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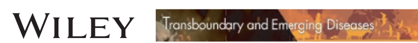

H7N6 low pathogenic avian influenza outbreak in commercial turkey farms in Chile caused by a native South American Lineage

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Summary

In December 2016, low pathogenic avian influenza (LPAI) caused by an H7N6 subtype was confirmed in a grow-out turkey farm located in Valparaiso Region, Chile. Depopulation of exposed animals, zoning, animal movement control and active surveillance were implemented to contain the outbreak. Two weeks later, a second grow-out turkey farm located 70 km north of the first site was also infected by H7N6 LPAI, which subsequently spilled over to one backyard poultry flock. The virus involved in the outbreak shared a close genetic relationship with Chilean aquatic birds' viruses collected in previous years. The A/turkey/Chile/2017(H7N6) LPAI virus belonged to a native South American lineage. Based on the H7 and most of the internal genes' phylogenies, these viruses were also closely related to the ones that caused a highly pathogenic avian influenza outbreak in Chile in 2002. Results from this study help to understand the regional dynamics of influenza outbreaks, highlighting the importance of local native viruses circulating in the natural reservoir hosts.

KEYWORDS
disease outbreaks, Influenza in birds, phylogeny, poultry, Viruses

1 | INTRODUCTION

Outbreaks caused by avian influenza viruses (AIV) have major impacts in the poultry industry. Each year, events of highly pathogenic avian influenza (HPAI), and low pathogenic avian influenza (LPAI) are reported by different countries regardless of their overall animal health status and strategies to prevent the introduction of foreign animal diseases. These outbreaks cause substantial economic losses to the poultry industry and governments due to the elimination of all exposed animals, deployment of resources for surveillance and disease control strategies, as well as international trade restrictions (Djunaidi & Djunaidi, 2007; Otte, Hinrichs, Rushton, Roland-Holst, & Zilberman, 2008). Disease prevention is complex because many wild aquatic birds are a natural reservoir of influenza A viruses (Vandegrift, Sokolow, Daszak, & Kilpatrick, 2010).

The H5 and H7 subtypes are known for their potential to mutate into HPAI, typically after infecting poultry, resulting in severe disease and high mortality (Alexander, 2000). These subtypes are often

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found in their natural reservoir. Of the H7 subtypes that have caused epidemics in poultry globally, the Asian H7N9 lineage emergence is one of the most relevant events. The H7N9 Asian lineage emerged in China in 2013. This virus is highly poultry adapted and spread widely in China, mutating to a highly pathogenic form during late 2016. The H7N9 virus that caused this outbreak is particularly concerning because, besides causing severe disease in poultry, it has been responsible for more than 1,600 cases of illness in humans (CDC, 2018; Jiao et al., 2018; Ke et al., 2017). In the Americas, H7 subtypes have caused sporadic outbreaks of HPAI in poultry (Afanador-Villamizar, Gomez-Romero, Diaz, & Ruiz-Saenz, 2017; Krauss et al., 2015). These include an H7N3 outbreak in Chile in broiler farms in 2002, an H7N3 outbreak in broiler farms in British Columbia, Canada in 2004, and an H7N3 outbreak also in a broiler breeding farm, in Saskatchewan, Canada in 2007 (Berhane et al., 2009; Max, Herrera, Moreira, & Rojas, 2007; Pasick, Berhane, & Hooper-McGrevy, 2009). More recently, an H7N3 subtype caused an HPAI epidemic in Jalisco, Mexico in commercial layer farms, in 2012–2013, an H7N8 infected a commercial turkey flock, in Indiana, US, in 2016 and an H7N9 caused an outbreak in commercial broiler farms in Tennessee, US, in 2017 (Brown, 2018; Kapczynski et al., 2013; Lee, Torchetti, Killian, Berhane, & Swayne, 2017).

The genetic diversity of AIV in South America has not been described in detail compared to that in North America. Regional dynamics of AIV have been associated to South American and North American lineages and their transmission through migration routes that connect North and South America, especially the Pacific, Central and Mississippi Flyways. However, the information and reports of subtypes and lineages circulating, does not exist in detail for all countries in South America. Of the previously reported AIVs in Latin America, 43.7% belong to migratory birds, 28.1% to local wild birds and 28.1% to poultry (Afanador-Villamizar et al., 2017). Additionally, several influenza subtypes have been detected in Chilean wild birds, including H5 and H7 subtypes, highlighting the risk for poultry production (Bravo-Vasquez et al., 2016; Jimenez-Bluhm et al., 2018; Mathieu et al., 2015).

As mentioned above, Chilean poultry has been affected by HPAI only once, in 2002. The outbreak was caused by HPAI A/chicken/Chile/2002(H7N3) virus that generated economic losses of over 31 million USD and ~635,000 animals dead or culled (Afanador-Villamizar et al., 2017; Max et al., 2007; Spackman, McCracken, Winker, & Swayne, 2006). Other AIV impacting poultry in Chile includes an infection in a turkey farm in Valparaiso region in 2009, which was caused by the H1N1 pandemic (pH1N1) human virus. This event was evidenced by decreased egg production and shell quality and was reported only 2 months after the first pH1N1 detection in humans in Chile (Mathieu et al., 2010). In 2011, an LPAI H4N8 was found by serologic surveillance in a grow-out turkey farm, also in the region of Valparaiso. The presence of this AIV was confirmed by isolation in embryonated eggs. There was no evident clinical disease at the farm and rapid detection prevented further spread of the virus (SAG, 2011).

In this study, we describe the epidemiological investigations of a LPAI H7N6 outbreak in Chilean poultry during late 2016 and early 2017. We characterized the viruses using whole genome sequencing and reconstructed their phylogeny using Bayesian time divergence estimation, to infer their genetic relationship with North and South American AIVs. These results highlight the importance of characterization and molecular surveillance of local South American AIV strains to understand the viruses that pose a major risk to domestic poultry.

2 | METHODS

2.1 | Description of the outbreak

On 26 December 2016 a grow-out turkey house (Farm A) in the region of Valparaiso reported clinical respiratory disease. On the following day, respiratory signs increased in severity. Noticeable, this farm had undergone serological (ELISA) AIV testing (n = 60) on December 20, yielding negative results. On December 29 and following the diagnosis of clinical disease, the poultry company notified positive AIV ELISA test results to the Chilean Agricultural and Livestock Service (Servicio Agricola Ganadero; SAG).

On December 30, SAG officially confirmed the presence of AIV by real time RT-PCR (AI matrix gene). Subsequently, H5 and H7 real time RT-PCR subtyping assays were performed, confirming an H7 subtype at the Virology Unit of SAG laboratory. The samples were initially sent to a private laboratory (Macrogen, Inc.) for partial sequencing of the HA cleavage site. The amino acid sequence was consistent with a LPAI virus (corresponding to the translated amino acid site: NVPEKPRTR/GLF). Characterization of the neuraminidase gene subtype was carried out by neuraminidase-inhibition assay at SAG laboratory, revealing a N6 subtype. Furthermore, characterization of the virus included sequencing and in vivo testing to confirm the pathotype, performed at the United States Department of Agriculture's National Veterinary Services Laboratory (NVSL; Ames, Iowa, United States) and SAG laboratories.

Once the LPAI H7N6 outbreak was officially confirmed, immediate control measures were established. Control areas consisting of two zones surrounding the infected farm were defined: (a) an infected zone within a 3 km radius of the infected premises where animal movement was restricted; and (b) a buffer zone within a 7 km radius of the infected premises, where increased biosecurity and surveillance for avian influenza were carried out (USDA, 2015). Additionally, all breeders and grow-out farms belonging to the affected company throughout the country were tested for influenza (including the ones outside the buffer zone).

