentioned *S.* Typhimurium variants have host tropism to particular wild birds, they occasionally infect humans, domestic animals, and other host species.

In the past decades, passerine-associated *S.* Typhimurium have been linked to salmonellosis outbreaks in both humans and wild birds. In New Zealand, a decade-long (1998-2012) outbreak of *S.* Typhimurium DT160 affected >3,000 people and killed passerines (7). In 2008, *S.* Typhimurium DT160 was identified in Tasmania, Australia, where it infected ≈ 50 people and caused passerine mortality (8). In Europe, outbreaks of passerine-associated human infections have been reported in the United Kingdom (5, 9) and Sweden (10). These outbreaks were caused by *S.* Typhimurium DT40 and DT56(v). In the United States, *S.* Typhimurium isolates associated with the 2009 pine siskin outbreak were implicated in a human salmonellosis outbreak in the same year (11). More recently, a 2021 *S.* Typhimurium outbreak linked to passerines resulted in 29 illnesses and 14 hospitalizations in 12 US states (12).

The emergence of passerine-associated *S.* Typhimurium and corresponding outbreaks worldwide raises the questions about their origin, evolution, and genetic relationship. In this study, we conducted comparative genomic analysis of passerine-associated *S.* Typhimurium from Europe, New Zealand, and the United States over a 40-year period. The genetic relationship and emergence of passerine-associated *S.* Typhimurium from different locations were inferred by phylogenetic analysis and Bayesian inference, respectively. Further, we investigated the genetic content of passerine-associated *S.* Typhimurium by profiling their virulence factors, plasmids, and antimicrobial resistance (AMR) determinants. We also compared the whole-genome sequences of *S.* Typhimurium from passerines and other diverse hosts (*e.g.*, humans, cattle, pigs, poultry, other birds such as pigeons and ducks) to determine if passerine-associated *S.* Typhimurium had distinct phylogenetic and genetic signatures.

RESULTS

Phylogenetic relationship of geographically dispersed *S.* Typhimurium from passerines. A maximum-likelihood phylogenetic tree (Figure 1) was built based on 2,253 single nucleotide polymorphisms (SNPs) in the core genomic regions of 84 publicly available passerine isolates (Table 1; New Zealand: n = 25, isolated year: 2000–2009; United States: n = 33, isolated year: 1978–2019; European countries: United Kingdom: n = 11, Sweden: n = 14, Germany: n = 1, isolated year: 2001–2016) against reference genome of *S.* Typhimurium strain LT2 (RefSeq NC_003197.1). We found that passerine isolates from Europe clustered to form a lineage (henceforth referred to as the EU passerine lineage). Further, we observed that passerine isolates from the United States clustered to form a lineage (henceforth referred to as the US passerine lineage), and *S.* Typhimurium DT160 isolates from New Zealand clustered to form a sublineage

(henceforth referred to as the NZ passerine lineage) of the US passerine lineage (Figure 1). The EU, US, and NZ passerine lineages were supported by robust bootstrap values of 100%. The average SNP distance in the core genome between isolates in the US and NZ passerine lineages was 81, while the average SNP distance in the core genome between isolates in the US, NZ, and EU passerine lineages was 265. Multilocus sequence typing (MLST) indicated that isolates from NZ and US passerine lineages belonged to ST19, whereas the European passerine isolates presented variable STs (*i.e.*, ST19, 568, and 7075).

Emergence time of passerine-associated *S.* Typhimurium lineages in Europe, New Zealand, and the United States. A time-scaled Bayesian phylogenetic tree was built using BEAST2 (v2.6.5) to infer the emergence time of the passerine lineages (Figure 2). The most recent common ancestor (MRCA) of the passerine isolates was estimated to originate in *ca.* 1840 [95% highest probability density (HPD): 1784–1887]. Based on the Bayesian inference, the MRCA evolved to form the US and EU passerine lineages in *ca.* 1952 (95% HPD: 1942–1960) and *ca.* 1970 (95% HPD: 1960–1978), respectively (Figure 2). The NZ passerine lineage formed two sublineages, which emerged in *ca.* 1995 (95% HPD: 1992–1997) and *ca.* 1997 (95% HPD: 1994–1999) (Figure 2). We estimated that the median substitution rate for the 84 passerine isolates was 3.2×10^{-7} substitutions/site/year (95% HPD: $1.8–5.0 \times 10^{-7}$ substitutions/site/year). Median substitution rates for the isolates from the EU and US passerine lineages were 3.2×10^{-7} substitutions/site/year (95% HPD: $1.8–4.6 \times 10^{-7}$ substitutions/site/year) and 3.6×10^{-7} substitutions/site/year (95% HPD: $2.3–5.5 \times 10^{-7}$ substitutions/site/year), respectively. The isolates from the NZ passerine lineage mutated at the same rate as the US passerine isolates.

Antimicrobial resistance, plasmid, and virulence gene profiles of passerine-associated *S.* Typhimurium. Antimicrobial resistance (AMR) profiling (Dataset S1) by ResFinder 2.0 detected

no AMR genes in isolates from the EU, US, and NZ passerine lineages, except the unexpressed gene *aac(6')-Iaa* (13). Plasmid profiling (Dataset S1) by PlasmidFinder 2.0 suggested that all of the EU passerine isolates (26/26) lacked the *S.* Typhimurium-specific virulence plasmid pSLT (Figure 1). However, all of the passerine isolates (25/25) from New Zealand and one third (11/33) of the US passerine isolates carried this plasmid (Figure 1). Virulence gene profiling by ABRicate against the Virulence Factor Database (VFDB) database detected an average number of 107, 110, and 116 virulence genes in the EU, US, and NZ passerine isolates, respectively (Dataset S1). The absent virulence genes in the EU and US passerine isolates were primarily plasmid mediated [*i.e.*, pSLT-mediated virulence genes: *pefABCD* (plasmid-encoded fimbriae), *rck* (resistance to complement killing), and *spvBCR* (*Salmonella* plasmid virulence)] (Dataset S1; Figure 1). Isolates from the EU, US, and NZ passerine lineages possessed the same chromosomal pseudogenes (*i.e.*, *lpfD* and *ratB*) (Figure 1; Table 2). In addition, isolates from the NZ and US passerine lineages had a single-base deletion mutation in type 1 fimbrial gene *fimC*, which was intact in the EU passerine isolates (Figure 1; Table 2). In contrast, single-base deletion mutation in type 3 secretion system (T3SS) effector genes (*i.e.*, *gogB*, *sseJ*, and *sseK2*) was detected in all of the European passerine isolates, however, most of the US and NZ passerine isolates had a single-base substitution rather than deletion mutation in the *gogB* gene, and their *sseJ* and *sseK2* genes were intact (Figure 1; Table 2).

