



Salmonella enterica Serovar Typhimurium Isolates from Wild Birds in the United States Represent Distinct Lineages Defined by Bird Type

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ABSTRACT *Salmonella enterica* serovar Typhimurium is typically considered a host generalist; however, certain isolates are associated with specific hosts and show genetic features of host adaptation. Here, we sequenced 131 *S. Typhimurium* isolates from wild birds collected in 30 U.S. states during 1978–2019. We found that isolates from broad taxonomic host groups including passerine birds, water birds (Aequornithes), and larids (gulls and terns) represented three distinct lineages and certain *S. Typhimurium* CRISPR types presented in individual lineages. We also showed that lineages formed by wild bird isolates differed from most isolates originating from domestic animal sources, and that genomes from these lineages substantially improved source attribution of Typhimurium genomes to wild birds by a machine learning classifier. Furthermore, virulence gene signatures that differentiated *S. Typhimurium* from passerines, water birds, and larids were detected. Passerine isolates tended to lack *S. Typhimurium*-specific virulence plasmids. Isolates from the passerine, water bird, and larid lineages had close genetic relatedness with human clinical isolates, including those from a 2021 U.S. outbreak linked to passerine birds. These observations indicate that *S. Typhimurium* from wild birds in the United States are likely host-adapted, and the representative genomic data set examined in this study can improve source prediction and facilitate outbreak investigation.

IMPORTANCE Within-host evolution of *S. Typhimurium* may lead to pathovars adapted to specific hosts. Here, we report the emergence of disparate avian *S. Typhimurium* lineages with distinct virulence gene signatures. The findings highlight the importance of wild birds as a reservoir for *S. Typhimurium* and contribute to our understanding of the genetic diversity of *S. Typhimurium* from wild birds. Our study indicates that *S. Typhimurium* may have undergone adaptive evolution within wild birds in the United States. The representative *S. Typhimurium* genomes from wild birds, together with the virulence gene signatures identified in these bird isolates, are valuable for *S. Typhimurium* source attribution and epidemiological surveillance.

KEYWORDS *Salmonella* Typhimurium, host adaptation, machine learning, virulence gene signatures, wild birds

Salmonella enterica is a major zoonotic and foodborne pathogen, and one of the leading bacterial causes of foodborne illnesses in the United States. The Centers for Disease Control and Prevention (CDC) estimates that *Salmonella enterica* is responsible

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for approximately 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths each year in the United States (1). Food products such as eggs, livestock, and poultry meat are the main sources for most of these illnesses (1). Source attribution studies have documented that food-producing animals such as pigs, cattle, and chickens are the main reservoirs for *Salmonella* bacteria that infect humans (2). Although transmission of nontyphoidal *Salmonella* to humans through food and food-producing animals is well described, the contribution of nonfood sources such as wild birds is less defined. Nonfood-producing animals are less explored in most source-attribution studies mainly due to a lack of representative and routinely collected data on these animals (3).

There have been several reports indicating transmission of *S. enterica* between wild birds and humans in recent years. For instance, possible interspecies transmission of *S. enteritidis* among gulls, poultry, and humans has been reported in Chile (4). Isolates of *S. Hvittingfoss* from a 2016 multi-state outbreak originating from tainted cantaloupes in Australia closely matched *S. Hvittingfoss* isolates from bar-tailed godwits (*Limosa lapponica*) (5). Although there is no direct evidence indicating that the above-mentioned outbreaks originated from charadriiform birds (shorebirds and larids), it highlights the potential role of charadriiform birds in dissemination of *S. enterica* involved in human infections. Investigations focused on passerine birds (songbirds) identified a total of 337 human infections with passerine-associated *S. Typhimurium* DT40, DT56 (v), and DT160 isolates during 2000–2010 (6). In 2021, an *S. Typhimurium* outbreak linked to passerines led to 29 illnesses and 14 hospitalizations in over 12 U.S. states (6).

As wild birds may serve as a reservoir of *S. enterica* that cause human salmonellosis, characterizing isolates from wild birds is important so that isolates from human cases can be properly traced to the appropriate source and potential transmission routes can be identified. A public portal integrating pathogen genomic sequences from various sources and geographic regions can facilitate source attribution studies with large data sets in real-time. Although the NCBI Pathogen Detection (PD) provides such a data portal for traceback investigation of an outbreak (<https://www.ncbi.nlm.nih.gov/pathogens/>), the sequencing data are skewed toward isolates from clinical samples, foods, or food-producing animals (i.e., livestock and poultry). The scanty data from nonfood sources such as wild birds may hinder source attribution during an outbreak investigation, thus affecting subsequent prevention and mitigation measures. Besides direct sequence comparisons, machine learning algorithms such as Random Forest (RF) classifier have shown promise for predicting *Salmonella* host specificity (7) or zoonotic sources (8). Zhang et al. reported an RF classifier for genomic source prediction of *S. Typhimurium* in the United States that showed a marked difference in prediction accuracies between food-producing animals (prediction accuracy ~90%) and wild birds (prediction accuracy ~50%). The difference was presumably due to a lack of representative and available wild bird isolates (8). Therefore, including *S. enterica* isolates beyond food or food-producing animals in a public data portal such as PD would help better resolve complex *Salmonella* epidemiology.

In this study, we sequenced 131 *S. Typhimurium* isolates from wild birds. Isolates were collected by the U.S. Geological Survey—National Wildlife Health Center during 1978–2019 in 30 U.S. states. We used these data to test the following hypotheses. (1) Isolates would be phylogenetically segregated by bird host; (2) additional *S. Typhimurium* genomes from wild bird isolates would improve the performance of machine learning-based source attribution; (3) allelic variation in virulence genes would contribute to *S. Typhimurium* host specificity; and (4) inclusion of these data in the PD database would better define cross-species transmission of *S. Typhimurium* between wild birds, humans, and other hosts.

RESULTS

Distinct *S. Typhimurium* lineages are carried by passerine birds, water birds, and larids. Metadata (isolate name, Sequence Read Archive [SRA] accession number, collection year, state, source/host species, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) type, and classic 7-gene MLST sequence type) for the 131 isolates are listed in Data Set S1 in the supplemental material. Based on single

nucleotide polymorphism (SNP) phylogenetic analyses, isolates from wild birds formed three major lineages (Fig. 1A), each supported by robust bootstrap values of 100%. The largest branch included 42 isolates, all of which originated from passerine birds (order Passeriformes; e.g., cardinal, finches, and sparrows). The second major lineage included 40 isolates predominantly originating from water birds of the clade Aequornithes (e.g., cormorants, pelicans, and herons). This lineage also included occasional isolates from other birds that commonly share the same aquatic habitats or prey/scavenge on the carcasses of the above-mentioned species: terns ($n = 2$), gull ($n = 1$), grebe ($n = 1$), goose ($n = 1$), and bald eagle ($n = 1$). The third major *S. Typhimurium* lineage consisted of 32 isolates primarily from larids (terns and gulls; order Charadriiformes). This lineage also included isolates originating from a plover (also a member of Charadriiformes) and an owl. Subclades formed by *S. Typhimurium* isolates within each of the three major lineages did not show clear patterns of host specificity (e.g., isolates from terns and gulls did not form unique subclades but rather were interspersed within the core larid lineage). Isolates from raptors (e.g., owls, bald eagles, and hawks) did not cluster together, and are highlighted in red in Fig. 1A. Based on Bayesian phylogenetic inference (Fig. 1B), we were able to estimate that the most recent common ancestor (MRCA) of the water bird lineage was from around 1915 (95% highest posterior distribution [HPD]: 1902–1926). The MRCAs of the passerine lineage and larid lineage were from approximately 1941 (95% HPD: 1934–1947) and 1938 (95% HPD: 1929–1947), respectively. The passerine lineage and the larid lineage were estimated to have split from each other around 162 years ago (median: 1857; 95% HPD: 1841–1873).