By January 2, clinical respiratory disease was present in flocks in five out of eight house barns within Farm A. The animals presented a variety of gross lesions including bursitis, catarrhal to mucopurulent tracheitis, caseous airsacculitis, polyserositis, pericarditis/hydropericardium, congestion and pulmonary oedema, mucopurulent to caseous pneumonia, localized subcutaneous emphysema, mild

splenomegaly and pancreatitis. Perihepatitis was only found in one house in the Farm A.

Surveillance following the outbreak in Farm A was performed between 29 December 2016 and 13 January 2017, and consisted in blood sample collection and testing by agar gel immunodiffusion (AGID) assay. A total of 327,000 exposed animals in Farm A were culled between December 30 and 31 (index barn), and animals in the remaining barns were culled between January 9 and 10.

At the national level, recent records with productivity data from all commercial farms were reviewed, focusing in mortality, meat production and egg production curves. The objective was to determine any abnormalities and potential undetected cases using syndromic surveillance. The parameters from all commercial farms yielded within expected production values.

On 17 January 2017, 70 km north from the first affected farm (Valparaiso), a male turkey grow-out farm (Farm B) belonging to the same company, was visited by a veterinarian due to increased respiratory disease signs. SAG confirmed the presence of AIV and culled 35,572 exposed animals in the farm. All animals in farm B were culled on January 18, and the farm was subsequently cleaned and disinfected. Infected and buffer zones were put in place around the infected premises, and surveillance testing was finished by June 20. Disposal of all carcasses was done by burial. In Farm B clinical gross lesions observed consisted of caseous sinusitis, catarrhal tracheitis, petechiae and ecchymosis focused on epicardium and coronary fat, fibrin-purulent pericarditis, purulent airsacculitis, pulmonary congestion/mucopurulent to caseous pneumonia, pleuritis and mild splenomegaly.

Epidemiological investigations concluded that the initial entry route of the virus into Farm A was through a breach in biosecurity, which resulted in transmission from wild birds through faeces or contaminated water into one of the grow-out sites. Lateral transmission between Farm A and Farm B likely occurred due to an unreported breach in biosecurity by personnel, supplies or vehicles that were shared between Farm A and Farm B.

On January 28, a backyard poultry farm within a household located in the defined buffer zone of Farm B tested positive to AIV by AGID but was negative to PCR testing. This farm had direct links through personnel with Farm B. All poultry in the household were culled on January 29.

Vaccination against seasonal avian influenza is mandatory for all workers in commercial poultry farms. This avian influenza outbreak was reported to the Pan American Health Organization (Regional Office for the Americas of the World Health Organization). The Animal Health authorities in coordination with the Ministry of Health, immediately triggered a protocol to investigate potential influenza infection in exposed humans. All directly or indirectly exposed personnel in Farms A and B, as well as contacts from the backyard operation were clinically evaluated for respiratory symptoms. A total of nine individuals with respiratory disease (six from Farm A and three from Farm B/backyard operation) were sampled. Samples were tested for AIV and for the specific H7 subtype at the Public Health Institute Laboratories. All samples were negative for influenza. Therefore, no human cases of A/turkey/Chile/2017(H7N6) infection were reported during this outbreak.

Thereafter, all testing conducted in commercial farms inside and outside the control zones were negative to AIV. On June 9, 3 months after finalizing stamping out of exposed and infected animals, Chile regained the OIE free status (OIE, 2017).

2.2 | Viral sequencing and phylogenetic analysis

Complete genome sequencing attempts were made from oral and tracheal swab pools at the NVSL. Briefly, viral RNA was extracted from samples using the MagMAX Viral RNA Isolation Kit (Ambion/ThermoFisher Scientific). Complementary DNA was synthesized by reverse transcription reaction using SuperScript III (Invitrogen/ThermoFisher Scientific). All eight segments were amplified by PCR and complete genome sequencing was conducted using the Illumina Miseq system. The Nextera XT DNA Sample Preparation Kit (Illumina) was used to generate multiplexed paired-end sequencing libraries, according to the manufacturer's instructions. The dsDNA was fragmented and tagged with adapters by Nextera XT transposase and 12-cycle PCR amplification. Fragments were purified on Agencourt AMpure XP beads (Beckman Coulter). The barcoded multiplexed library sequencing was performed using the 500 cycle MiSeq Reagent Kit v2 (Illumina). De Novo and directed assembly of genome sequences were carried out using the SeqMan NGen v.4 program. Viral sequences were submitted to GenBank (accession numbers MK424141–MK424219).

Phylogenetic analyses were performed separately for each segment. The closest references viruses were obtained by a BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search and the 100 closest hits were included to reconstruct the phylogenetic tree. Sequences obtained for each segment and the corresponding reference sequences were aligned using MUSCLE (Edgar, 2004). Redundant reference sequences were discarded (identical or near identical sequences with similar collection dates and locations). Bayesian time divergence estimation using the HKY+G[4] nucleotide substitution model and an uncorrelated relaxed clock was performed for all segments. Coalescent Bayesian Skyline tree prior was used to reconstruct the phylogeny of H7 (segment 4) to allow for population size changes over time. For N6 (segment 6) exponential growth and Bayesian skyline coalescent tree priors were compared using path sampling and stepping-stone sampling marginal likelihood estimation (Baele, Li, Drummond, Suchard, & Lemey, 2013). For the remaining segments 1, 2, 3, 5, 7 and 8 (coding for PB2, PB1, PA, NP, M and NS, respectively) a coalescent exponential growth tree prior was initially run. If the 95% high posterior density (95% HPD) of the growth parameter included 0 (meaning that the population size is not growing in time) a coalescent constant population tree prior was run and used for the final phylogeny, whereas if the parameter did not include 0, the original run with the exponential growth tree prior used was used. The analyses were run in

BEAST 1.8.4 (Drummond, Suchard, Xie, & Rambaut, 2012). A total of 500,000,000 iterations were run, sampling every 50,000 trees using Cipres computational resources (Miller, Pfeiffer, & Schwartz, 2010). Traces of the parameters were checked for convergence and >200 effective sample size. The maximum clade credibility (MCC) tree was annotated, burning the first 10% sampled trees, using TreeAnnotator and visualized using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Amino acid sequences were compared to the closest references to identify relevant mutations using MEGA7 (Kumar, Stecher, & Tamura, 2016).

2.3 | Genetic markers for human transmission

To determine if there was a risk of infection in humans, we analysed the outbreak sequences to determine the presence of potential genetic markers of AIV adaptation in humans (Lloren, Lee, Kwon, & Song, 2017). The specific amino acid changes queried were in PB2: K526R using reference A/Zhejiang/DTID-ZJU01/2013(H7N9) (KC885962.1); A588V using reference A/chicken/Guangdong/V/2008(H9N2) (JQ639783.1); Q591K, D701N, M535L and T271A using reference A/Shanghai/02/2013(H7N9) (NC_026422.1). As well as potential markers in HA: A135T (A143T) using reference A/Netherlands/33/03, Q226L using reference A/Shanghai/02/2013(H7N9) (NC_026425.1) and K404R using reference A/Netherlands/33/03(H7N7) (AY338457.1).

3 | RESULTS

Phylogenies were estimated using coalescent constant population tree priors for PB2, PA, NP, M and NS coding segments. Bayesian skyline tree prior was used for reconstructing HA and NA phylogenies, while exponential growth was used to estimate the phylogeny of PB1 (Table 1). The estimated nucleotide substitution rate per site per year for each coding segment ranged between 1.96×10^{-3} (95% HPD 1.52×10^{-3}–2.44×10^{-3}) and 3.98×10^{-3} (95% HPD 2.83×10^{-3}–5.26×10^{-3}), which were the lowest and highest rates estimated for segment 7 (M) and 4 (HA), respectively.