Population structure of *S.* Typhimurium from passerines and other diverse hosts. A maximum-likelihood phylogenetic tree (Figure 3A) was built based on 10,065 SNPs in the core genomic regions of passerine isolates (n = 84) and context isolates (n = 112; Dataset S2) from multiple hosts to represent a broader collection of *S.* Typhimurium. The context isolates formed nine context lineages in the tree (Figure 3A). Six out of the nine context lineages were associated

with specific hosts, *i.e.*, DT2 (n = 13) and DT99 (n = 6) lineages adapted to pigeons, DT8 lineage (n = 10) associated with ducks, ST313 lineage (n = 10) causing invasive nontyphoidal *Salmonella* diseases in humans, DT204 complex lineage (n = 9) primarily infecting cattle, and U288 complex lineage (n = 20) majorly found in pigs. Additionally, three out of the nine context lineages had broad host range, which included DT104 complex (n = 14), DT193 complex (n = 9), and ST34 (n = 21). We found that the EU (n = 26), US (n = 33), and NZ (n = 25) passerine lineages clustered in a large lineage that was distinct from the nine major *S.* Typhimurium lineages circulating globally in different hosts (Figure 3A). The three passerine lineages had the closest genetic relatedness with DT204 complex lineage (primary host: cattle) in the tree (average SNP distance in the core genome ≈ 308). We also generated a neighbor joining (NJ) tree (Figure 3B) of the 84 passerine and 112 context isolates based on EnteroBase whole-genome MLST. The lineages present in the NJ tree were congruent with those formed in the maximum-likelihood phylogenetic tree based on SNPs (Figure 3B).

Genetic comparison of *S.* Typhimurium from different lineages. The average number of virulence genes, plasmid replicons, and AMR genes per isolate from a specific *S.* Typhimurium lineage is shown in Figure 4. Isolates from most of the individual *S.* Typhimurium lineages had an average number of 115–116 virulence genes. However, the average number of virulence genes per isolate from the EU, US passerine lineages and ST34 lineage was less than 110 (Figure 4). Similarly, isolates from these three lineages carried fewer plasmid replicons (average number <1) compared to isolates from other lineages (average number >1). In fact, we identified the absent virulence genes were mostly located on pSLT (*i.e.*, *pefABCD*, *rck*, and *spvBCR*) (Table 3). Moreover, all of the isolates from the EU, NZ, US passerine lineages and DT99 lineage (host: pigeon) lacked identifiable AMR genes (average number = 1; the only AMR gene was *aac(6')*-

183 *Iaa*) (Figure 4). However, isolates from lineages with broad host range (average number >4),
184 adapted to humans (ST313: average number ≈ 7), or associated with specific livestock (DT204
185 complex: average number ≈ 2; U288 complex: average number ≈ 8) had more AMR genes (Figure
186 4).

187 We also identified the virulence gene signatures that can discriminate passerine isolates from
188 isolates of other hosts. Compared to isolates from other lineages, pseudogenization of *fimC* (full
189 length: 708 bp; deletion of G at position 87) was unique to isolates from the US and NZ passerine
190 lineages, while pseudogenization of *gogB* (full length: 1,498 bp; deletion of T at position 1,125),
191 *sseJ* (full length: 1,234 bp; deletion of C at position 976), and *sseK2* (full length: 1,047 bp; deletion
192 of A at position 522) only concurred in isolates from the EU passerine lineages (Table 3).

193

194 **DISCUSSION**

195 Passerine-associated *S.* Typhimurium have a global distribution and are linked to human
196 salmonellosis outbreaks in recent decades. In this study, we explored the emergence, genetic
197 relationship, and evolution of passerine-associated *S.* Typhimurium from Europe, New Zealand,
198 and the United States, and compared the passerine isolates with isolates from diverse hosts. Our
199 study revealed that the EU and US passerine isolates formed two distinct lineages, while the NZ
200 passerine isolates clustered as a sublineage of the US passerine lineage. Further, the emergence of
201 the EU, NZ, and US passerine lineages were relatively recent events. Although the EU and US
202 passerine lineages identified in this study were distinct from each other with different virulence
203 genetic signatures, they clustered in a lineage that was distantly related to the major *S.*
204 Typhimurium lineages formed by isolates from multiple hosts (*i.e.*, humans, cattle, pigs, poultry,
205 pigeons, ducks). One of the caveats of the study is a general paucity of whole-genome sequences

of passerine isolates from Asia, Africa, South America in public database (*e.g.*, NCBI). Integration of passerine isolates from these locations will improve the robustness of the genomic analysis.

Previous epidemiologic survey indicated that passerine salmonellosis was caused by specific *S.* Typhimurium variants. For examples, *S.* Typhimurium DT40 and DT56(v) were the dominant *S.* Typhimurium isolated from passerines suffering from salmonellosis in European countries such as the United Kingdom and Sweden (5, 9, 10). Additionally, *S.* Typhimurium DT160 were demonstrated to be responsible for the human and passerine salmonellosis outbreaks in New Zealand and Australia (7, 8). In the United States, *S.* Typhimurium PFGE (pulsed-field gel electrophoresis) type A3 from pine siskins was identified as the cause of salmonellosis outbreaks both in humans and passerines (11). Although these epidemiologic studies were able to link specific passerine-associated *S.* Typhimurium variants to human and passerine salmonellosis outbreaks through phage typing or PFGE typing, the genetic relationship of these pathovariants from different locations remains unknown. The availability of whole-genome sequences through public database facilitates a high-resolution investigation on the genetic relatedness of globally sourced passerine isolates.

The 84 passerine isolates from the United Kingdom, Sweden, Germany, New Zealand, and the United States clustered in a lineage distinct from the *S.* Typhimurium lineages that have broad host range (*e.g.*, humans, livestock, poultry), such as DT104 complex (1), monophasic *S.* Typhimurium ST34 (2), and DT193 complex (14). The passerine lineage also differed from those narrow-host-range lineages such as U288 complex lineage primarily circulating in pigs (15), DT204 complex lineage majorly infecting cattle (16), DT2 (17) and DT99 (3) lineages adapted to pigeons, DT8 lineage adapted to ducks (4), and ST313 lineage (18, 19) causing invasive human salmonellosis in sub-Saharan Africa. The distinct phylogenetic lineage formed by geographically dispersed

passerine isolates agrees with a previous study, in which Mather et al. (2016) demonstrated that the UK passerine isolates formed a lineage distinct from representative isolates of diverse hosts (*e.g.*, humans, cattle, horses, chicken, pigeons) and geographical regions (20). The phylogenetic signature presented by the passerine isolates from different countries supported the hypothesis that certain *S.* Typhimurium variants have undergone evolution towards a passerine-adapted lifestyle.

Host adaptation is often accompanied by loss-of-function mutation or genome degradation (18, 21–23). For example, loss of virulence for secondary hosts have been observed in *S.* Typhimurium adapted to pigeons (17). In this study, pseudogenization of *lpfD* and *ratB* due to deletion mutation was found in host-adapted *S.* Typhimurium isolates from the DT2, DT8, DT99, DT204 complex, ST313, and the passerine lineages, except isolates from U288 complex lineage possibly adapted to pigs (Table 3). Therefore, the two virulence genes may segregate host-adapted *S.* Typhimurium lineages from lineages with broad host range (*i.e.*, ST34, DT104 complex, DT193 complex). In addition, the passerine isolates accumulated virulence pseudogenes other than *lpfD* and *ratB*. The US and NZ passerine isolates had a single-base deletion in type 1 fimbrial gene *fimC*, while the EU passerine isolates had a concurrent single-base deletion in T3SS effector genes *gogB*, *sseJ*, and *sseK2*. It should be noted these virulence genes were intact in almost all of the isolates from other lineages (Table 3). Therefore, they may be genetic signatures that can discriminate passerine isolates from other-sourced isolates. Further, the *fimC* gene is required for the biosynthesis of type 1 fimbriae, which is involved in adhesion to host cells (24). It has been reported that allelic variation in type 1 fimbriae affected *Salmonella* host specificity (22). In addition, a recent study reported that loss-of-function mutations in T3SS effector genes attenuated pathogenicity of *S.* Typhimurium to humans or mammals but maintained virulence to avian hosts (25). Taken together,

pseudogenization of fimbrial or T3SS effector genes due to frameshift mutation may lead to loss of virulence and contribute to host adaptation of *S.* Typhimurium to the passerine hosts.