Defined *S. Typhimurium* lineages contain disparate CRISPR types. We further classified isolates by two established *Salmonella* typing methods, i.e., classic 7-gene multilocus sequence typing (MLST) and CRISPR typing, to determine the sequence type (ST) and *S. Typhimurium* CRISPR type (STCT) of our isolates. Six STs were detected and 93.9% of the isolates (123/131) belonged to ST19 ($n = 90$) and ST99 ($n = 33$). All of the ST99 isolates belonged to the water bird lineage. However, ST did not always distinguish between the lineages. Specifically, ST19 isolates were dispersed among the larid and passerine bird lineages (Fig. 1A and Data Set S1). On the other hand, CRISPR analysis identified 37 STCTs within the 131 isolates (Fig. 1A and Data Set S1), several of which only appeared in certain lineages. For examples, STCT10 ($n = 12$), STCT11 ($n = 14$), and STCT34 ($n = 10$) were unique to the passerine bird lineage; STCT18 ($n = 24$), STCT19 ($n = 5$), and STCT20 ($n = 6$) only occurred in the water bird lineage; and STCT7 ($n = 9$), STCT8 ($n = 10$), and STCT36 ($n = 6$) were exclusive to the larid lineage.

Genetic relatedness of U.S. wild bird isolates with isolates from other reservoirs and geographic regions. We examined whether wild bird isolates of *S. Typhimurium* were related to those from other host sources and geographic regions. Fig. 2 and Data Set S2 show that wild bird isolates were related to other isolates in the PD database from diverse sources (e.g., humans, water, fish/shellfish, horses, cats, and food) and different geographic regions. The PD SNP Tree Viewer showed that our isolates from the passerine lineage, water bird lineage, and larid lineage formed six, four, and four SNP clusters with other isolates in the large database, respectively (Data Set S2). Each SNP cluster contains isolates whose genomes are within 50 SNP distance of each other. We summarized the source niches and geographic regions of all the isolates in the SNP clusters formed by different *S. Typhimurium* lineages, and the results are represented in Fig. 2. As shown in Fig. 2A and B, isolates from both the water bird and larid lineages had close genetic relatedness to isolates from water, fish/shellfish, etc. In contrast, isolates of the passerine lineage were not related to fish/shellfish isolates in the PD database; rather, these isolates were more closely related to isolates originating from horses and cats (Fig. 2C). Notably, isolates from the three major wild bird lineages were genetically similar to a number of clinical isolates from humans. The numbers of clinical isolates clustered with isolates from water bird, larid, and passerine lineages in the PD database were 37, 84, and 495, respectively. Interestingly, isolates from food-producing animals such as livestock and poultry rarely clustered with wild bird isolates. Only two

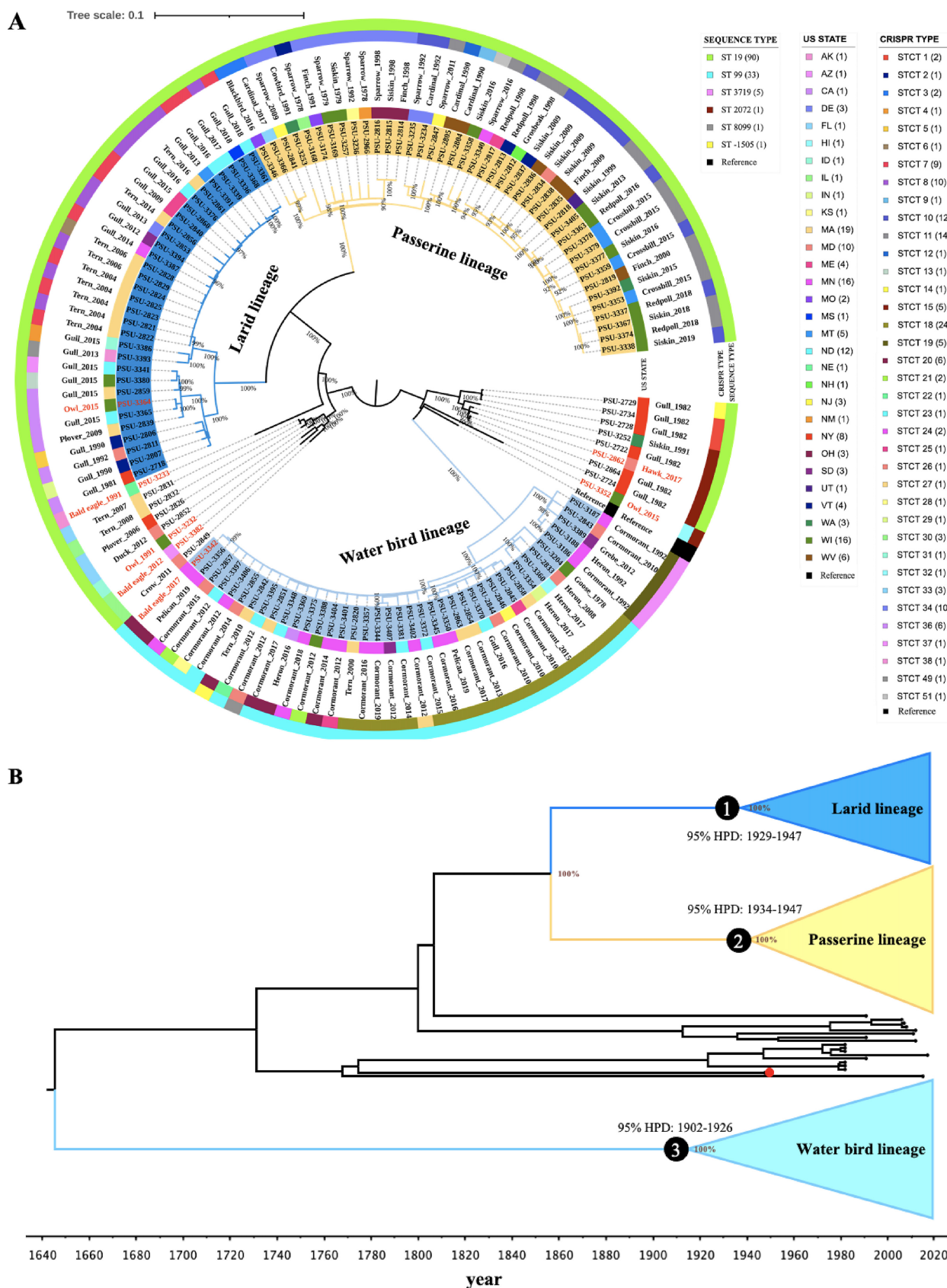


FIG 1 Phylogenetic analyses reveal distinct *S. Typhimurium* lineages in passerine birds, water birds, and larids. (A) Maximum-likelihood phylogenetic tree of 131 *S. Typhimurium* isolates from wild birds collected during 1978–2019 in 30 U.S. states. The tree is visualized using iTOL. (Continued on next page)