3.1 | HA coding segment

All viruses from the 2017 Chilean turkey LPAI outbreak viruses were included in a monophyletic clade, which shared the closest ancestor with two viruses (A/yellow_billed_pintail/Chile/10/2014(H7N3) and A/yellow_billed_teal/Chile/9/2013(H7N6)) collected from wild local water birds. The tMRCA shared between the closest Chilean wild bird virus and the A/turkey/Chile/2017(H7N6) was estimated at 2012 (95% HPD 2011–2013) (Figure 1). The A/turkey/Chile/2017(H7N6) LPAI viruses were also related to AIV found in wild aquatic birds in Chile and Bolivia, and with the A/Chicken/Chile 2002 (that had caused an HPAI outbreak in Chile) with a tMRCA estimated at 1995 (95% HPD 1989–1999). Based on

TABLE 1 Results of Bayesian time estimation analyses for each protein-coding segment. Tree prior used for Bayesian time divergence estimation analysis for each segment alignment is shown. The estimated nucleotide substitution rate per site per year for each coding segment was highest for HA. The time to the most recent common ancestor (tMRCA) with the closest reference virus, with A/chicken/Chile/2002(H7N3) and with equine influenza virus are indicated for related viral segments, as well as the characteristics of the lineage to which they belong

Protein-coding segment	Tree prior used (coalescent)	Nucleotide substitution/site/year × 10^{-3} (95% HPD)	tMRCA closest reference sequence(s) (95% HPD)
PB2	Constant population	2.19 (1.63, 2.82)	2008 (2006–2011)
PB1	Exponential growth	2.70 (2.30, 3.11)	2011 (2009–2013)
PA	Constant population	2.60 (1.87, 3.40)	2012 (2010–2013)
HA	Bayesian Skyline	3.79 (2.98, 4.67)	2012 (2011–2013)
NP	Constant population	2.90 (1.82, 4.18)	2014 (2012–2015)
NA	Bayesian Skyline	2.01 (1.67, 2.36)	2004 (2000–2008)
M	Constant population	2.02 (1.60, 2.49)	2012 (2010–2014)
NS	Constant population	2.38 (1.55, 3.29)	2011 (2010–2013)

Note. [a]Reference viruses from Chilean/chicken or equine influenza references were not included as they were more distant than any other reference included to reconstruct the phylogeny.

the HA phylogeny, these South American viruses were grouped in a unique cluster. The remaining closest reference, belonging to a North American avian lineage, that recently caused an HPAI outbreak in the United States, shared an earlier common ancestor estimated in 1944 (95% HPD 1913–1969). Specific amino acid changes fixed in the LPAI A/turkey/Chile/2017(H7N6) compared to the closest references were in HA amino acid positions V42I, V96I, I191V, P524S, and a fixed mutation in Farm B sequences R316K (relative to HPAI A/chicken/Chile2002, which has a 10-amino acid insertion in positions 338–347; Table 2). Three of these specific amino acid changes are located in antigenic sites C, D and E (Supplementary File 1), which are not related to additional glycosylation sites.

3.2 | NA coding segment

Similar to HA coding segments, NA sequences from the A/turkey/Chile/2017(H7N6) LPAI outbreak are genetically related to a distinct group that includes viruses collected from Chilean wild birds in 2013 (H7 and H3 subtypes being the closest ones) with a tMRCA estimated at 2004 (95% HPD 2000–2008) (Figure 2). This group is more genetically distant to viruses from North American avian lineages compared to the HA and most internal genes' phylogenies, having a tMRCA estimated at 1869 (95% HPD 1823–1911). Sequences from A/turkey/Chile/2017(H7N6) LPAI had three amino acid substitutions fixed compared to the closest references (amino acid positions T42P, V333A and N359S). We included only one sequence per sample to depict the phylogeny, however, a mixed population of NA sequences with and without stalk deletions (ranging between 23 and 27 amino acid long) were found in (pooled) samples collected from Farm A. All four samples from Farm B had sequences with the same 26 amino acid nucleotide stalk deletion between amino acid positions 32–57. This deletion was not associated with additional glycosylation sites in HA.

3.3 | Phylogeny of internal segments PB2, PB1, PA, NP, M and NS

For the reconstructed phylogeny of internal segments, the closest reference sequences to the A/turkey/Chile/2017(H7N6) LPAI viruses were viruses collected from Chilean aquatic birds (Figure 3). The closest relationships for each segment of A/turkey/Chile/2017(H7N6) LPAI were with different viruses from a variety of HA and NA types (Table 1). PB1, PA, HA and NP internal genes coding segments of A/turkey/Chile/2017(H7N6) LPAI shared a similar tMRCA with A/chicken/Chile/2002(H7N3) HPAI estimated between 1992 and 1995. In terms of regional lineages, the A/turkey/Chile/2017(H7N6) LPAI segments PB2, NP and M were grouped with avian viruses sampled mostly from South America and few from North America, whereas segments PA, HA and NS clustered

tMRCA with A/Chicken/Chile/2002 H7N3 (95% HPD)	Closest viral sequence/clade from reference sequences	Geographic related lineage	tMRCA Equine Influenza (95% HPD)
1986 (1975–1994)	A/yellow_billed_pintail/Chile/C1267/2015(H5N3), A/yellow_billed_pintail/Chile/10/2014(H7N3), A/mallard_duck/Chile/C4079/2015(H1N1), A/yellow_billed_pintail/Chile/C4256/2015(H10N1), A/yellow_billed_teal/Chile/C4131/2015(H1N1)	Mostly South American avian, few North American avian	[a]
1994 (1993–1996)	A/mallard_duck/Chile/C4079/2015(H1N1), A/mallard/Chile/C948/2015(H4N2)	South American and North American avian	[a]
1995 (1989–1999)	A/yellow_billed_pintail/Chile/10/2014(H7N3), A/blackish_oystercatcher/Chile/C6534/2016(H2N2)	South American avian	1942 (1989–1999)
1995 (1989–1999)	A/yellow_billed_pintail/Chile/10/2014(H7N3)	South American avian	[a]
1992 (1986–1997)	A/yellow_billed_teal/Chile/C5750/2016(H12N5)	Mostly South American avian, few North American avian	1955 (1986–1997)
[a]	A/yellow_billed_teal/Chile/9/2013(H7N6), A/yellow_billed_teal/Chile/8/2013(H7N6), A/red_fronted_coot/Chile/5/2013(H3N6)	Only Chilean avian	[a]
1988 (1982–1994)	A/yellow_billed_teal/Chile/C5750/2016(H12N5)	Mostly South American avian, few North American avian	[a]
1826 (1678–1953)	A/red_fronted_coot/Chile/5/2013(H3N6)	South American avian	1942 (1921–1955)