The plasmid pSLT is an *S.* Typhimurium-specific virulence plasmid that harbors virulence genes such as *pefABCD*, *rck*, and *spvBCR* (26). These virulence genes are important for *S.* Typhimurium survival and replication in human and mouse macrophages (25, 27, 28). All of the EU passerine isolates (26/26) and two thirds (22/33) of the US passerine isolates lacked pSLT, while most of the isolates from other lineages except monophasic *S.* Typhimurium ST34 harbored this virulence plasmid (Table 3), indicating that the pSLT-mediated virulence was dispensable for *S.* Typhimurium pathogenesis in passerine hosts. The loss of pSLT in passerine isolates is likely an ongoing process as pSLT is only absent in a partial number of the US and NZ passerine isolates. In addition to lack of pSLT, the EU, US, and NZ passerine isolates also lacked AMR genes with the exception of *aac(6')-Iaa*. The absence of AMR genes occurred in DT99 isolates from feral pigeons as well. In contrast, AMR genes were identified in isolates from other diverse hosts, especially those with broad host range (Figure 4). The lack of AMR genes in globally distributed passerine isolates is consistent with our previous study, which revealed the low occurrence of AMR in *S.* Typhimurium from wild birds in the United States (29). A plausible explanation for the observation is that environments utilized by passerine birds are less exposed to antibiotics compared to those utilized by domestic animals and humans. Therefore, isolates from passerines are rarely subjected to antibiotic selection pressure and thus less likely to develop AMR.

The passerine isolates from European countries and the United States formed two lineages closely related to each other (Figure 3; average SNP distance in the core genome ≈ 265), suggesting common ancestry for the two lineages (30). Further, Bayesian inference suggested that the MRCA of the two lineages originated from *ca.* 1840 (Figure 2). As the EU and US passerine lineages were

more closely related (average SNP distance in the core genome ≈ 308) to DT204 complex lineage (primary host: cattle) compared to other lineages in the tree (Figure 3), it is possible that passerine birds acquired the MRCA from domestic animals, potentially cattle. However, more evidence is required to determine the original host of the MRCA and the directionality of transmission. In addition, the emergence of the US, EU, and NZ passerine lineages were estimated after 1950 over short timescales, indicating that host adaptation of *S.* Typhimurium to passerines may be a relatively recent ongoing process driven by anthropogenic influences. The passerine isolates (*i.e.*, *S.* Typhimurium DT160) from New Zealand clustered as a sublineage of the US passerine lineage. Further, the NZ passerine lineage was estimated to emerge in *ca*. 1995–1997 in this study. In a previous study, Bloomfield et al. (2017) reported that the salmonellosis outbreak caused by *S.* Typhimurium DT160 resulted from a single introduction into New Zealand between 1996 and 1998 (7). However, the origin and source of the outbreak have not been identified. Our study reveals the closely genetic relatedness (average SNP distance in the core genome ≈ 81) between isolates from the NZ and US passerine lineages, suggesting that DT160 isolates in New Zealand may have originated from passerines outside Oceania. However, whole-genome sequences of passerine isolates from other locations (*i.e.*, Asia, South America, and Africa) are necessary to conduct further genomic analysis to test this hypothesis.

In conclusion, our study demonstrates the importance of whole-genome sequencing and genomic analysis of historical microbial collections. The findings provide insights into host adaptation of *S.* Typhimurium in passerines and are helpful for modern day epidemiologic surveillance. Host-specific genetic signatures identified in this study can aid source attribution of *S.* Typhimurium to avian hosts in outbreak investigation. As passerines are highly mobile and can spread zoonotic pathogens over a large spatial scale, it is important to raise our awareness of

297 passerines as reservoirs of specific *S.* Typhimurium variants. Although these pathovariants only
298 account for a small number of human salmonellosis cases worldwide, control strategies, for
299 example washing hands after contact with wild birds, would be taken to reduce potential
300 transmission between passerines and humans.
301
302 **MATERIALS AND METHODS**
303 **Dataset selection and quality assessment for raw reads.** Passerine-associated *S.*
304 Typhimurium isolates (Table 1; New Zealand: n = 25, isolated year: 2000–2009; United States: n
305 = 33, isolated year: 1978–2019; European countries: n = 26, isolated year: 2001–2016) were
306 derived from wild birds with confirmed salmonellosis over broad temporal and spatial scales, and
307 some of these isolates also had closely genetic relatedness with human clinical isolates (7, 10, 20,
308 31). Therefore, the isolates were chosen to represent *S.* Typhimurium pathovariants emerging
309 worldwide that caused salmonellosis both in humans and passerines. Context *S.* Typhimurium
310 isolates (Dataset S2; n = 112) were selected to represent the phylogenetic diversity of this serovar
311 across different hosts and geographic locations, and to compare the genomic differences between
312 isolates from passerines and other multiple hosts. The Illumina paired-end reads of the selected
313 isolates were available at the NCBI database (accession number provided in Table 1 and Dataset
314 S2). The quality of the sequence data was assessed using the MicroRunQC workflow in
315 GalaxyTrakr v2 (32). Raw reads meeting the quality control requirements (*i.e.*, average coverage
316 >30, average quality score >30, number of contigs <400, total assembly length between 4.4–5.1
317 Mb) were used for genomic analysis in this study.
318 **Phylogenetic analysis.** The phylogenetic relationship of the 84 passerine isolates was inferred
319 from their core genomes. Snippy (Galaxy v4.5.0) (https://github.com/tseemann/snippy) was used

to generate a whole-genome alignment and find SNPs between the reference genome LT2 (RefSeq NC_003197.1) and the genomes of passerine isolates. Snippy-core (Galaxy v4.5.0) (https://github.com/tseemann/snippy) was used to convert the Snippy outputs (*i.e.*, whole-genome alignment) into a core-genome alignment. The resultant core-genome alignment (2,253 SNPs in the core genomic regions) was used to construct a maximum-likelihood phylogenetic tree by MEGA X (v10.1.8) (33) using the Tamura-Nei model and 500 bootstrap replicates. The SNP phylogenetic tree was visualized and annotated using the Interactive Tree of Life (iTOL v6; https://itol.embl.de). SNP distance between sequences was calculated using snp-dists (Galaxy v0.6.3) (https://github.com/tseemann/snp-dists). Sequence type (ST) of the *S.* Typhimurium isolates was identified using 7-gene (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) MLST at EnteroBase (34). STs were then annotated in the SNP phylogenetic tree. We also generated a maximum-likelihood phylogenetic tree of the 84 passerine isolates (Table 1) and 112 context isolates (Dataset S2) from diverse hosts to represent the genetic diversity within serovar Typhimurium. The tree was created based on 10,065 SNPs in the core genomic regions of the 196 passerine and context isolates with reference to *S.* Typhimurium LT2 using the EnteroBase SNP project (34). In addition, a NJ tree of the 196 passerine and context isolates based on the *Salmonella* whole-genome MLST (21,065 loci) scheme at EnteroBase (34) was built to complement the core-genome SNP-based phylogenetic analysis.