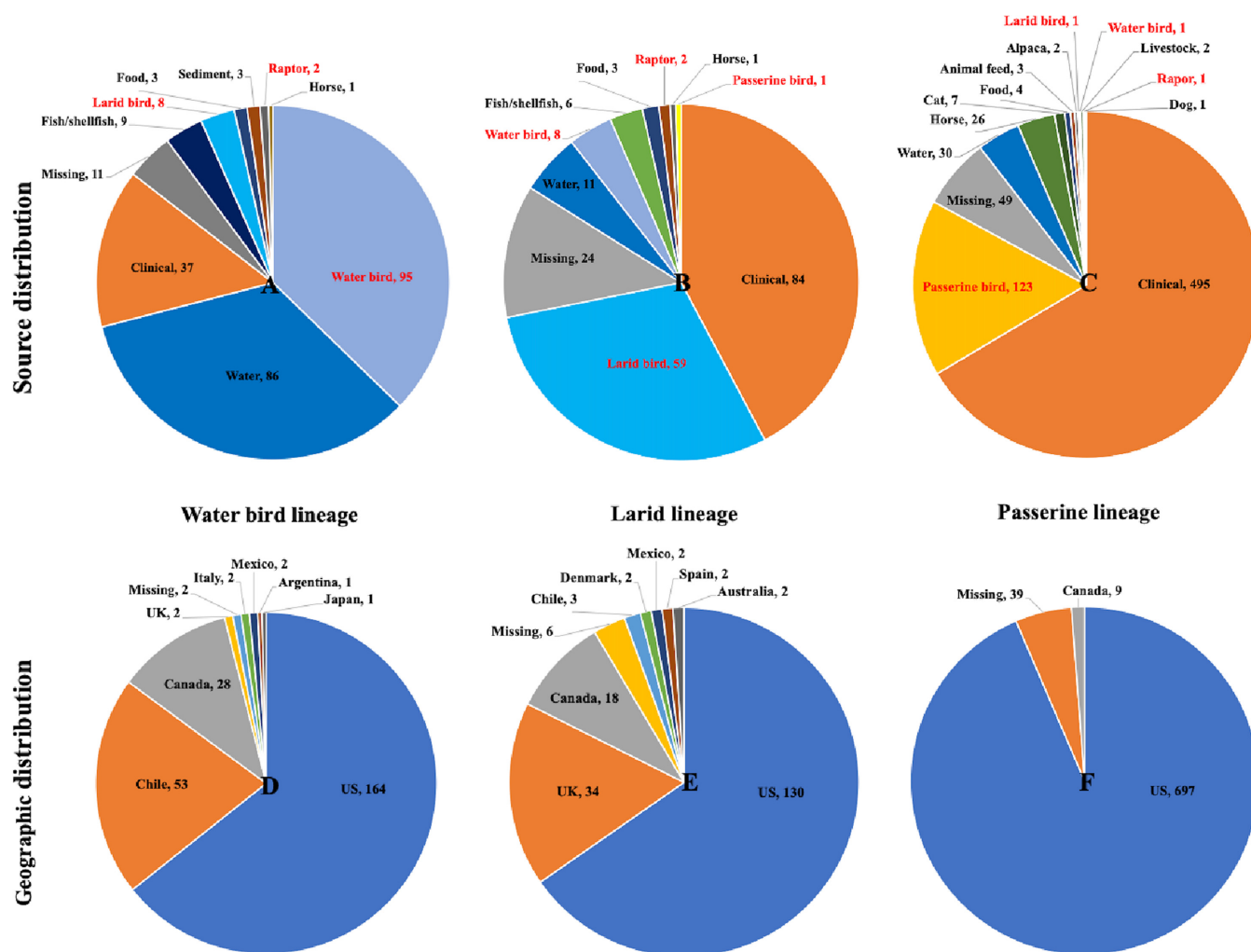


FIG 2 Distribution of source niches and geographic regions for the isolates in the Pathogen Detection (PD) database that cluster with *S. Typhimurium* isolates from different wild bird lineages. (A–C) Source distribution of isolates in the PD database clustering with isolates from water bird lineage, larid lineage, and passerine lineage. (D–F) Geographic distribution of isolates in the PD database clustering with isolates from water bird lineage, larid lineage, and passerine lineage. The number following the source niche or geographic region represents the number of isolates from that specific source or geographic region in the PD database. Complete data used to generate the pie charts are given in Data Set S2.

isolates from livestock were related to passerine-derived isolates, while none were closely associated with water bird or larid isolates (Fig. 2A to C). Geographically, isolates in both the water bird and larid lineages were closely related to isolates originating from outside of the United States. Isolates from Canada, Chile, Mexico, United Kingdom, Japan, and Australia clustered with isolates from the water bird and larid lineages from the United States (Fig. 2D and E). Isolates from the passerine lineage only demonstrated close genetic relatedness with isolates from sources within North America (Fig. 2F).

FIG 1 Legend (Continued)

Three large lineages are defined by bird types, i.e., passerine lineage (yellow), larid lineage (blue), and water bird lineage (light blue). Bootstrap values are displayed as percentage on tree branch. The labels at tips represent the isolate name, the inner color strip represents the U.S. state from which the sample originated, the outmost color strips represent the *S. Typhimurium* CRISPR type and MLST sequence type, and the text labels between the color strips represent the bird host and isolation year. The legend fields at the right of the tree represent the *S. Typhimurium* MLST sequence type (number of isolates), U.S. state abbreviation (number of isolates), and the *S. Typhimurium* CRISPR type (number of isolates). The labels highlighted in red represent isolates from raptors. (B) Time-scaled Bayesian phylogenetic tree of 131 *S. Typhimurium* isolates from wild birds collected during 1978–2019 in 30 U.S. states. The tree is visualized using FigTree v1.4.4. Numbers in black circles at the nodes represent the 95% highest posterior probability density (HPD) for the times of most recent common ancestor for the water bird lineage (light blue), larid lineage (blue), and passerine lineage (yellow). The red circle at the tree tip represents the reference strain LT2 (isolation year: 1948). Posterior probability values are displayed as percentage on tree nodes of the three lineages.

TABLE 1 Confusion matrix showing the prediction accuracy of a Random Forest classifier for source attribution of *S. Typhimurium* to bovine, porcine, poultry, and wild bird sources

Original training dataset					
Out-of-bag prediction accuracy: 82.9%					
Actual class	Predicted class				Class accuracy
	Bovine	Porcine	Poultry	Wild bird	
Bovine	126	38	29	2	64.62%
Porcine	24	300	10	4	88.76%
Poultry	24	11	401	4	91.14%
Wild bird	10	8	14	36	52.94%
Original training data set with addition of the 131 wild bird isolates					
Out-of-bag prediction accuracy: 84.3%					
Actual class	Predicted class				Class accuracy
	Bovine	Porcine	Poultry	Wild bird	
Bovine	122	41	28	4	62.56%
Porcine	26	300	8	4	88.76%
Poultry	24	12	400	4	90.91%
Wild bird	12	7	14	166	83.42%