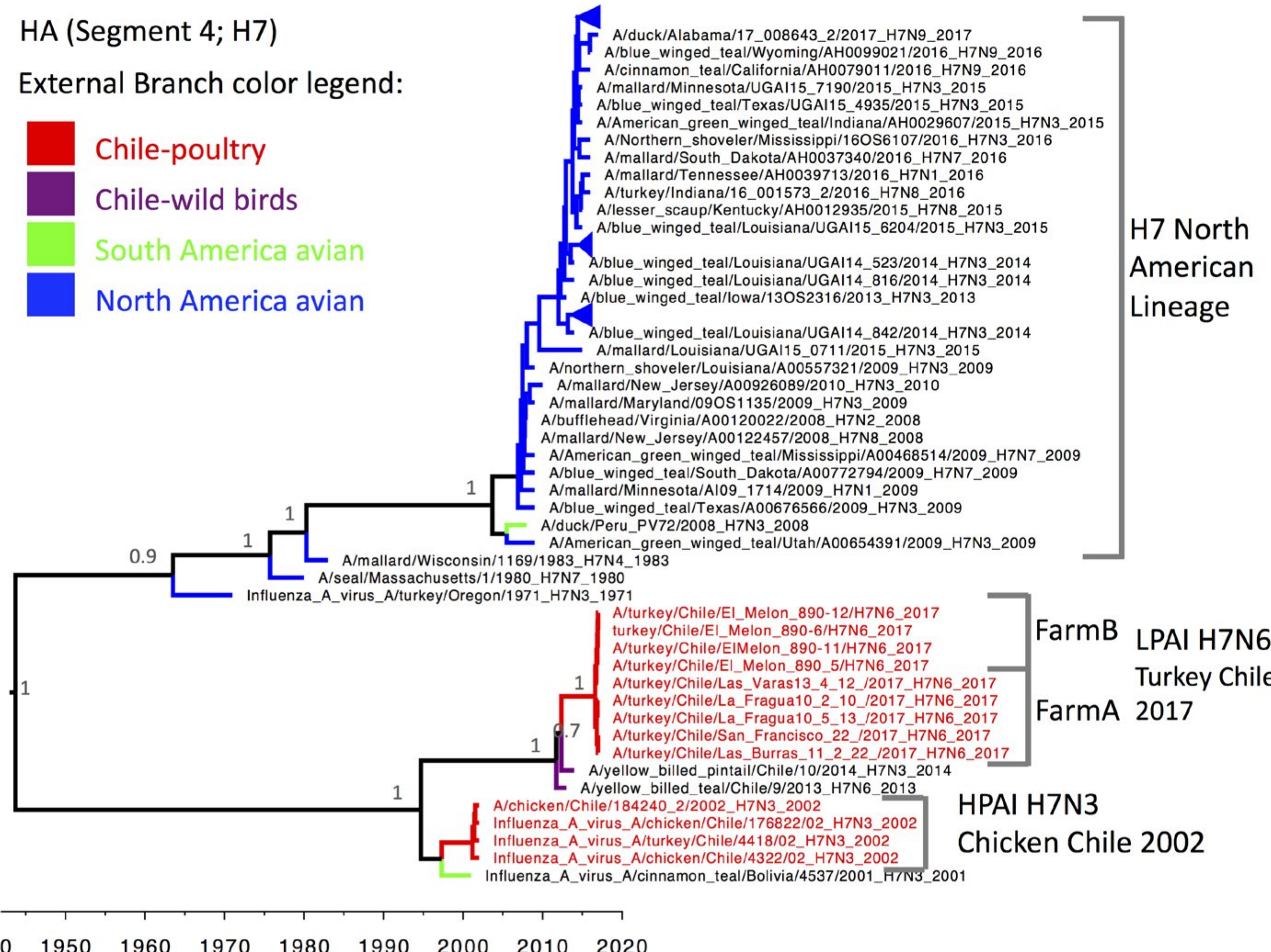


FIGURE 1 Maximum clade credibility tree of HA segment. The South American lineage shared a common ancestor in 1944 (95% HPD 1913–1969) with the closest reference viruses (North American Lineage). The viruses that caused the A/turkey/Chile/2017(H7N6) low pathogenic avian influenza (LPAI) viruses were genetically related to the those that caused an highly pathogenic avian influenza (HPAI) outbreak in 2002 in Chile (A/chicken/Chile/2002[H7N3]) [Colour figure can be viewed at wileyonlinelibrary.com]

with sequences that were exclusively South American. The PA, NP and NS lineages to which A/turkey/Chile/2017(H7N6) LPAI belong, were related to equine viruses as a result from a potential reassortment event that occurred sometime between 1942 and 1955 (Table 1). Interestingly, one of the M segments from the A/chicken/Chile/2002(H7N3) outbreak was distinct from other viruses collected during that epidemic (Figure 3).

Nucleotide mutations fixed in the A/turkey/Chile/2017(H7N6) LPAI viruses compared to close reference from local aquatic birds were found in PB1 (V344I, V444I and T683I), in M2 (K18R and V27I) and in NP (V193I, T196I and M221V) as well as a Farm B specific fixed mutation in PB1 (R101G and N573H) and NP (M123I).

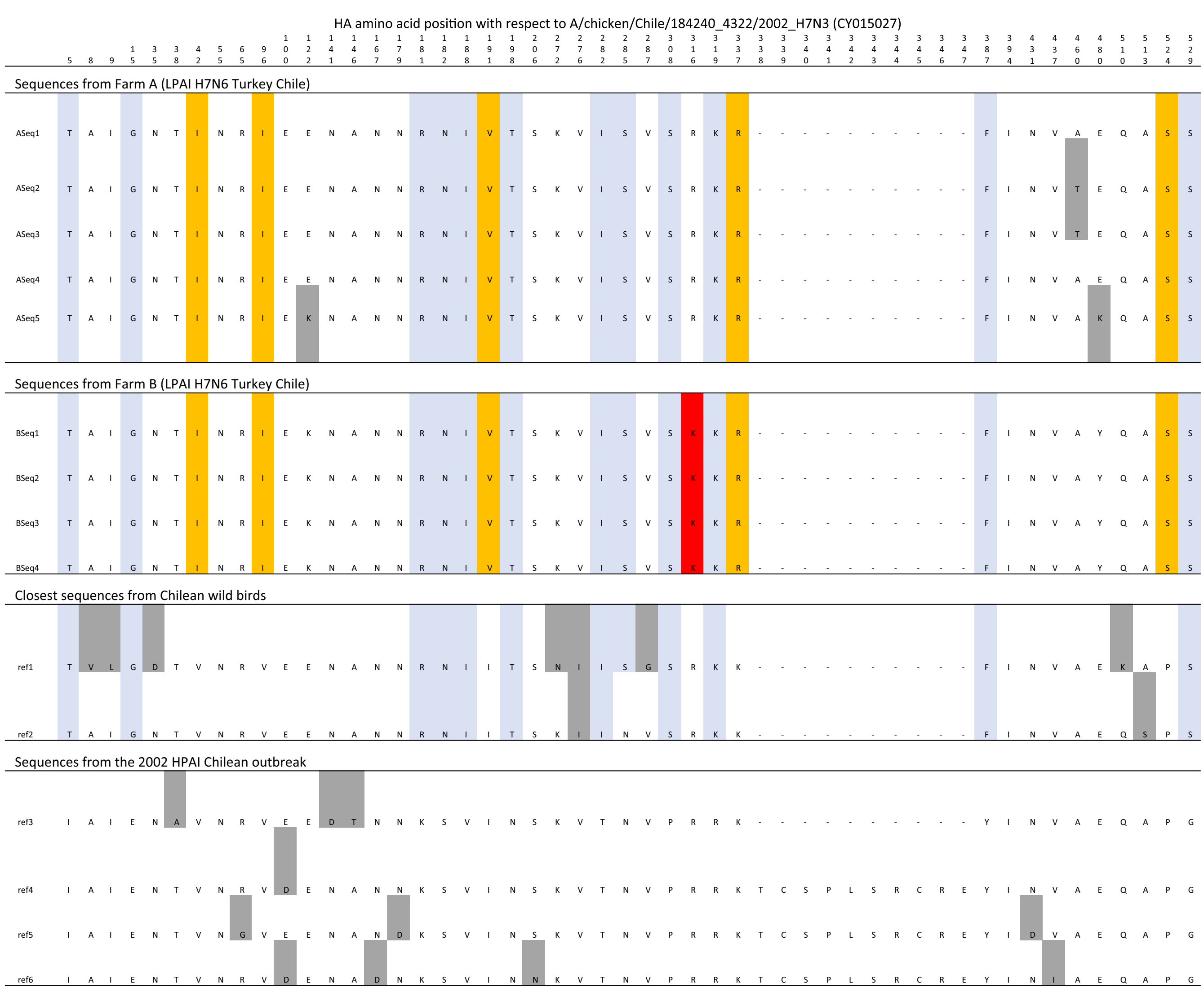
None of the segments were related to the pH1N1 viruses that caused an outbreak in turkeys during 2010, and which HA and NA were related to the 2009 pandemic virus.