Bayesian inference. A time-scaled Bayesian phylogenetic tree was constructed to determine the divergence times of the *S.* Typhimurium lineages from passerines. The temporal signal of the sequence data was examined using TempEst (35) before phylogenetic molecular clock analysis (Figure S1). The core-genome alignment (2,253 SNPs in the core genomic regions) of passerine isolates (n = 84; Table 1) generated previously was used as the input for the time-scaled tree

343 construction. The parameters for constructing the Bayesian phylogenetic tree were set in BEAUti
344 (v2.6.5) (36) as follows: Prior assumption-coalescent Bayesian skyline; Clock model-relaxed
345 clock log normal with the default clock rate value of 1.0; and Markov chain Monte Carlo (MCMC)
346 at chain length-100 million, storing every 1,000 generations. Two independent runs with the same
347 parameters were performed in BEAST2 (v2.6.5) (36) to ensure convergence. The resultant log
348 files were viewed in Tracer (v1.7.2) to check if the effective sample sizes of all parameters were
349 more than 200 and the MCMC chains were converged. A maximum clade credibility tree was
350 created using TreeAnnotator (v2.6.4) (36) with a burn-in percentage of 10% and node option of
351 median height. Finally, the tree was visualized using FigTree v1.4.4
352 (https://github.com/rambaut/figtree/releases). To determine the substitution rate for the genome of
353 passerine isolates, we multiplied the substitution rate estimated by BEAST2 (v2.6.5) by the
354 number of analyzed core-genome SNPs (2,253 bp), and then divided the product by the average
355 genome size of the analyzed passerine isolates (4,951,383 bp).

356 **Antimicrobial resistance, virulence, and plasmid profiling**. Raw reads of each isolate were
357 *de novo* assembled using Shovill (Galaxy v1.0.4) (37). ABRicate (Galaxy v1.0.1) (38) was used
358 to identify the AMR genes, virulence factors, and plasmid replicons by aligning each draft genome
359 assembly against the ResFinder database (39), VFDB (40), and PlasmidFinder database (41),
360 respectively. For all searches using ABRicate, minimum nucleotide identity and coverage
361 thresholds of 80% and 80% were used, respectively. Virulence genes that were not 100% identical
362 or covered with the reference virulence gene from VFDB may have deletions, insertions, or
363 substitutions of interest. We then manually checked the mutation type by aligning the virulence
364 gene of interest against the reference virulence gene from VFDB using BLAST
365 (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

366 **Data availability**. Sequence data of the *S.* Typhimurium strains are publicly available in the
367 NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra). Accession numbers are
368 available in Table 1 and Dataset S2.
369
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376
377 **AUTHOR CONTRIBUTION**
378 Y.F. designed the study, sequenced the US passerine isolates, collected the globally sourced
379 data from EnteroBase and NCBI, performed the comparative genomic analysis of the data,
380 interpreted the data, and wrote the draft manuscript; N.M.M. and E.G.D. contributed to
381 interpretation of the data and manuscript revision.
382
383 **CONFLICTS OF INTEREST**
384 The authors declare no competing interests.
385
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562 **Table 1.** Metadata information of the 84 *Salmonella enterica* serovar Typhimurium isolates from

563 passerines.