Inclusion of the 131 *S. Typhimurium* isolates from wild birds improves source prediction accuracy of a Random Forest classifier. Given the distinct clustering of wild bird isolates, we next explored whether our data set would improve a previously published RF classifier for source prediction of *S. Typhimurium* isolates. With the original training data set (195 bovine isolates, 338 porcine isolates, 440 poultry isolates, and 68 wild bird isolates), the RF classifier had a prediction accuracy of 64.62%, 88.76%, 91.14%, and 52.94% for bovine, porcine, poultry, and wild birds, respectively, and an out-of-bag prediction accuracy of 82.9%. After adding our 131 wild bird isolates into the training data set, the out-of-bag prediction accuracy was increased to 84.3% (Table 1). Notably, there was a substantial increase in prediction accuracy for wild birds from 52.94% to 83.42%. In contrast, the prediction accuracies for bovine and poultry remained largely unaffected. To explain the prediction results using phylogeny, an SNP tree was built that included 1,604 isolates from different sources (171 human clinical isolates, 261 isolates from food and other sources, and 1,172 isolates used for the RF classifier training [i.e., 195 bovine isolates, 338 porcine isolates, 440 poultry isolates, and 199 wild bird isolates in which 131 were from this study]). These different sourced isolates excluding the 131 wild bird isolates were characterized in a previous *S. Typhimurium* source attribution study (8). The wild bird isolates formed three lineages that did not overlap with those formed by other animal-sourced isolates (Fig. 3), which corresponded to the source prediction performance of the RF classifier that the number of wild bird isolates falsely attributed to porcine, bovine, or poultry sources was 32 out of 68 in the original training data set, and 33 out of 199 after adding our bird isolates into the original training data set (Table 1). It should be noted that isolates from bovines were more dispersed and overlapped with other sourced isolates in the phylogenetic tree (Fig. 3). This was consistent with the source prediction performance of the RF classifier that 37.44% (73/195) of the bovine isolates were assigned to porcine, poultry, or wild bird sources (Table 1).

Genetic signatures related to virulence differ between isolates originating from different types of wild birds. We next took a more focused approach to examine whether presence of antimicrobial resistance genes, virulence genes, and/or plasmids were associated with the major wild bird lineages. All 131 wild bird *S. Typhimurium* isolates were predicted to be sensitive to major types of antimicrobials (beta-lactam, tetracycline, phenicol, fluoroquinolone) (Data Set S3). These isolates carried common virulence determinants found in *S. Typhimurium*. These included fimbrial/nonfimbrial

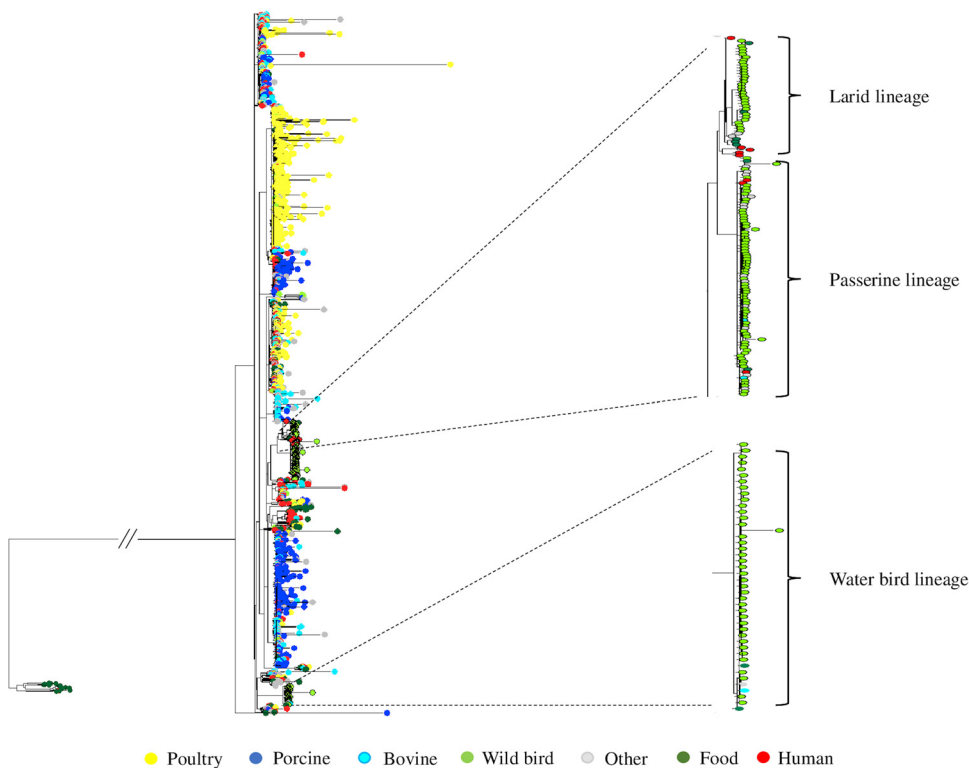


FIG 3 Maximum-likelihood phylogenetic tree of 1,604 *S. Typhimurium* isolates from different sources for zoonotic source prediction. The maximum-likelihood phylogenetic tree for zoonotic source prediction includes *S. Typhimurium* isolates from seven source classes: (i) bovine ($n = 195$), including isolates from cattle, beef, and raw milk; (ii) porcine ($n = 338$), including isolates from pigs and pork; (iii) poultry ($n = 440$), including isolates from chickens, turkeys, ducks, and their eggs; (iv) wild bird ($n = 199$), including 131 isolates from this study; (v) human ($n = 171$), including human clinical isolates; (vi) food ($n = 83$), including seafood, plant-based food, and other ready-to-eat and/or processed food; and (vii) other ($n = 178$), including any isolates not belonging to the aforementioned classes. The larid, passerine, and water bird lineages formed by the *S. Typhimurium* isolates from wild birds are enlarged and highlighted in the phylogenetic tree.

adherence determinants, genes promoting bacterial survival in activated macrophage, type three secretion system (TTSS) encoded by *Salmonella* pathogenicity island I and II (SPI-1 and SPI-2, respectively), and genes associated with magnesium uptake, serum resistance, stress adaptation, and toxin production (Data Set S3).

Isolates from the water bird, larid, and passerine lineages each had defining mutations that may abolish the function of specific virulence genes. The *fimC* gene (full length: 708 bp) encoding the type 1 pilus chaperone FimC had a single base-pair deletion at position 87 in all isolates (100%; 42/42) belonging to the passerine lineage. Only two isolates (i.e., PSU-2849 and PSU-3252) from passerine birds were predicted to encode the full length *fimC*; however, these isolates were divergent from the core passerine lineage. Conversely, all the isolates (100%; 72/72) from the water bird and larid lineages possessed an intact *fimC* (Fig. 4A). On the other hand, all the isolates (100%; 40/40) in the water bird lineage had a single base-pair insertion in the *safB* gene (full length: 714 bp; insertion at position 601) encoding the Saf pilus chaperone SafB, a 60 base-pair deletion in the *sthC* gene (full length: 2538 bp; deletion at 481–540) encoding the Sth pilus usher protein SthC, and a single base-pair insertion and substitution in the *sseL* gene (*Salmonella* secreted effector L; full length: 962 bp; insertion at position 474, substitution at position 823). These three genes occurred in all the isolates from passerine and larid lineages (Fig. 4A). However, there was a substitution at position 867 of the *sseL* gene in isolates from the larid lineage that led to a premature stop codon (Fig. S1). The organization of the fimbrial operons containing *fimC*, *safB*, and *sthC* in isolates from the three major wild bird lineages, and reference isolate *S.*