None of the genetic markers for AIV adaptation in humans were found in the A/turkey/Chile/2017(H7N6) sequences.

4 | DISCUSSION

In Chile, an outbreak of H7N6 LPAI (A/turkey/Chile/2017(H7N6)) was detected shortly after initial signs of respiratory disease were reported in a commercial turkey grow-out farm in December 2016. Epidemiological investigations and phylogenetic analyses of viral sequence data were used to understand the source of the outbreak and characterize the lineage and the genetic relationships with reference viruses. All segments from the A/turkey/Chile/2017(H7N6) LPAI virus were related to viruses collected in previous years from wild birds in Chile. The A/turkey/Chile/2017(H7N6) LPAI virus grouped within the same lineage as the Chicken/Chile/2002(H7N3) HPAI, based on the phylogeny of HA and most genetic segments coding for internal proteins. The A/turkey/Chile/2017(H7N6) LPAI virus belongs to a native South American lineage, and is not closely related to other recent viruses reported from poultry in the Americas, such as the recent H7N9 subtype that had caused outbreaks during 2017 in commercial chicken farms in Tennessee and

TABLE 2 Sites with amino acid variants in HA alignment of A/turkey/Chile/2017(H7N6), the closest viruses collected from wild birds, and viruses from the previous Chicken/Chile/2002 H7N3 outbreak. Sites with at least one amino acid difference in the alignment are shown. Changes fixed in viruses collected after the 2002 outbreak are coloured in blue, whereas changes fixed only in A/turkey/Chile/2017(H7N6) LPAI are highlighted in orange. Non-fixed mutations are highlighted in grey. One nucleotide substitution was fixed in Farm B R316K (highlighted in red) . Viruses indicated as references correspond to the following sequences: ref1=A/yellow billed pintail/Chile/10/2014(H7N3), ref2=A/yellow billed teal/Chile/9/2013(H7N6), ref3=A/chicken/Chile/176822/02(H7N3), ref4=A/chicken/Chile/184240-4322/2002(H7N3), ref5=A/turkey/Chile/4418/02(H7N3), ref6=A/chicken/Chile/4322/02(H7N3)



HA amino acid position with respect to A/chicken/Chile/184240_4322/2002_H7N3 (CY015027)

	5	8	9	15	35	38	42	55	65	96	100	122	141	146	167	179	181	182	188	191	198	206	272	276	282	285	287	308	316	319	337	338	339	340	341	342	343	344	345	346	347	387	394	431	437	460	480	510	513	524	529
Sequences from Farm A (LPAI H7N6 Turkey Chile)																																																			
ASeq1	T	A	I	G	N	T	I	N	R	I	E	E	N	A	N	N	R	N	I	V	T	S	K	V	I	S	V	S	R	K	R	-	-	-	-	-	-	-	-	-	-	F	I	N	V	A	E	Q	A	S	S
ASeq2	T	A	I	G	N	T	I	N	R	I	E	E	N	A	N	N	R	N	I	V	T	S	K	V	I	S	V	S	R	K	R	-	-	-	-	-	-	-	-	-	-	F	I	N	V	T	E	Q	A	S	S
ASeq3	T	A	I	G	N	T	I	N	R	I	E	E	N	A	N	N	R	N	I	V	T	S	K	V	I	S	V	S	R	K	R	-	-	-	-	-	-	-	-	-	-	F	I	N	V	T	E	Q	A	S	S
ASeq4	T	A	I	G	N	T	I	N	R	I	E	E	N	A	N	N	R	N	I	V	T	S	K	V	I	S	V	S	R	K	R	-	-	-	-	-	-	-	-	-	-	F	I	N	V	A	E	Q	A	S	S
ASeq5	T	A	I	G	N	T	I	N	R	I	E	K	N	A	N	N	R	N	I	V	T	S	K	V	I	S	V	S	R	K	R	-	-	-	-	-	-	-	-	-	-	F	I	N	V	A	K	Q	A	S	S
Sequences from Farm B (LPAI H7N6 Turkey Chile)																																																			
BSeq1	T	A	I	G	N	T	I	N	R	I	E	K	N	A	N	N	R	N	I	V	T	S	K	V	I	S	V	S	K	K	R	-	-	-	-	-	-	-	-	-	-	F	I	N	V	A	Y	Q	A	S	S
BSeq2	T	A	I	G	N	T	I	N	R	I	E	K	N	A	N	N	R	N	I	V	T	S	K	V	I	S	V	S	K	K	R	-	-	-	-	-	-	-	-	-	-	F	I	N	V	A	Y	Q	A	S	S
BSeq3	T	A	I	G	N	T	I	N	R	I	E	K	N	A	N	N	R	N	I	V	T	S	K	V	I	S	V	S	K	K	R	-	-	-	-	-	-	-	-	-	-	F	I	N	V	A	Y	Q	A	S	S
BSeq4	T	A	I	G	N	T	I	N	R	I	E	K	N	A	N	N	R	N	I	V	T	S	K	V	I	S	V	S	K	K	R	-	-	-	-	-	-	-	-	-	-	F	I	N	V	A	Y	Q	A	S	S
Closest sequences from Chilean wild birds																																																			
ref1	T	V	L	G	D	T	V	N	R	V	E	E	N	A	N	N	R	N	I	I	T	S	N	I	I	S	G	S	R	K	K	-	-	-	-	-	-	-	-	-	-	F	I	N	V	A	E	K	A	P	S
ref2	T	A	I	G	N	T	V	N	R	V	E	E	N	A	N	N	R	N	I	I	T	S	K	I	I	N	V	S	R	K	K	-	-	-	-	-	-	-	-	-	-	F	I	N	V	A	E	Q	S	P	S
Sequences from the 2002 HPAI Chilean outbreak																																																			
ref3	I	A	I	E	N	A	V	N	R	V	E	E	D	T	N	N	K	S	V	I	N	S	K	V	T	N	V	P	R	R	K	-	-	-	-	-	-	-	-	-	-	Y	I	N	V	A	E	Q	A	P	G
ref4	I	A	I	E	N	T	V	N	R	V	D	E	N	A	N	N	K	S	V	I	N	S	K	V	T	N	V	P	R	R	K	T	C	S	P	L	S	R	C	R	E	Y	I	N	V	A	E	Q	A	P	G
ref5	I	A	I	E	N	T	V	N	G	V	E	E	N	A	N	D	K	S	V	I	N	S	K	V	T	N	V	P	R	R	K	T	C	S	P	L	S	R	C	R	E	Y	I	D	V	A	E	Q	A	P	G
ref6	I	A	I	E	N	T	V	N	R	V	D	E	N	A	D	N	K	S	V	I	N	N	K	V	T	N	V	P	R	R	K	T	C	S	P	L	S	R	C	R	E	Y	I	N	I	A	E	Q	A	P	G

Alabama in the United States (Lee et al., 2017), and the H7N3 that has caused sporadic HPAI outbreaks in Mexico since 2015 (OIE, 2018a, 2018b). This South American lineage is also unrelated to the poultry-adapted Asian H7N9 virus (CDC, 2018).