Isolate name	Collection year	Continent	Country	Accession number	Source	Source detail	Sequence type
054/01	2001	Europe	United Kingdom	ERS217365	Passeriformes	House sparrow	568
062/01	2001	Europe	United Kingdom	ERS217362	Passeriformes	European greenfinch	19
065/01	2001	Europe	United Kingdom	ERS217360	Passeriformes	House sparrow	19
100/01	2001	Europe	United Kingdom	ERS217364	Passeriformes	European greenfinch	19
108/01	2001	Europe	United Kingdom	ERS217358	Passeriformes	European greenfinch	568
132/06	2006	Europe	United Kingdom	ERS217361	Passeriformes	European greenfinch	568
1356/06	2006	Europe	United Kingdom	ERS217366	Passeriformes	House sparrow	19
1377/06	2006	Europe	United Kingdom	ERS217363	Passeriformes	House sparrow	568
1402/06	2006	Europe	United Kingdom	ERS217356	Passeriformes	European greenfinch	19
1422/05	2005	Europe	United Kingdom	ERS217359	Passeriformes	European greenfinch	568
20-SA01024-0	2020	Europe	Germany	SRS9340518	Passeriformes	Eurasian blue tit	19
S00228-16	2016	Europe	United Kingdom	ERS2028946	Passeriformes	Finch	19
STm_SE_01	2016	Europe	Sweden	ERR2617828	Passeriformes	Common redpoll	19
STm_SE_02	2016	Europe	Sweden	ERR2617829	Passeriformes	Common redpoll	19
STm_SE_03	2016	Europe	Sweden	ERR2617830	Passeriformes	Common redpoll	19
STm_SE_04	2016	Europe	Sweden	ERR2617831	Passeriformes	Common redpoll	19
STm_SE_05	2016	Europe	Sweden	ERR2617832	Passeriformes	Eurasian bullfinch	19
STm_SE_06	2016	Europe	Sweden	ERR2617833	Passeriformes	Eurasian bullfinch	19
STm_SE_08	2016	Europe	Sweden	ERR2617835	Passeriformes	Eurasian bullfinch	7075
STm_SE_09	2016	Europe	Sweden	ERR2617836	Passeriformes	Eurasian siskin	19
STm_SE_10	2016	Europe	Sweden	ERR2617837	Passeriformes	Eurasian siskin	19
STm_SE_11	2016	Europe	Sweden	ERR2617838	Passeriformes	Eurasian bullfinch	19
STm_SE_12	2016	Europe	Sweden	ERR2617839	Passeriformes	Eurasian bullfinch	19
STm_SE_13	2016	Europe	Sweden	ERR2617840	Passeriformes	Eurasian bullfinch	19
STm_SE_14	2016	Europe	Sweden	ERR2617841	Passeriformes	Eurasian bullfinch	19
STm_SE_15	2016	Europe	Sweden	ERR2617842	Passeriformes	Eurasian bullfinch	19
PSU-2812	1998	North America	United States	SRS7318189	Passeriformes	Evening grosbeak	19
PSU-2813	1998	North America	United States	SRS7318190	Passeriformes	Redpoll	19
PSU-2814	1998	North America	United States	SRS7318191	Passeriformes	American goldfinch,	19
PSU-2816	1998	North America	United States	SRS7318193	Passeriformes	White-throated sparrow	19
PSU-2817	1998	North America	United States	SRS7417389	Passeriformes	Redpoll	19
PSU-2819	2000	North America	United States	SRS7318196	Passeriformes	Gold finch	19
PSU-2835	2009	North America	United States	SRS7611982	Passeriformes	Purple finch	19
PSU-2838	2009	North America	United States	SRS7611983	Passeriformes	Pine siskin	19
PSU-2841	2009	North America	United States	SRS7417441	Passeriformes	House sparrow	19
PSU-2847	2011	North America	United States	SRS7417395	Passeriformes	House sparrow	19
PSU-2966	1978	North America	United States	SRS7417415	Passeriformes	House sparrow	19
PSU-3168	1978	North America	United States	SRS7449455	Passeriformes	House sparrow	19
PSU-3169	1979	North America	United States	SRS7718306	Passeriformes	House sparrow	19
PSU-3174	1991	North America	United States	SRS7449460	Passeriformes	Goldfinch	19
PSU-3234	1992	North America	United States	SRS7612006	Passeriformes	Cardinal	19
PSU-3235	1992	North America	United States	SRS7718330	Passeriformes	House sparrow	19
PSU-3236	1992	North America	United States	SRS7612008	Passeriformes	House sparrow	19
PSU-3253	1991	North America	United States	SRS7611956	Passeriformes	Brown-headed cowbird	19
PSU-3337	2018	North America	United States	SRS7840968	Passeriformes	Redpoll	19
PSU-3338	2019	North America	United States	SRS7840979	Passeriformes	Pine siskin	19
PSU-3340	2016	North America	United States	SRS7840996	Passeriformes	House sparrow	19
PSU-3346	2016	North America	United States	SRS7840969	Passeriformes	Red-winged blackbird	19
PSU-3353	2015	North America	United States	SRS7840976	Passeriformes	Red crossbill	19
PSU-3358	2016	North America	United States	SRS7840981	Passeriformes	Pine siskin	19
PSU-3359	2015	North America	United States	SRS7840982	Passeriformes	Red crossbill	19
PSU-3363	2016	North America	United States	SRS7840986	Passeriformes	Redpoll	19
PSU-3367	2018	North America	United States	SRS7840991	Passeriformes	Pine siskin	19
PSU-3374	2018	North America	United States	SRS7841677	Passeriformes	Redpoll	19
PSU-3377	2016	North America	United States	SRS7841693	Passeriformes	Pine siskin	19
PSU-3378	2015	North America	United States	SRS7841694	Passeriformes	Red crossbill	19
PSU-3379	2015	North America	United States	SRS7841695	Passeriformes	Red crossbill	19
PSU-3405	2013	North America	United States	SRS7841689	Passeriformes	Pine siskin	19
PSU-4760	2012	North America	United States	SRS9461411	Passeriformes	American goldfinch	19
DT160_01	2000	Oceania	New Zealand	ERS1456804	Passeriformes	Passerine bird, not specified	19
DT160_02	2007	Oceania	New Zealand	ERS1456799	Passeriformes	Passerine bird, not specified	19
DT160_03	2000	Oceania	New Zealand	ERS1456794	Passeriformes	Passerine bird, not specified	19
DT160_04	2006	Oceania	New Zealand	ERS1456793	Passeriformes	Passerine bird, not specified	19
DT160_05	2008	Oceania	New Zealand	ERS1456792	Passeriformes	Passerine bird, not specified	19
DT160_06	2003	Oceania	New Zealand	ERS1456791	Passeriformes	Passerine bird, not specified	19
DT160_07	2004	Oceania	New Zealand	ERS1456790	Passeriformes	Passerine bird, not specified	19
DT160_08	2003	Oceania	New Zealand	ERS1456787	Passeriformes	Passerine bird, not specified	19
DT160_09	2007	Oceania	New Zealand	ERS1456781	Passeriformes	Passerine bird, not specified	19
DT160_10	2003	Oceania	New Zealand	ERS1456778	Passeriformes	Passerine bird, not specified	19
DT160_11	2005	Oceania	New Zealand	ERS1456773	Passeriformes	Passerine bird, not specified	19
DT160_12	2001	Oceania	New Zealand	ERS1456770	Passeriformes	Passerine bird, not specified	19
DT160_13	2007	Oceania	New Zealand	ERS1456766	Passeriformes	Passerine bird, not specified	19
DT160_14	2009	Oceania	New Zealand	ERS1456765	Passeriformes	Passerine bird, not specified	19
DT160_15	2008	Oceania	New Zealand	ERS1456760	Passeriformes	Passerine bird, not specified	19
DT160_16	2002	Oceania	New Zealand	ERS1456758	Passeriformes	Passerine bird, not specified	19
DT160_17	2009	Oceania	New Zealand	ERS1456757	Passeriformes	Passerine bird, not specified	19
DT160_18	2004	Oceania	New Zealand	ERS1456756	Passeriformes	Passerine bird, not specified	19
DT160_19	2002	Oceania	New Zealand	ERS1456755	Passeriformes	Passerine bird, not specified	19
DT160_20	2002	Oceania	New Zealand	ERS1456754	Passeriformes	Passerine bird, not specified	19
DT160_21	2001	Oceania	New Zealand	ERS1456749	Passeriformes	Passerine bird, not specified	19
DT160_22	2001	Oceania	New Zealand	ERS1456748	Passeriformes	Passerine bird, not specified	19
DT160_23	2005	Oceania	New Zealand	ERS1456744	Passeriformes	Passerine bird, not specified	19
DT160_24	2005	Oceania	New Zealand	ERS1456734	Passeriformes	Passerine bird, not specified	19
DT160_25	2006	Oceania	New Zealand	ERS1456733	Passeriformes	Passerine bird, not specified	19

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565 **Table 2.** Mutation of specific chromosome-encoded virulence genes in *Salmonella enterica*
566 serovar Typhimurium isolates from passerines[a].

	Chromosome-encoded virulence gene					
	lpfD (length = 1,087 bp)	*ratB* (length = 7,315 bp)	*fimC* (length = 708 bp)	*gogB* (length = 1,498 bp)	*sseJ* (length = 1,234 bp)	*sseK2* (length = 1,047 bp)
US passerine lineage (*n* = 33)	Deletion of GTTTGAGAAT at position 406-415 (33/33)	Deletion of T at position 5,814 (33/33)	Deletion of G at position 87 (33/33)	Substitution: T → C at position 238 (30/33); Gene absence (3/33)	Intact gene (30/33); Substitution: A → C at position 1,110 (2/33); Gene absence (1/33)	Intact gene (30/33); Gene absence (1/33); Deletion of A at position 522 (2/33)
NZ passerine lineage (*n* = 25)	Deletion of GTTTGAGAAT at position 406-415 (25/25)	Deletion of T at position 5,814 (25/25)	Deletion of G at position 87 (25/25)	Substitution: T → C at position 238 (23/23); Deletion of A at position 1,006 (1/23)	Intact gene (23/23)	Intact gene (25/25)
EU passerine lineage (*n* = 26)	Deletion of GTTTGAGAAT at position 406-415 (26/26)	Deletion of T at position 5,814 (24/26); Gene absence (2/26)	Intact gene (26/26)	Deletion of T at position 1,125 (26/26)	Deletion of C at position 976 (26/26)	Deletion of A at position 522 (26/26)

567 [a]Mutation of specific chromosome-encoded virulence genes is identified by aligning draft genomes from passerine
568 isolates against reference genes from *S.* Typhimurium LT2. Mutation position is determined on the plus strand of the
569 reference genes.

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Table 3. Differences in chromosome- and plasmid-mediated virulence genes between *Salmonella enterica* serovar Typhimurium isolates from diverse lineages[a].