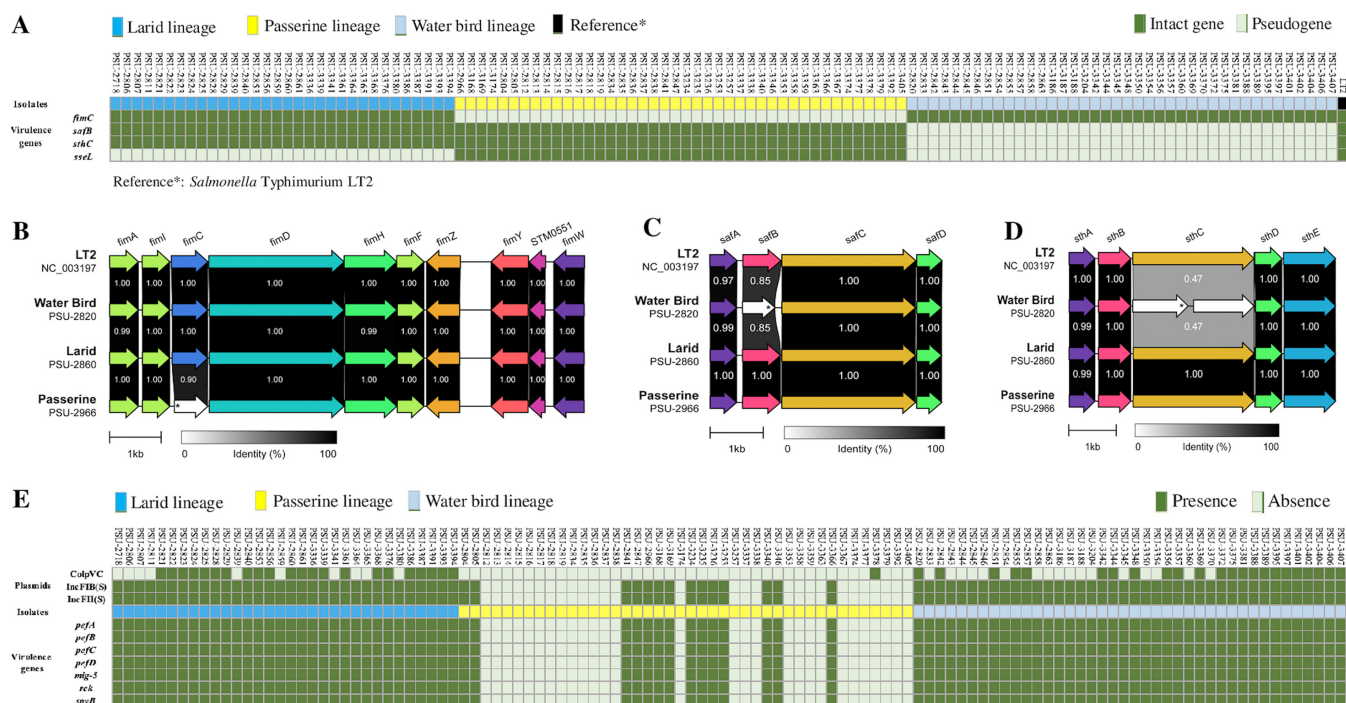


FIG 4 Chromosomal and plasmid-associated virulence gene signatures that differ among the larid, passerine, and water bird lineages. (A) Distribution of chromosomal virulence gene signatures (*fimC*, *safB*, *sthC*, and *sseL*) among the larid, passerine, and water bird lineages. (B–D) Organization of the fimbrial operons containing *fimC*, *safB*, and *sthC* in isolates from the larid, passerine, and water bird lineages, and reference strain *S. Typhimurium* LT2. (E) Distribution of plasmids and plasmid virulence genes among the larid, passerine, and water bird lineages. In the heatmaps (A) and (E): light green = pseudogene in (A), and absence of the gene or plasmid in (E); dark green = intact gene in (A), and presence of the gene or plasmid in (E); blue = isolates from larid lineage; yellow = isolates from passerine lineage; light blue = isolates from water bird lineage; dark = reference strain (*S. Typhimurium* LT2). Isolate names are represented on the x axis, virulence genes and plasmid names are represented on the y axis. Complete data used to generate the heatmaps is given in Data Set S3. In the fimbrial operons (B), (C), and (D), the pseudogenes are marked as white arrows, and the locations of indels in the pseudogenes are represented by asterisks.

Typhimurium LT2 are shown in Fig. 4, B to D. Gene *sseL* was monocistronic and its alignment within different bird isolates is shown in Fig. S1. Overall, the mutations (frameshift and premature stop codon) of these virulence genes resulted in pseudogene accumulation that may inactivate their functions (Fig. 4B to D).

Because of known concurrence, we conducted plasmid profiling to examine the correlation between certain virulence/resistance genes and plasmids in different wild bird lineages. As shown in Fig. 4E, four plasmids of the incompatibility groups ColpVC, IncFIB(S), IncFII(S), and IncI1 were present in these isolates. None of the plasmids carried antimicrobial resistance genes. However, three plasmids (IncI1, IncFIB(S), and IncFII(S)) carried virulence genes. IncI1 was present in a single isolate (PSU-2832); this plasmid carried the type IV pili genes (*pilUQRS*). IncFIB(S) and IncFII(S) carried virulence genes including *pefABCD* (plasmid encoded fimbriae), *mig-5* (macrophage induced gene), *rck* (resistance to complement killing), and *spvB* (*Salmonella* plasmid virulence). We found that 100% (72/72) of isolates from both water bird and larid lineages carried these two virulence plasmids, while only 33.3% (14/42) of isolates from the passerine lineage had these two plasmids. Isolates from passerine birds without the two plasmids also lacked the corresponding virulence genes (i.e., *pefABCD*, *mig-5*, *rck*, and *spvB*) (Fig. 4E).

DISCUSSION

In this study, we explored the genetic diversity of 131 *S. Typhimurium* isolates collected from wild birds over 4 decades. Whole genome sequence phylogeny revealed three major lineages of *S. Typhimurium* largely defined by bird host taxonomic group; these lineages possessed certain STCTs and virulence gene signatures. Potential transmission of isolates between the three lineages and non-avian host species was variable as was the extent of their geographic distribution. Our study also shows that the wild

bird *S. Typhimurium* lineages did not overlap with major lineages formed by domestic animal-sourced isolates. The addition of these wild bird isolates into a training data set improved source prediction accuracy of a RF classifier among bovine, porcine, poultry, and wild birds.

Different host physiologies, habitats, and life histories may explain the divergence of *S. Typhimurium* lineages infecting different groups of wild birds. The three major lineages of *S. Typhimurium* in our study each corresponded to taxonomically disparate host groups. The water bird lineage was primarily associated with birds in the clade Aequornithes (e.g., cormorants, pelicans, herons), the larid lineage with birds in the order Charadriiformes (e.g., gulls, terns, plover), and the passerine lineage with birds in the order Passeriformes (e.g., finches, sparrows). Correspondence of these lineages to bird taxonomic group indicates a degree of host adaptation by *S. Typhimurium* infecting wild birds, similar to what has been described in isolates infecting sea turtles (9) and pigeons (10). Some evidence indicates that certain *S. Typhimurium* isolates maintained within passerine birds in the United Kingdom are host-adapted (11, 12). However, passerine-adapted *S. Typhimurium* isolates in the United Kingdom belong to two definitive phage types (DT), i.e., DT40 and DT56(v) (the STCTs are 745, 7743, or 9520) (13, 14) and were apparently unrelated to the passerine isolates in this study, which mostly have the STCTs of 10, 11, and 34 (Fig. 1A). To the best of our knowledge, there are no readily available systematic studies reporting *S. Typhimurium* isolates adapted to water birds or larids. Although the three lineages we describe herein were associated with particular bird groups at broad host taxonomic levels, we did not observe evidence of further specialization occurring at finer taxonomic scales. Specifically, *S. Typhimurium* isolates from the same host species did not form subclades within their major lineages (e.g., cormorant isolates did not form a subclade unique from pelican isolates within the water bird lineage). However, we sampled a relatively small number of wild bird hosts relative to the avian diversity that exists in North America. Specifically, our sampling was biased toward species that regularly experience outbreaks of salmonellosis such as cormorants, gulls, and finches (15). Outbreaks may be more prevalent in these bird species because colonial nesting (cormorants, pelicans, herons, gulls, and terns) and congregation at feeding and water sites such as bird feeders and bird baths (songbirds) facilitate transmission. Thus, a greater diversity of host-adapted lineages of *S. Typhimurium* may be discovered by sampling additional avian groups that harbor cryptic infections.