Control of AIV in poultry is complex and challenging. High biosecurity measures must be accomplished to prevent viruses that continuously circulate in wild birds from entering poultry operations. A wide diversity of influenza A viruses exist among water birds, which may persist in geographical areas where commercial and backyard poultry farms are located. A large number of wetlands throughout the extensive Chilean coast, provides an ecosystem where migratory and local wild water birds (including the main avian influenza reservoirs) co-exist (Jimenez-Bluhm et al., 2018). Small backyard production systems are also constantly exposed to transmission from wild birds and are, likely, sporadically infected (Bravo-Vasquez et al., 2016), representing another potential source of AIV for commercial farms. Additionally, transmission from human or other species also pose a risk for influenza outbreaks in poultry, such as the pH1N1 introduced from human into a commercial turkey farm in Chile in 2009 (Mathieu et al., 2010).

The specific mechanism in which the virus first entered Farm A is still unclear. Sequence analysis indicates that this virus is very similar to the ones circulating in wild birds. The most likely origin of the outbreak is a breach in the farm's biosecurity that allowed viruses from wild birds entering Farm A. Genetic similarity with viruses collected from wild birds support this hypothesis. Additionally,

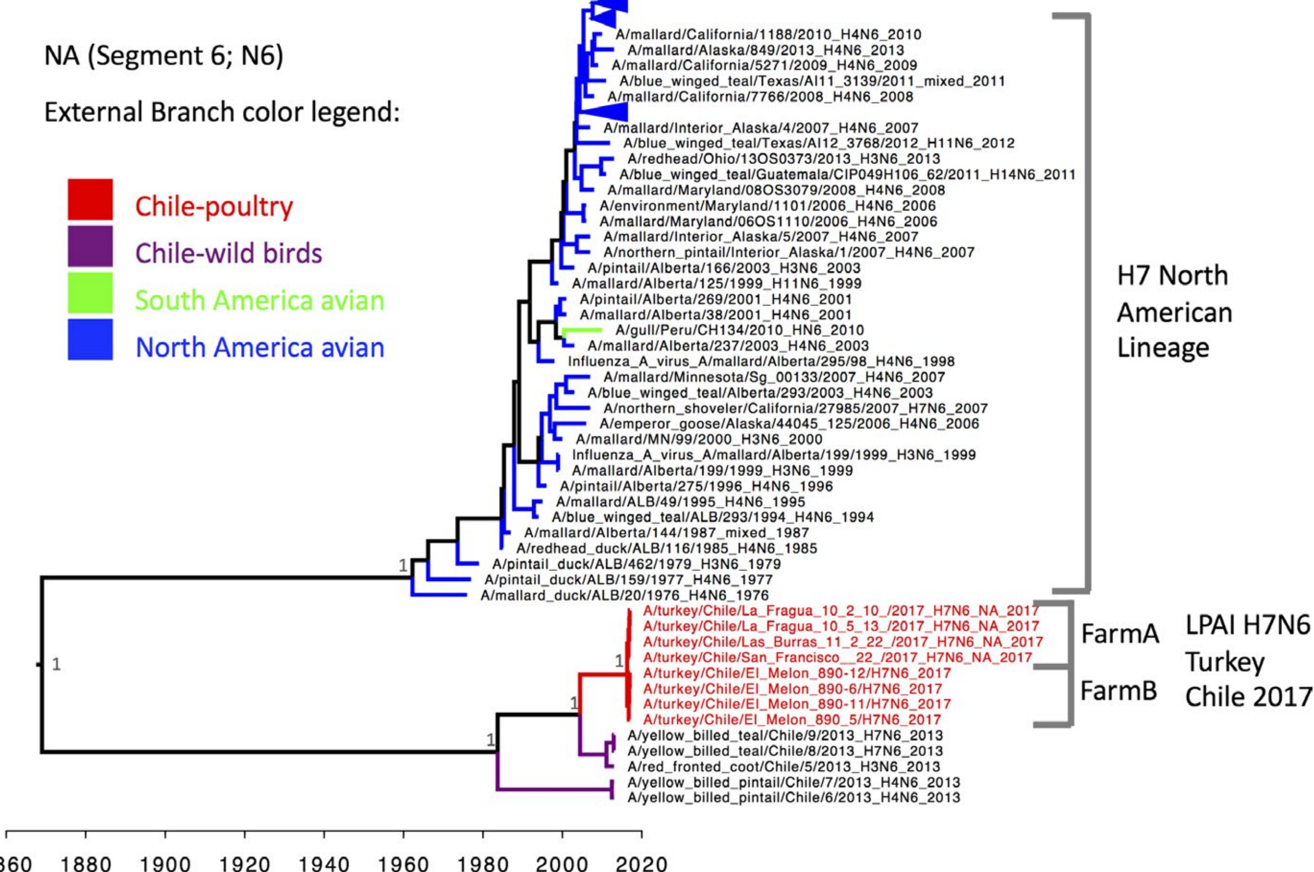


FIGURE 2 Maximum clade credibility tree of NA segment. A/turkey/Chile/2017(H7N6) low pathogenic avian influenza (LPAI) viruses are grouped in a unique cluster unrelated to other references and sharing a common ancestor in 1869 (95% HPD 1823–1911) with the closest North American lineage [Colour figure can be viewed at wileyonlinelibrary.com]

no further infected farms were found during surveillance activities, so it is unlikely that the virus was transmitted from another farm.

A close ancestral relationship between viruses that caused the A/chicken/Chile/2002(H7N3) HPAI and the A/turkey/Chile/2017(H7N6) LPAI revealed the importance of this specific South American lineage virus as a risk for commercial poultry farms. Aquatic bird reservoirs have maintained this virus throughout the years, posing a risk that was not evidenced until the recent 2017 outbreak. Considering this H7N6 event, as well as the previous H7N3 in Chile, this local viral lineage has been the main source of AIV poultry outbreaks. However, the risk of introduction of other lineages into Chile should not be underestimated as there is a wide variety of local and North American lineages circulating in wild water birds.

Previous studies have supported that A/chicken/Chile/2002(H7N3), as well as other American AIV H7 subtype strains, need a lower infectious dose to cause clinical disease in turkey, compared to that needed to affect chicken (Spackman et al., 2006, 2010). It is relevant to design targeted surveillance, as turkey may act as sentinels for emerging and more pathogenic AIV strains. In this particular event, clinical disease caused by LPAI in two turkey farms was rapidly detected and preventive measures were timely put in place to contain viral spread. Some viruses in Farm A and all viruses from Farm B had already acquired amino acid stalk deletion in its NA segment. These genetic changes have been associated to an adaptation from the natural reservoir (aquatic birds) to the poultry host and confer increased virulence and transmission (Campitelli et al., 2004; Li, Zu Dohna, Cardona, Miller, & Carpenter, 2011; Sorrell, Song, Pena, & Perez, 2010). Therefore, this virus was rapidly adapting to commercial turkey.

The genetic changes occurred in the viral genome within a short time window (less than a month) allowed to correctly group Farm A and B clusters in the phylogeny reconstructed for segments HA, NA, PB1 and M. However, the reconstruction of the remaining segments did not allow to discern between Farm A and B. This is information is relevant to understand the level of resolution to reconstruct interfarm transmission networks in based on phylogenies. The overall substitution rates reported here reflect the phylogeny of all the viruses used for the analysis, including North American lineages, as well as equine and swine viruses included in the internal segment analyses. The estimates were within ranges described previously for H7 AIV subtype (Chen & Holmes, 2010; Lewis et al., 2015; Lu, Lycett, & Leigh Brown, 2014; Rejmanek, Hosseini, Mazet, Daszak, & Goldstein, 2015).