	Chromosome-encoded virulence gene (n/N)[c]						pSLT-mediated virulence gene (n/N)[d]					pSLT replicon (n/N)[e]	
***S.* Typhimurium lineage (N)[b]**	***lpfD***	***ratB***	***fimC***	***gogB***	***sseJ***	***sseK2***	***pefABCD***	***spvB***	***spvC***	***spvR***	***rck***	**IncFIB(S)**	**IncFII(S)**
EU passerine lineage (26)	26/26	24/26	26/26	26/26	26/26	26/26	0/26	0/26	0/26	0/26	0/26	0/26	0/26
US passerine lineage (33)	33/33	33/33	33/33	30/33	30/33	30/33	11/33	11/33	11/33	11/33	11/33	11/33	11/33
NZ passerine lineage (25)	25/25	25/25	25/25	25/25	25/25	25/25	25/25	25/25	25/25	25/25	25/25	25/25	25/25
DT204 complex (9)	9/9	9/9	9/9	9/9	9/9	9/9	8/9	8/9	8/9	9/9	9/9	8/9	9/9
ST313 (10)	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
DT99 (6)	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
DT8 (10)	10/10	10/10	10/10	9/10	10/10	10/10	9/10	9/10	9/10	9/10	9/10	9/10	9/10
DT2 (13)	13/13	13/13	13/13	13/13	13/13	11/13	12/13	12/13	12/13	12/13	12/13	12/13	12/13
DT104 complex (14)	14/14	14/14	14/14	14/14	14/14	14/14	13/14	14/14	14/14	14/14	13/14	13/14	13/14
DT193 complex (9)	9/9	9/9	9/9	9/9	9/9	9/9	5/9	7/9	7/9	7/9	6/9	0/9	6/9
U288 complex (20)	20/20	16/20	20/20	19/20	20/20	20/20	15/20	18/20	18/20	11/20	20/20	15/20	20/20
S. 4,[5],12:i:- ST34 (21)	21/21	21/21	21/21	20/21	19/21	20/21	0/21	0/21	0/21	0/21	0/21	0/21	0/21

[a]Green: intact gene; Yellow: deletion mutation; Orange: substitution mutation; Blue: presence; Red: absence; Grey: partial absence.

[b]N: the total number of isolates belonging to a specific lineage.

[c]n/N: the number of isolates carrying an intact or specific mutant virulence gene/the total number of the isolates belonging to a specific lineage.

[d]n/N: the number of isolates carrying a pSLT-mediated virulence gene/the total number of the isolates belonging to a specific lineage.

[e]n/N: the number of isolates carrying a pSLT-associated replicon/the total number of the isolates belonging to a specific lineage.

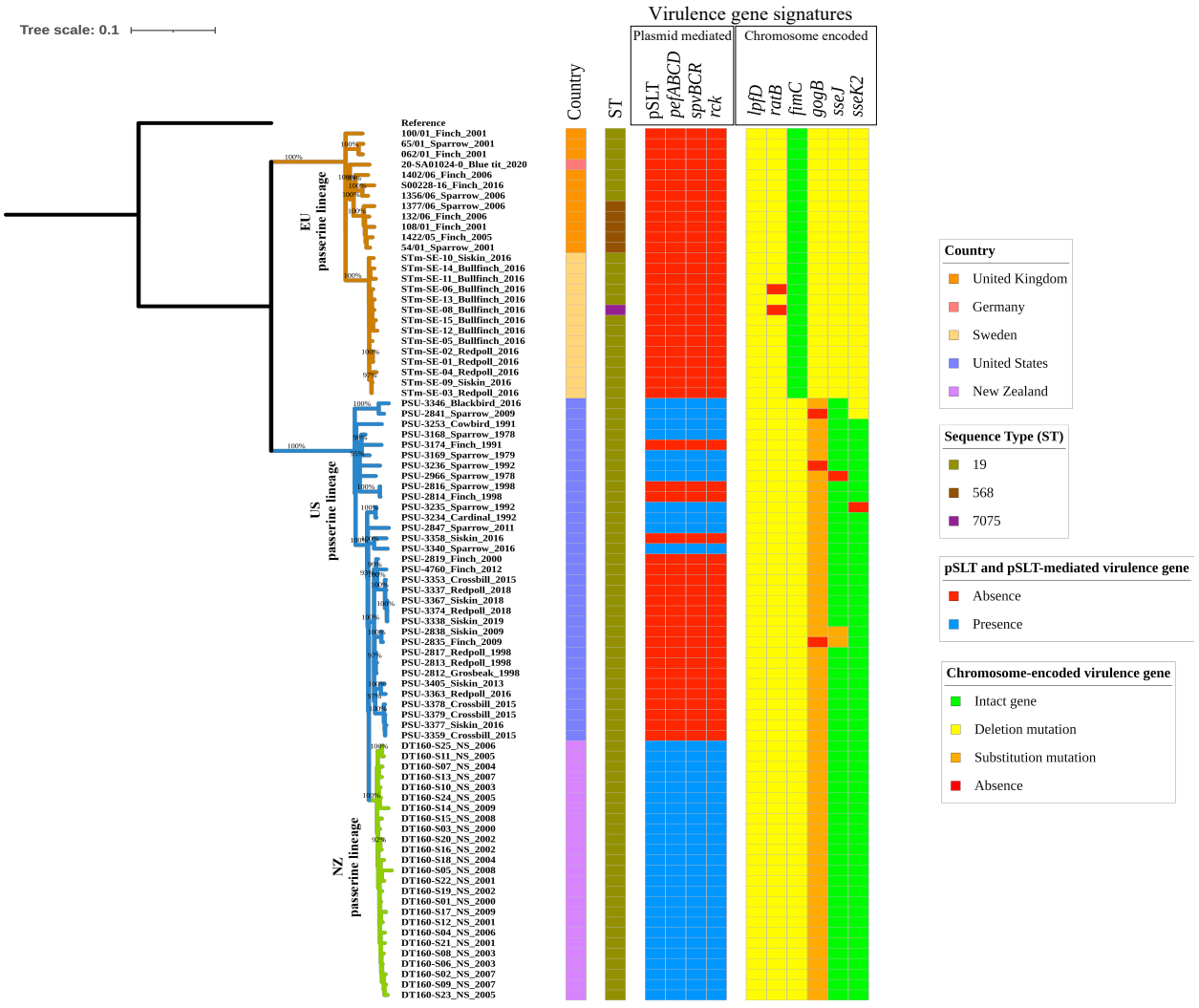

Figure 1. Maximum-likelihood phylogenetic tree of the 84 *Salmonella enterica* serovar Typhimurium isolates from passerines (New Zealand: n = 25, United States: n = 33, United Kingdom: n = 11, Sweden: n = 14, Germany: n = 1). The tree is created based on 2,253 core-genome single nucleotide polymorphisms (SNPs) with reference to *S.* Typhimurium LT2. Three lineages are defined in the tree, *i.e.*, EU (European countries) passerine lineage (brown), US (the United States) passerine lineage (blue), and NZ (New Zealand) passerine lineage (green). Bootstrap values are displayed as percentage on the tree branches. The labels at the tree tips represent the "isolate name_bird host_collection year". "NS" is used to replace "bird host" if the bird host is not specified in the NCBI database. The color strip "country" represents the isolation

location. The color strip "ST" represents the *S.* Typhimurium multilocus sequence type. The virulence gene signatures identified in this study are categorized into plasmid-mediated and chromosome-encoded, and represented in different color strips.