Spatial patterns, including habitat partitioning by different bird hosts, do not fully explain the *S. Typhimurium* phylogenetic patterns we observed. For example, cormorants, pelicans, and gulls often occupy the same habitats and occur close to one another. However, isolates from these birds tended to fall into two distinct lineages based on bird phylogeny, indicating that factors such as host physiology may be the key factor driving *S. Typhimurium* host adaptation. Notably, the passerine lineage is sister to the larid lineage, and both are more distantly related to the water bird lineage despite water birds and larids being much more closely related than either group is to the passerines (16). The Bayesian phylogenetic inference indicates the three lineages formed sometime after 1900. This implies that host adaptation occurred well after the divergence of the avian host groups. Similarly, a lineage of *S. Typhimurium* adapted to sea turtles across the Pacific Ocean evolved from an MRCA only a few decades ago (9). Taken together, these findings indicate that spillover of *S. Typhimurium* into wildlife and subsequent host adaptation may be a relatively recent phenomenon, likely driven by anthropogenic influences.

While the presence of three major lineages indicated host adaptation by bird-infecting isolates of *S. Typhimurium*, evidence indicated that isolates within a lineage could infect bird species outside of its typical host group. Infections in aberrant hosts usually corresponded with hosts from different groups that share similar environments to one another. For example, *S. Typhimurium* isolates in the water bird lineage were occasionally from other birds (i.e., terns, gulls, grebe, and goose) that share aquatic habitats with cormorants, pelicans, and herons. Interestingly, isolates of *S. Typhimurium* from raptors (e.g., eagles, hawks, and owls) were phylogenetically dispersed. The diversity of

isolates harbored by raptors may be due to acquisition of *S. Typhimurium* through consumption of other birds and animals that may be infected with the bacterium rather than possessing their own unique isolates.

While *S. Typhimurium* isolates clustered by host species, evidence of geographic patterns was scant based on collection locations within the United States (Fig. 1A). This may be explained by the high mobility of wild birds, which travel long distances and across political boundaries. Isolates from the three major lineages did, however, cluster with isolates from different global regions in the PD database. Isolates from the larid lineage were genetically similar to isolates in the database originating from South America, Oceania, and Europe. This is consistent with the long-distance migratory capacity for terns and gulls, which makes them capable of spreading certain strains of *S. Typhimurium* across the world. Isolates from the water bird lineage exhibited a similar geographic pattern. In contrast, isolates from the passerine lineage did not closely match isolates outside of North America, which is consistent with the more typical intracontinental migratory patterns for the passerine species. It should be noted that the PD database is heavily skewed toward isolates from the United States. Isolates from other geographic regions are underrepresented because many countries do not sequence isolates or actively submit data to the NCBI PD database for surveillance purposes. Additional sampling in other parts of the world may reveal that all three major lineages are more widely distributed.

Host adaptation of *S. Typhimurium* in various groups of wild birds may affect its transmission among different hosts or sources. For example, isolates from the water bird and larid lineages clustered with isolates from aquatic animals (fish/shellfish), which is understandable given their overlap in habitats. However, no isolates from aquatic animals were closely related with isolates from the passerine lineage. Instead, the passerine lineage was more likely to contain *S. Typhimurium* isolates from horses and cats. Studies have reported that cats can acquire *S. Typhimurium* by catching infected songbirds or spending time around bird feeders (17, 18). However, few studies explored the potential spread of *S. Typhimurium* between wild birds and horses, which may be exposed to isolates of the passerine lineage in open pastures or stables that are also occupied by avian species such as finches and house sparrows.

Interestingly, isolates from the three wild bird lineages were rarely related to isolates from livestock and poultry, but were frequently associated with clinical isolates from human beings (Fig. 2 and Data Set S2). Our addition of isolates to the database will help facilitate attribution of *S. Typhimurium* isolates from wild birds recovered from other hosts or substrates to the appropriate source. Although the exact transmission route and direction are difficult to determine, studies such as this are important in raising awareness of wild birds as a reservoir of *S. Typhimurium* that may contribute to preventable cases of human salmonellosis. For example, several studies have reported the concurrence of *S. Typhimurium* outbreaks in both passerine birds and humans in the United Kingdom, United States, and Sweden during late winter or early spring (18–20), and cats are considered to be a possible intermediate vehicle for *S. Typhimurium* transmission between passerine birds and human beings during outbreaks (18, 21, 22). This is consistent with our observation that passerine bird isolates cluster with isolates from cats in the PD database (Fig. 2C). In 2021, an *S. Typhimurium* outbreak that caused at least 29 illnesses and 14 hospitalizations in the United States was traced back to isolates from songbirds (6). In fact, clinical isolates from this outbreak formed an SNP cluster with passerine isolates in our study (Fig. 5; three pine siskin isolates: PSU-3338, PSU-3367, and PSU-3392; two redpoll isolates: PSU-3337 and PSU-3374; and one crossbill isolate: PSU-3353, highlighted in red). These bird isolates belonged to STCT10 and STCT11. Our passerine isolates differed from clinical isolates by <10 SNPs, indicating a recent common ancestor (23). None of our passerine isolates were collected earlier than the clinical isolates from the 2021 outbreak (Fig. 5), and although we cannot say with certainty, it is plausible that isolates transmitted from passerine birds to humans were responsible for the outbreak. Humans may get infected via direct contact with songbirds, contaminated bird feeders, or infected cats;