In conclusion, because of the rapid diagnostic and intervention, the LPAI outbreak that affected Chilean turkeys in 2017 was

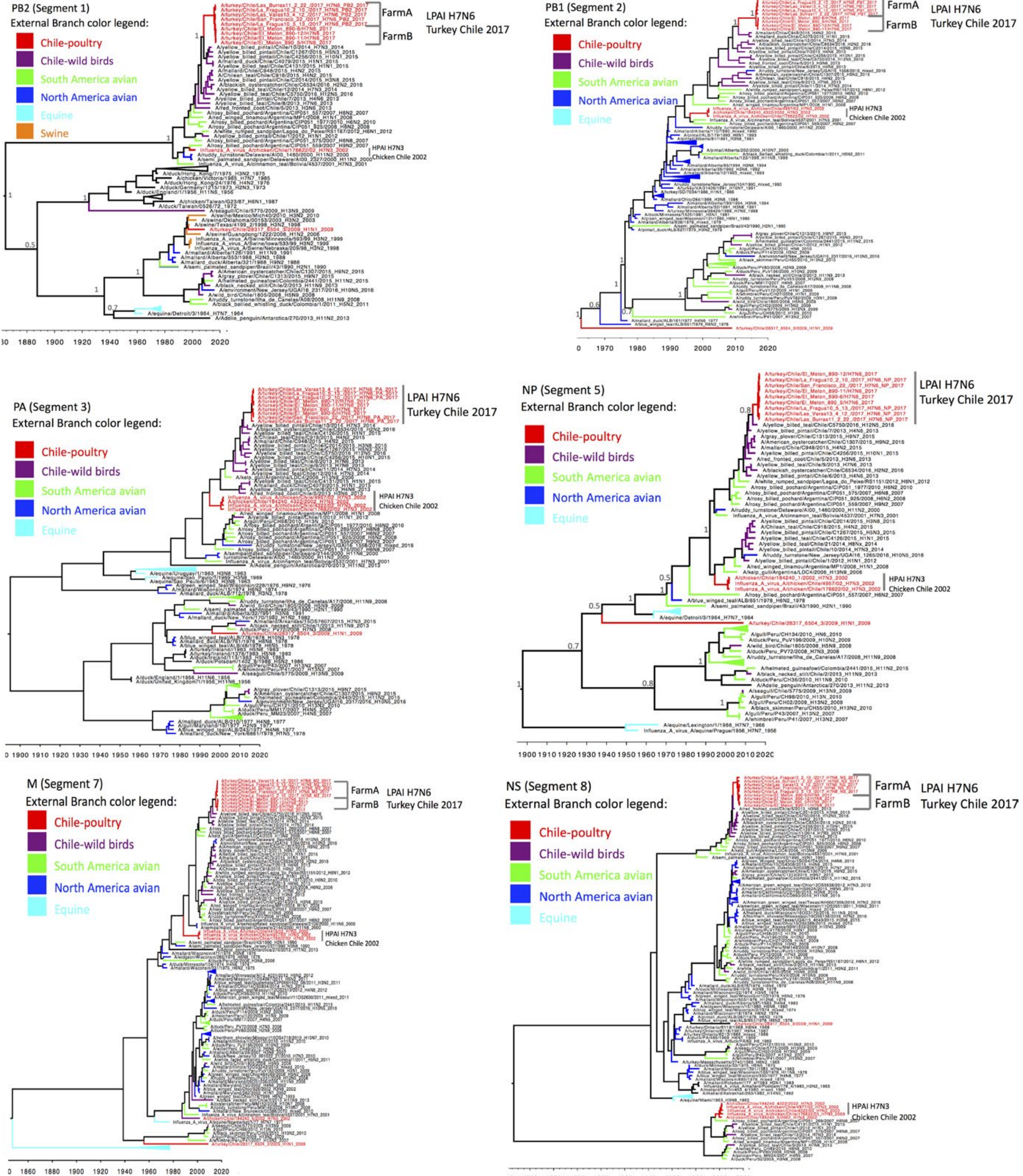


FIGURE 3 Maximum clade credibility tree of segments PB2, PB1, PA, NP, M and NS. All segments are closely related to viruses previously collected from Chilean wild birds. All Segments, except for NS, are related to the previous Chilean 2002 highly pathogenic avian influenza (HPAI) outbreak [Colour figure can be viewed at wileyonlinelibrary.com]

contained and limited to only two premises of the same company. The outbreak was caused by a South American lineage that is unrelated to the 2017 North American H7N9 outbreak in the United States in March. The lack of systematic molecular AIV surveillance in South American countries highlights the need to coordinate regional efforts to better understand the genetic diversity and viral

dynamics in the region, and to be prepared for future outbreaks in domestic species (Afanador-Villamizar et al., 2017). This report contributes to understanding this particular clinical LPAI event in turkey and the role of local South American lineages circulating in the wild reservoirs as a major source of AIVs that are pathogenic to domestic poultry.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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REFERENCES

Afanador-Villamizar, A., Gomez-Romero, C., Diaz, A., & Ruiz-Saenz, J. (2017). Avian influenza in Latin America: A systematic review of serological and molecular studies from 2000-2015. *PLoS ONE*, *12*(6), e0179573. https://doi.org/10.1371/journal.pone.0179573

Alexander, D. J. (2000). A review of avian influenza in different bird species. *Veterinary Microbiology*, *74*(1–2), 3–13. https://doi.org/10.1016/S0378-1135(00)00160-7

Baele, G., Li, W. L., Drummond, A. J., Suchard, M. A., & Lemey, P. (2013). Accurate model selection of relaxed molecular clocks in bayesian phylogenetics. *Molecular Biology and Evolution*, *30*(2), 239–243. https://doi.org/10.1093/molbev/mss243

Berhane, Y., Hisanaga, T., Kehler, H., Neufeld, J., Manning, L., Argue, C., … Pasick, J. (2009). Highly pathogenic avian influenza virus A (H7N3) in domestic poultry, Saskatchewan, Canada, 2007. *Emerging Infectious Diseases*, *15*(9), 1492–1495. https://doi.org/10.3201/eid1509.080231

Bravo-Vasquez, N., Di Pillo, F., Lazo, A., Jimenez-Bluhm, P., Schultz-Cherry, S., & Hamilton-West, C. (2016). Presence of influenza viruses in backyard poultry and swine in El Yali wetland, Chile. *Preventive Veterinary Medicine*, *134*, 211–215. https://doi.org/10.1016/j.prevetmed.2016.10.004

Brown, J. A. (2018). Public health response to an avian influenza A(H7N8) virus outbreak in commercial Turkey flocks — Indiana, 2016. *MMWR. Morbidity and Mortality Weekly Report*, *67*, 1339–1341. https://doi.org/10.15585/mmwr.mm6748a2

Campitelli, L., Mogavero, E., De Marco, M. A., Delogu, M., Puzelli, S., Frezza, F.,… Donatelli, I. (2004). Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in Italy. *Virology*, *323*(1), 24–36. https://doi.org/10.1016/j.virol.2004.02.015

CDC (Centers for Disease Control and Prevention). (2018). Asian lineage avian influenza A (H7N9) virus. Retrieved from https://www.cdc.gov/flu/avianflu/h7n9-virus.htm

Chen, R., & Holmes, E. C. (2010). Hitchhiking and the population genetic structure of avian influenza virus. *Journal of Molecular Evolution*, *70*(1), 98–105. https://doi.org/10.1007/s00239-009-9312-8

Djunaidi, H., & Djunaidi, A. (2007). The economic impacts of avian influenza on world poultry trade and the U.S. poultry industry: A spatial equilibrium analysis. *Journal of Agricultural and Applied Economics*, *39*(2), 313–323. https://doi.org/10.1017/s1074070800023014

Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, *29*(8), 1969–1973. https://doi.org/10.1093/molbev/mss075

Edgar, R. C. (2004). MUSCLE: A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics*, *5*, 113. https://doi.org/10.1186/1471-2105-5-113