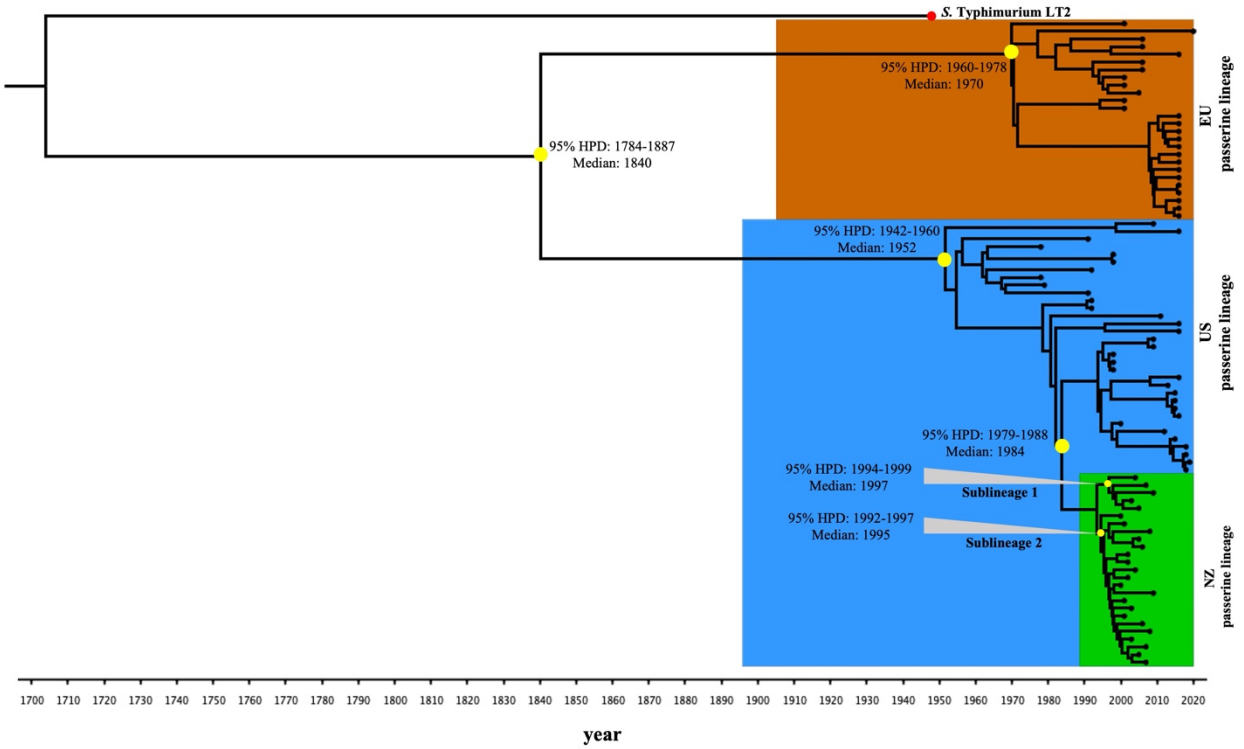

Figure 2. Time-scaled Bayesian phylogenetic tree of the 84 *Salmonella enterica* serovar Typhimurium isolates from passerines. The EU, US, and NZ passerine lineages are colored in brown, blue, and green in the tree, respectively. Median years or range of years at the tree nodes (yellow circles) represent the 95% highest posterior probability density (HPD) for the times of most recent common ancestor for representative divergent events. The red circle at the tree tip represents the reference strain LT2 (collection year: *ca.*1948). The posterior probability values of representative divergent events (yellow circles at tree nodes) are >95%.

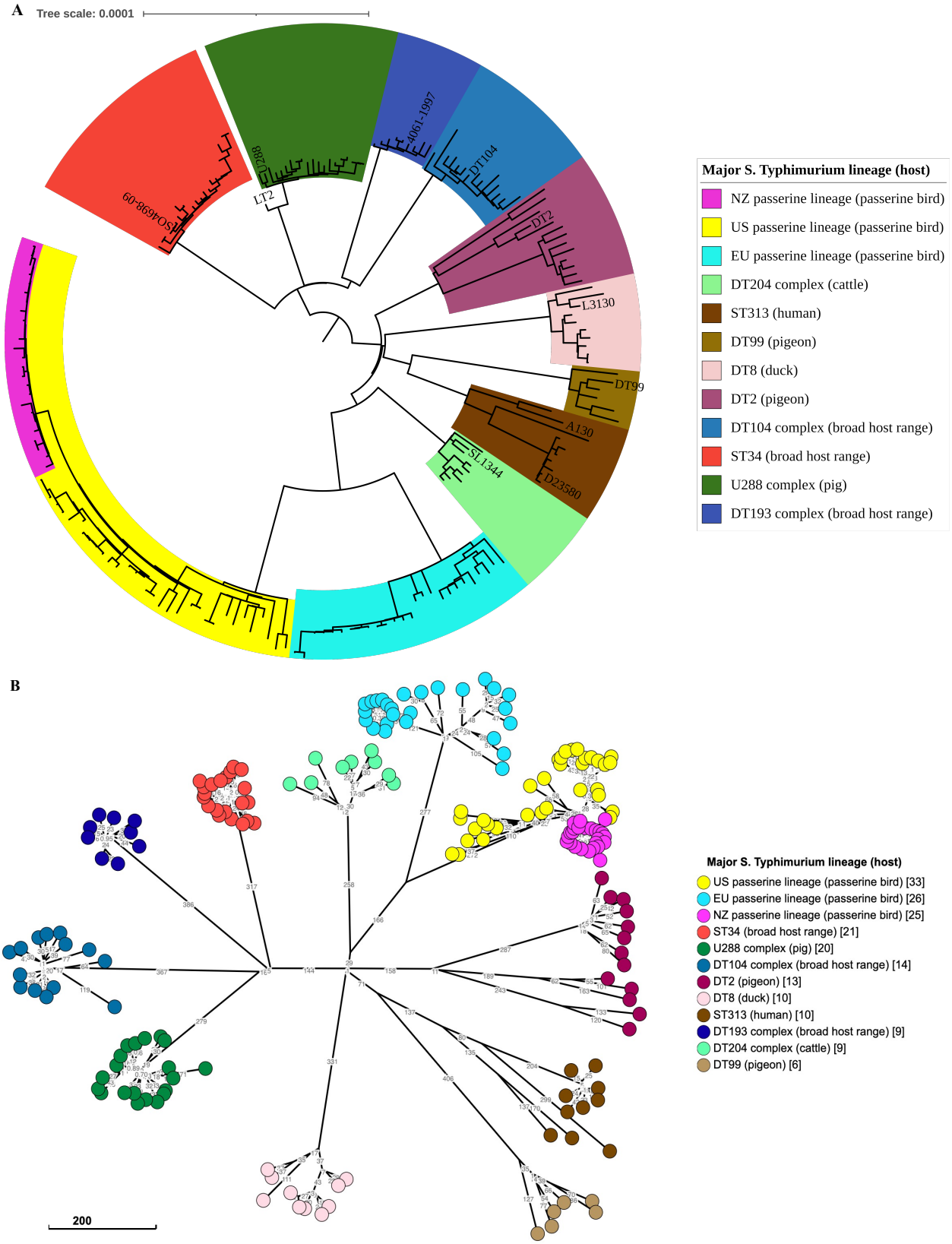

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Figure 3. (**A**) Maximum-likelihood phylogenetic tree of the 196 *Salmonella enterica* serovar Typhimurium isolates from various hosts representing the genetic diversity within the serovar. The tree is created based on 10,065 core-genome single nucleotide polymorphisms (SNPs) with reference to *S.* Typhimurium LT2 and rooted at midpoint. Color ranges in the tree represent the major *S.* Typhimurium lineages identified in the literature and in this study. Labels at the tree tips represent the representative isolates from individual lineages. The legend field at the right of the tree represents the *S.* Typhimurium lineage (primary host). Broad host range in parentheses indicates that isolates from the corresponding lineage are commonly identified among humans, cattle, pigs, poultry, and other hosts or environmental niches. The specific host in parentheses indicates that isolates from the corresponding lineage are primarily from that specific host. (**B**) Neighbor joining tree of the 196 *S.* Typhimurium isolates from various hosts. The tree is created based on allelic differences in the 21,065 loci of the whole-genome multilocus sequence typing (wgMLST) *Salmonella* scheme with GrapeTree at EnteroBase. The major *S.* Typhimurium lineages are highlighted in colors in the tree. The legend field at the right of the tree represents the *S.* Typhimurium lineage (primary host) [number of isolates in the lineage]. The scale bar indicates 200 wgMLST alleles. Allele differences between isolates are indicated by numbers on the connecting lines.