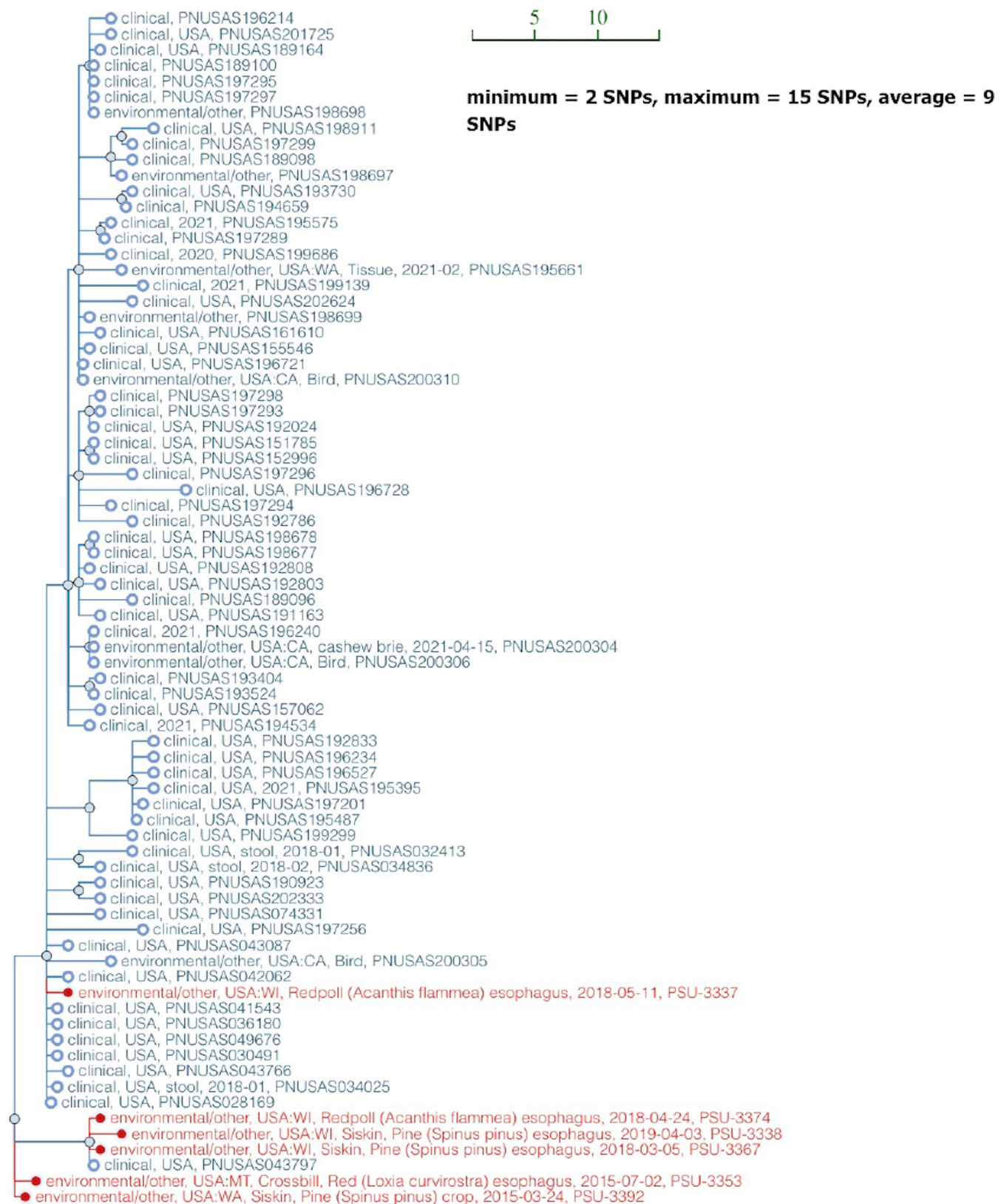


FIG 5 Close genetic relatedness between passerine isolates from this study and clinical isolates from a 2021 salmonellosis outbreak in the United States. Single nucleotide polymorphism (SNP) tree is automatically generated in the NCBI Pathogen Detection database when sequencing data are submitted to the database. The scale bar represents the unit SNP distance. The average SNP distance for all isolates in the tree is nine. *S. Typhimurium* isolates in the SNP tree from this study are highlighted in red.

or there may be additional unknown sources not represented in the PD database. To further elucidate routes of transmission, collecting contemporaneous *S. Typhimurium* isolates from humans, wild birds, and environmental samples through outbreak investigations would be beneficial. Such efforts could offer insights to guide preventative measures to reduce the health risk for future outbreaks.

Inclusion of the wild bird isolates in the PD database not only facilitates outbreak investigation, but also improves source attribution. The RF classifier in this study performed much better to predict isolates from wild birds with the addition of our 131 isolates to a training data set from porcine ($n = 338$), bovine ($n = 195$), poultry ($n = 440$), and wild birds ($n = 68$) (Table 1). The substantial increase in wild bird isolates from 68 to 199 may contribute to the improved prediction accuracy for wild bird sources. It should be noted, however, that prediction accuracy not only depends on sampling intensity but also presence of representative isolates from different sources. Even though the count of isolates from bovine sources ($n = 195$) is almost equal to that from wild birds ($n = 199$), prediction accuracy for bovine sources (62.56%) is lower than that for wild bird sources (83.42%). The high prediction accuracy for wild birds may also be attributed to the consistent presence of genetic signatures such as pseudogene accumulation in fimbrial genes, which may help discriminate wild bird isolates from other sourced isolates. This is consistent with the observation that wild bird isolates form three unique lineages, while bovine isolates are more phylogenetically dispersed in the SNP phylogenetic tree (Fig. 3). Overall, an improvement in prediction accuracy is crucial to help identify root sources of *S. Typhimurium* outbreaks, especially when the outbreak is linked to nonfood sources.

Genome degradation contributing to *Salmonella* host adaptation is well documented (24–29). Our study shows pseudogene accumulation in specific chromosomal fimbrial genes in certain wild bird *S. Typhimurium* lineages (i.e., *fimC* in passerine lineage, *saftB* and *sthC* in water bird lineage). The inactivation of these genes due to frame-shift and premature stop codons may help *S. Typhimurium* adapt to a particular bird host. Furthermore, passerine bird isolates tended to lose the plasmid carrying virulence genes (i.e., *pefABCD*, *mig-5*, *rck*, and *spvB*). These virulence genes are important for *S. Typhimurium* survival and replication in human and mouse macrophages (30–32), which may indicate that isolates from the passerine lineage are more host-adapted to birds than those from water bird and larid lineages.

To conclude, the findings in this study highlight the importance of wild birds as a reservoir for *S. Typhimurium*. Source attribution can be more precise if more representative wild bird isolates were included in *S. Typhimurium* databases. In addition, the distinct lineages defined by bird type, together with the presence of virulence gene signatures, imply host adaptation of *S. Typhimurium* to wild birds. Considering wild birds may spread *S. Typhimurium* in a very different manner than domestic animals, alternative management strategies would be beneficial to prevent their transmission to human beings. While wild birds serve as potential reservoirs of *S. Typhimurium*, these species also provide critical ecological and economic services to humans and are of great cultural significance. Activities aimed at preventing zoonotic transmission would focus on strategies that benefit both wild birds and humans alike.

MATERIALS AND METHODS

Bacterial isolates. *Salmonella* Typhimurium isolates ($n = 131$) were collected from sick or dead birds submitted to the U.S. Geological Survey—National Wildlife Health Center as part of wildlife mortality and morbidity investigations. The isolates were collected between 1978 and 2019 from 30 states in the United States and stored in cryogenic vials at -80°C before further use. All isolates were confirmed as *S. Typhimurium* by traditional *Salmonella* serotyping and reconfirmed by SeqSero2 v1.2.1 (33) from newly generated whole genome sequencing data (see below).

DNA extraction and whole genome sequencing. For DNA extraction, each isolate was streaked onto xylose lysine deoxycholate (XLD) agar plates and incubated for 18 h at 37°C . A single colony was then picked, transferred to Luria-Bertani broth (LB), and cultured overnight at 37°C with continuous agitation (250 rpm). Genomic DNA was extracted using the Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, California) following the manufacturer's instructions. Genomic DNA purity was confirmed via an A260/A280 measurement (target ≥ 1.8) using NanoDrop One (Thermo Scientific, Delaware), and DNA

concentration was quantified using Qubit 3.0 (Thermo Fisher Scientific Inc., Massachusetts) fluorometer. Isolated genomic DNA was stored at -20°C before use. For whole genome sequencing (WGS), genomic DNA was adjusted to $0.2\text{ ng}/\mu\text{L}$. DNA library was then prepared using the Nextera XT DNA Library Prep Kit (Illumina Inc., San Diego, California). The resulting library was normalized using a quantitation-based procedure and pooled together at equal volume. The pooled library ($600\text{ }\mu\text{L}$) was denatured and sequenced on an Illumina MiSeq sequencer (Illumina Inc., San Diego, California) using a MiSeq reagent v3 kit, with 500 (2×250) cycles.