Jiao, P., Song, Y., Huang, J., Xiang, C., Cui, J., Wu, S.,… Liao, M. (2018). H7N9 avian influenza virus is efficiently transmissible and induces an antibody response in chickens. *Frontiers in Immunology*, *9*, 789. https://doi.org/10.3389/fimmu.2018.00789

Jimenez-Bluhm, P., Karlsson, E. A., Freiden, P., Sharp, B., Di Pillo, F., Osorio, J. E.,… Schultz-Cherry, S. (2018). Wild birds in Chile Harbor diverse avian influenza A viruses. *Emerging Microbes & Infections*, *7*(1), 44. https://doi.org/10.1038/s41426-018-0046-9

Kapczynski, D. R., Pantin-Jackwood, M., Guzman, S. G., Ricardez, Y., Spackman, E., Bertran, K., … Swayne, D. E. (2013). Characterization of the 2012 highly pathogenic avian influenza H7N3 virus isolated from poultry in an outbreak in Mexico: Pathobiology and vaccine protection. *Journal of Virology*, *87*(16), 9086–9096. https://doi.org/10.1128/JVI.00666-13

Ke, C., Mok, C. K. P., Zhu, W., Zhou, H., He, J., Guan, W.,… Peiris, J. S. M. (2017). Human infection with highly pathogenic avian influenza A(H7N9) virus, China. *Emerging Infectious Diseases*, *23*(8), 1332–1340. https://doi.org/10.3201/eid2308.170600

Krauss, S., Stucker, K. M., Schobel, S. A., Danner, A., Friedman, K., Knowles, J. P., … Webster, R. G. (2015). Long-term surveillance of H7 influenza viruses in American wild aquatic birds: Are the H7N3 influenza viruses in wild birds the precursors of highly pathogenic strains in domestic poultry? *Emerging Microbes & Infections*, *4*(1), 1–9. https://doi.org/10.1038/emi.2015.35

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Lee, D. H., Torchetti, M. K., Killian, M. L., Berhane, Y., & Swayne, D. E. (2017). Highly pathogenic avian influenza A(H7N9) virus, Tennessee, USA, March 2017. *Emerging Infectious Diseases*, *23*(11). https://doi.org/10.3201/eid2311.171013

Lewis, N. S., Verhagen, J. H., Javakhishvili, Z., Russell, C. A., Lexmond, P., Westgeest, K. B., … de Graaf, M. (2015). Influenza A virus evolution and spatio-temporal dynamics in Eurasian wild birds: A phylogenetic and phylogeographical study of whole-genome sequence data. *The Journal of General Virology*, *96*(8), 2050–2060. https://doi.org/10.1099/vir.0.000155

Li, J., Zu Dohna, H., Cardona, C. J., Miller, J., & Carpenter, T. E. (2011). Emergence and genetic variation of neuraminidase stalk deletions in avian influenza viruses. *PLoS ONE*, *6*(2), e14722. https://doi.org/10.1371/journal.pone.0014722

Lloren, K. K. S., Lee, T., Kwon, J. J., & Song, M.-S. (2017). Molecular markers for interspecies transmission of avian influenza viruses in

mammalian hosts. *International Journal of Molecular Sciences*, *18*(12). https://doi.org/10.3390/ijms18122706
Lu, L., Lycett, S. J., & Leigh Brown, A. J. (2014). Reassortment patterns of avian influenza virus internal segments among different subtypes. *BMC Evolutionary Biology*, *14*(1), 16. https://doi.org/10.1186/1471-2148-14-16
Mathieu, C., Moreno, V., Pedersen, J., Jeria, J., Agredo, M., Gutierrez, C.,… Retamal, P. (2015). Avian Influenza in wild birds from Chile, 2007-2009. *Virus Research*, *199*, 42–45. https://doi.org/10.1016/j.virusres.2015.01.008
Mathieu, C., Moreno, V., Retamal, P., Gonzalez, A., Rivera, A., Fuller, J.,… Avalos, P. (2010). Pandemic (H1N1) 2009 in breeding turkeys, Valparaiso, Chile. *Emerging Infectious Diseases*, *16*(4), 709–711. https://doi.org/10.3201/eid1604.091402
Max, V., Herrera, J., Moreira, R., & Rojas, H. (2007). Avian influenza in Chile: A successful experience. *Avian Diseases*, *51*(1 Suppl), 363–365. https://doi.org/10.1637/7631-042806R1.1
Miller, M. A., Pfeiffer, W., & Schwartz, T. (2010, 14, Nov, 2010). *Creating the CIPRES Science Gateway for inference of large phylogenetic trees*. Paper presented at the Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans.
OIE (World Organization for Animal Health). (2017). Wahis, Follow-up report No. 8 (Final report). *World Animal Health Information Database*. Retrieved from https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=24038
OIE (World Organization for Animal Health). (2018a). *OIE situation report for avian influenza*. Retrieved from http://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/OIE_AI_situation_report/OIE_SituationReport_AI_January2018_01.pdf
OIE (World Organization for Animal Health). (2018b). Update on avian influenza in animals (types H5 and H7), avian influenza portal. Retrieved from http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/
Otte, J., Hinrichs, J., Rushton, J., Roland-Holst, D., & Zilberman, D. (2008). Impacts of avian influenza virus on animal production in developing countries. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *3*(80). https://doi.org/10.1079/pavsnnr20083080
Pasick, J., Berhane, Y., & Hooper-McGrevy, K. (2009). Avian influenza: The Canadian experience. *Revue Scientifique Et Technique (International Office of Epizootics)*, *28*(1), 349–358.
Rejmanek, D., Hosseini, P. R., Mazet, J. A. K., Daszak, P., & Goldstein, T. (2015). Evolutionary dynamics and global diversity of influenza A virus. *Journal of Virology*, *89*(21), 10993–11001. https://doi.org/10.1128/JVI.01573-15
SAG (Servicio Agricola y Ganadero). (2011). *Informe de Situacion Sanitaria de Chile Año 2011*. Retrieved from https://www2.sag.gob.cl/Pecuaria/bvo/BVO_15_I_semestre_2012/quehacer_unidades/descargables/situacion_sanitaria_animal_2011.pdf.
Sorrell, E. M., Song, H., Pena, L., & Perez, D. R. (2010). A 27-amino-acid deletion in the neuraminidase stalk supports replication of an avian H2N2 influenza A virus in the respiratory tract of chickens. *Journal of Virology*, *84*(22), 11831–11840. https://doi.org/10.1128/JVI.01460-10
Spackman, E., Gelb, J., Jr., Preskenis, L. A., Ladman, B. S., Pope, C. R., Pantin-Jackwood, M. J., & McKinley, E. T. (2010). The pathogenesis of low pathogenicity H7 avian influenza viruses in chickens, ducks and turkeys. *Virology Journal*, *7*, 331. https://doi.org/10.1186/1743-422X-7-331
Spackman, E., McCracken, K. G., Winker, K., & Swayne, D. E. (2006). H7N3 avian influenza virus found in a South American wild duck is related to the Chilean 2002 poultry outbreak, contains genes from equine and North American wild bird lineages, and is adapted to domestic turkeys. *Journal of Virology*, *80*(15), 7760–7764. https://doi.org/10.1128/JVI.00445-06
USDA (United States Department of Agriculture, Veterinary Services National Preparedness and Incident Coordination NPIC). (2015). *Highly pathogenic avian influenza (HPAI). Incidence 2014-2015, ready reference guide- overview of zones*. Retrieved from https://www.aphis.usda.gov/animal_health/emergency_management/downloads/hpai_zones.pdf.
Vandegrift, K. J., Sokolow, S. H., Daszak, P., & Kilpatrick, A. M. (2010). Ecology of avian influenza viruses in a changing world. *Annals of the New York Academy of Sciences*, *1195*, 113–128. https://doi.org/10.1111/j.1749-6632.2010.05451.x

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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