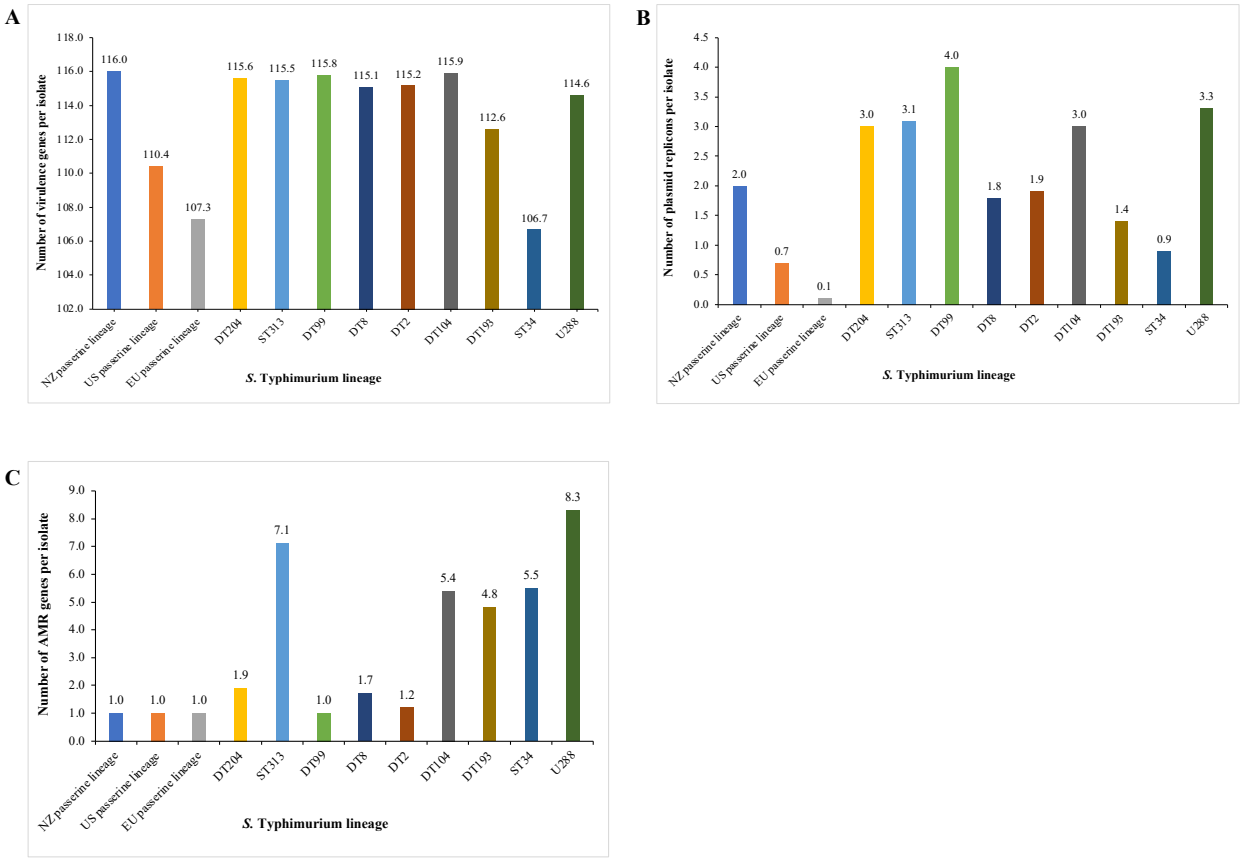

Figure 4. (**A**) Number of virulence genes per isolate detected in a specific *Salmonella enterica* serovar Typhimurium lineage. (**B**) Number of plasmid replicons per isolate detected in a specific *S.* Typhimurium lineage. (**C**) Number of antimicrobial resistance(AMR) genes per isolate detected in a specific *S.* Typhimurium lineage.

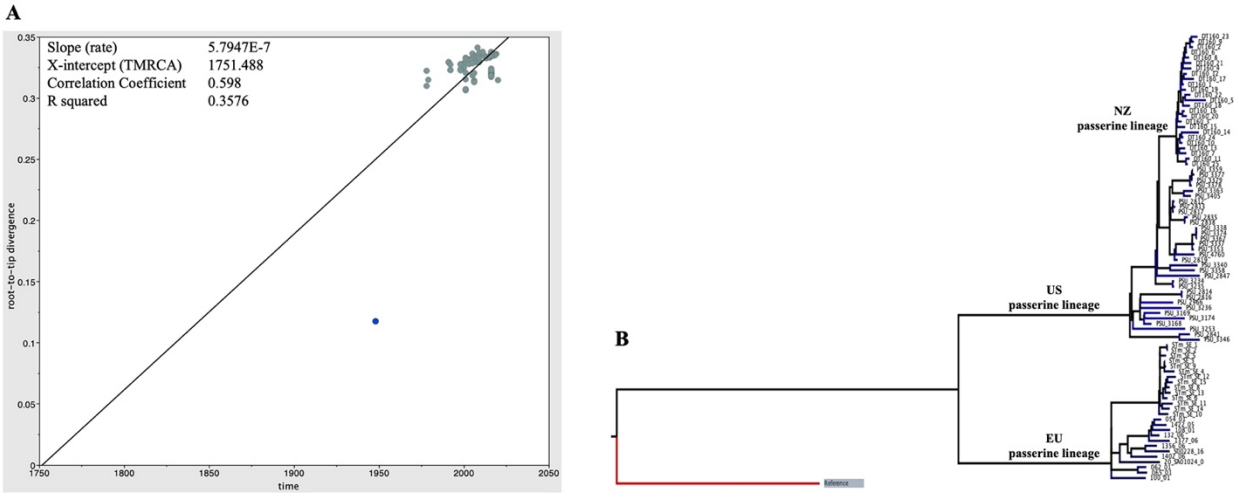

Figure S1. Temporal signal of the 84 *S.* Typhimurium genome sequences from passerine birds used for Bayesian inference. (**A**) Root-to-tip regression plot showing regression of genetic distance against sampling time. (**B**) Phylogeny of 84 *S.* Typhimurium genome sequences from passerine birds. The EU, US, and NZ passerine lineages are indicated on the phylogenetic tree branches. Reference genome from *S.* Typhimurium LT2 is highlighted in blue in (**A**) and shaded in grey in (**B**).

690 **Supplementary Dataset Legend**

691 **Dataset S1.** *In silico* virulence, antimicrobial resistance (AMR), and plasmid profiles of the public
692 available *Salmonella enterica* serovar Typhimurium isolates from passerine birds (n = 84) and
693 other diverse hosts (n = 112).

694

695 **Dataset S2.** Metadata information of the 112 context *Salmonella enterica* serovar Typhimurium
696 isolates from diverse hosts.