Quality assessment for raw reads. After sequencing, the quality of Illumina paired-end reads of all isolates was assessed using the MicroRunQC workflow in GalaxyTrakr v2 (34). Raw reads passing quality control thresholds (i.e., mean coverage >30 , mean quality score >30 , number of contigs <400 , total assembly length between 4.4 and 5.1 Mb) were submitted to the NCBI under BioProject [PRJNA357723](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA357723). Metadata information and Sequence Read Archive (SRA) accession numbers of these isolates are listed in Data Set S1.

Phylogenetic analysis. For genetic relatedness comparison between the 131 isolates, raw reads were uploaded to Enterobase (35). *S. Typhimurium* strain LT2 (RefSeq NC_003197.1) served as the reference genome, and a maximum-likelihood (ML) phylogenetic tree was created using the SNP project in Enterobase based on 5,839 SNPs in the core genomic regions of the 131 wild bird *S. Typhimurium* isolates. The ML phylogenetic tree was reconstructed by MEGA X v10.1.8 using the Tamura-Nei model and 500 bootstrap replicates (36). The SNP phylogenetic tree was visualized and annotated using the Interactive Tree of Life (iTOL v6; <https://itol.embl.de>). CRISPR arrays were identified using CRISPRviz (37). Spacers were aligned and unique arrays were given a unique allelic identifier as described by Shariat et al. (38). *S. Typhimurium* CRISPR Type (STCT) was then determined by the unique combination of CRISPR1 and CRISPR2 alleles. In addition, sequence type (ST) of these *S. Typhimurium* isolates was identified using 7-gene (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*) multilocus sequence typing (MLST) at Enterobase (35). STCTs and STs were annotated in the SNP phylogenetic tree.

Bayesian phylogenetic inference. The evolutionary history of the 131 isolates was inferred by Bayesian phylogenetic analysis. Snippy (Galaxy v4.5.0; <https://github.com/tseemann/snippy>) was used to generate a full alignment and find SNPs between the reference genome LT2 (RefSeq NC_003197.1) and the genomes of wild bird isolates, and Snippy-core (Galaxy v4.5.0; <https://github.com/tseemann/snippy>) was used to convert the Snippy outputs into a core SNP alignment. The resultant multiple sequence alignment (5,251 variant sites) was used to construct a time scale Bayesian phylogenetic tree by BEAUti v2.6.5 and BEAST2 v2.6.5 (39). The parameters were set as follows: prior assumption-coalescent Bayesian skyline; clock model-relaxed clock log normal; Markov chain Monte Carlo (MCMC): chain length 100 million, storing every 1,000 generations. A maximum clade credibility tree was created using TreeAnnotator v2.6.4 with burn-in percentage of 10% and node height option of median height (39). Finally, the tree was visualized using FigTree v1.4.4 (<https://github.com/rambaut/figtree/releases>).

Genetic relatedness between wild bird isolates and other isolates. The genetic relatedness between wild bird isolates and other isolates from environmental sources, food, or human hosts was inferred by using the NCBI Pathogen Detection <https://www.ncbi.nlm.nih.gov/pathogens/>. After uploading the raw reads of the 131 isolates to NCBI, NCBI Pathogen Detection assembled, annotated, and clustered the newly sequenced isolate genomes to other closely related isolates in the database. Clustering involved two steps: first, related isolates were clustered based on the whole genome MLST (wgMLST) scheme of *Salmonella* with a 25-allele cutoff; once wgMLST clusters were created, SNPs were called by aligning assemblies against a reference genome chosen from each wgMLST cluster of closely related isolates, and SNP clusters and phylogenetic trees were inferred. An SNP cluster was classified as a cluster of isolates where each isolate was less than or equal to 50 SNPs distant from other members of the cluster (40). Individual phylogenetic trees for each SNP cluster, together with the metadata information of isolates in the same cluster (Data Set S2), were used to examine the relationship between wild bird isolates and other isolates.

Random Forest-based source attribution. A machine learning Random Forest (RF) classifier was used for source attribution of *S. Typhimurium* to bovine, porcine, poultry, and wild bird sources. The RF classifier was built using the method described by Zhang et al. (8). Briefly, a set of 3,102 genetic features, including 1,850 SNPs, 147 indels, and 1,105 accessory genes, were used for the RF classifier. The randomForest package (v4.6-12) of R was used to build the RF classifier with the "ntree" argument set to 1,000 and other parameters being default. The original training data (195 bovine isolates, 338 porcine isolates, 440 poultry isolates, and 68 wild bird isolates) and the training data with the addition of the 131 isolates from wild birds were applied for classifier training. Tables of confusion were generated to describe the performance of the classifier. To explain the prediction performance of the classifier after adding our bird isolates into the training data set, a maximum-likelihood tree based on core genome alignment of 1,604 *S. Typhimurium* isolates from different sources was constructed using Parsnp v1.5.0 (41). The 1,604 *S. Typhimurium* isolates included 171 human clinical isolates, 83 food isolates, 178 isolates from miscellaneous sources, and 1,172 isolates used for the RF classifier training (i.e., 195 bovine isolates, 338 porcine isolates, 440 poultry isolates, and 199 wild bird isolates in which 131 isolates were from this study).

AMR, virulence, and plasmid profiling. Raw reads of each isolate were *de novo* assembled using Shovill with Trimmomatic in Galaxy (v1.0.4) (42). To identify the antimicrobial resistance (AMR) genes present in the isolates, each draft genome assembly was searched against the ResFinder database and CARD database using ResFinder 4.1 (43) and Resistance Gene Identifier (RGI) v5.2.0 (44). AMR genes that passed the default threshold values for ResFinder ($\geq 90\%$ nucleotide identity and $\geq 60\%$ coverage) and RGI ($\geq 95\%$ nucleotide identity) were considered to be present in the isolate. Virulence factors were

predicted by aligning the draft genome assembly for each isolate against the Virulence Factor database using VFAnalyzer (45). Virulence factors that passed the default threshold values for VFAnalyzer ($\geq 90\%$ amino acid identity and $\geq 50\%$ coverage) were considered to be present in the isolate. If a virulence factor was absent in the isolate identified by VFAnalyzer, the virulence gene encoding the specific virulence factor was then compared to its reference gene from strain *S. Typhimurium* LT2 by BLAST to confirm the mutation type (e.g., insertion, deletion, or substitution). Plasmid replicon sequences were identified by comparing the draft genome assembly against the PlasmidFinder database using PlasmidFinder 2.1 with default search settings of $\geq 95\%$ nucleotide identity and $\geq 60\%$ coverage (46).

Data availability. Sequence data of the 131 *S. Typhimurium* isolates are deposited in the NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) under BioProject [PRJNA357723](https://www.ncbi.nlm.nih.gov/sra). Accession numbers are available in Data Set S1. Isolates that form SNP clusters with wild bird isolates examined in this study are provided in Data set S2 and publicly available in the NCBI Pathogen Detection database (<https://www.ncbi.nlm.nih.gov/pathogens/>).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.2 MB.

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Y.F. whole genome sequenced the wild bird isolates, designed the study, performed the bioinformatics analysis of the data, interpreted the data, and wrote the draft manuscript. N.M.M., J.M.L., D.S.B., B.B.-Z., C.A.W., and E.G.D. assisted in collection of the isolates and contributed to interpretation of data and manuscript revision. S.L. and X.D. participated in Random Forest-based source attribution, interpretation of data, and manuscript revision. J.C.S. and N.W.S. participated in *S. Typhimurium* CRISPR typing, interpretation of data, and manuscript revision. E.M.N. contributed to identification of virulence gene mutation, interpretation of data, and manuscript revision. E.G.D. additionally designed and supervised the study.

We declare no conflicts of interest.

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