nature communications

Article

https://doi.org/10.1038/s41467-025-57338-z

Cross-species and mammal-to-mammal transmission of clade 2.3.4.4b highly pathogenic avian influenza A/H5N1 with PB2 adaptations

Received: 28 May 2024

Accepted: 17 February 2025

Published online: 06 March 2025

Check for updates

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Highly pathogenic H5N1 avian influenza viruses (HPAIV) belonging to lineage 2.3.4.4b emerged in Chile in December 2022, leading to mass mortality events in wild birds, poultry, and marine mammals and one human case. We detected HPAIV in 7,33% (714/9745) of cases between December 2022–April 2023 and sequenced 177 H5N1 virus genomes from poultry, marine mammals, a human, and wild birds spanning >3800 km of Chilean coastline. Chilean viruses were closely related to Peru's H5N1 outbreak, consistent with north-to-south spread down the Pacific coastline. One human virus and nine marine mammal viruses in Chile had the rare PB2 D701N mammalian-adaptation mutation and clustered phylogenetically despite being sampled 5 weeks and hundreds of kilometers apart. These viruses shared additional genetic signatures, including another mammalian PB2 adaptation (Q591K, $n = 6$), synonymous mutations, and minor variants. Several mutations were detected months later in sealions in the Atlantic coast, indicating that the pinniped outbreaks on the west and east coasts of South America are genetically linked. These data support sustained mammal-to-mammal transmission of HPAIV in marine mammals over thousands of kilometers of Chile's Pacific coastline, which subsequently continued through the Atlantic coastline.

Influenza A virus (IAV) is a segmented negative single-stranded RNA virus with a high capacity to evolve and spill over to new host species, resulting in epizootics in animals and occasional pandemics in humans[1,2]. Aquatic wild birds are considered the primary natural reservoir of IAV and harbor 16 HA subtypes and 9 NA subtypes. Endemic and migratory birds interact across established migratory flyways, which can serve as routes for long-distance IAV dispersal[3]. Highly pathogenic avian influenza viruses (HPAIV) present a major

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threat to animal health, causing important losses to the poultry industry and affecting wild birds and occasionally mammal populations. Zoonotic cases of HPAIV of the H5N1 subtype have high fatality rates in humans, upwards of 50%, and until recently have been concentrated in Asia[4]. The 1957, 1968, and 1977 influenza pandemics all originated in Asia, which has the world's largest poultry and swine populations and commonly serves as a global source of novel IAV strains. However, IAV ecology is complex and fast-changing, with the most recent 2009 H1N1 pandemic originated in Mexico, where the virus lurked undetected for years, reassorting and evolving in unexpected ways, before suddenly emerging in humans in early 2009[5]. South America continues to be a potential source for novel pandemic influenza viruses yet remains severely under-surveilled.

Since 2020, outbreaks caused by the HPAIV H5N1 2.3.4.4b clade have been reported in Europe, Asia, Africa, and North America[6]. In October 2022, HPAIV H5N1 emerged in South America[7] with cases identified in Peru, Venezuela, Chile, Mexico, and Ecuador[7,8]. To date, the reporting of HPAIV H5N1 in mammals, including humans, continues to be linked to high viral loads in positive wild birds[9,10]. Given the 2.3.4.4b virus's potential for reassortment, efficient transmission in avian populations, and the ability to transmit across species, this lineage was recently included in the World Health Organization's list of candidate vaccines for zoonotic influenza[11,12]. The 2.3.4.4b virus's rapid global expansion, frequent spillover into mammals, and unpredictable evolution, including genomic reassortment, raises concerns that the virus could evolve towards more efficient mammalian transmission and increase the risk of a global pandemic.

The first case of HPAIV H5N1 in Chile was confirmed in December 2022[13], resulting in large outbreaks in seabirds. The virus rapidly disseminated across the country, covering ~3800 kilometers over 4 months, from early December 2022 to April 2023. During this period, HPAIV of the H5N1 2.3.4.4b clade affected a diverse array of species, including but not limited to seabirds, bird scavengers, penguins, raptors, poultry, and marine mammals such as sea lions, sea otters, porpoises and dolphins. An estimated 1.900% increase (19 times) in the historical marine mammal strandings and deaths have been observed since the first detection of HPIAV H5N1 in Chile (National Fisheries Services; [SERNAPESCA] official records)[14]. The first detection of HPIAV H5N1 in poultry in Chile was reported on February 4th, 2023. Prior to the outbreak, Chile was considered free of avian influenza in poultry, and vaccination was not allowed due to commercial restrictions[15]. The H5N1 outbreak resulted in 100 positive backyard and 7 commercial poultry cases confirmed as HPAIV H5 positive, resulting in about 1,4 million poultry animals being culled to control the outbreak or which had died in major regions where commercial poultry farms are located. In March 2023, a positive human case was confirmed[16]. The patient developed difficult breathing (dyspnea) and was transferred to a regional hospital, where a bronchoalveolar (BAL) sample was collected and tested positive for Influenza A Virus and was later subtyped as A(H5N1) HPAIV at the National Influenza Center, the Instituto de Salud Pública Chile (ISP)[17]. The sample was sent to the WHO Collaborating Center at the Center for Disease Control and Prevention (CDC) in the United States for further characterization, and pathotyping in the ferret model, confirming its capacity to infect, produce severe disease and transmit by close direct contact in mammals[18,19].

A collaborative effort between governmental and academic institutions began in Chile in December 2022 to provide real-time active surveillance to monitor the outbreak and limit spillover events to other hosts. During December 2022–April 2023, we procured over 18,500 samples from 9745 cases reported in seabirds, birds of prey, backyard poultry, commercial poultry farms, marine mammals, terrestrial mammals, and humans, representing the largest and most comprehensive study of H5N1 HPAIV outbreak in Latin America to date. Using Bayesian phylodynamic approaches, we found that wild birds and poultry frequently exchanged H5N1 viruses back and forth in Chile. In contrast, the viruses found in Chilean marine mammals formed a discrete clade, including the human virus, that was defined by genetic signatures not found in any birds, raising the possibility that mammal-to-mammal transmission occurred in marine mammals over considerable time and space.

Results

HPAIV H5N1 outbreak in Chile

A large sampling effort was performed to monitor, contain, and characterize the Chilean HPAIV outbreak. 18,501 samples from 9745 submitted cases were tested, of which 7.33% (714 cases) were confirmed positive to H5N1 HPAIV by real-time RT-PCR (Supplementary Data). Most samples were collected from swabs taken from deceased animals in areas where HPAIV H5N1 had been detected, including samples taken in the context of mass mortality events, especially for pelicans, shags, sea lions, and penguins. The positive H5N1 cases were distributed across a 3800+ kilometer stretch of Chile's Pacific coastline extending from the Arica Region in Northern Chile (Lat: -18.419 long: -70.321) to the Magallanes Region in Southern Chile (Lat: -51.735 Long: -72.506) (Supplementary Fig. 1 and Supplementary Data). Positive cases were found in 18 of the 28 analyzed animal orders including wild birds, mammals, and backyard and commercial poultry. Here we report a 73.67% positivity rate of HPAIV in wild birds during the first 4 months of the outbreak in Chile, indicating an unprecedented high prevalence of the virus in wild avian populations, of which 121 (16.94%) occurred in Peruvian pelican (*Pelecanus thagus*), 119 (16.66%) in Peruvian booby (*Sula variegata*), 65 (9.10%) in Guanay shag (*Leucocarbo bougainvilliorum*), 43 (6.02%) in kelp gull (*Larus dominicanus*) and 73 (10.22 %) in other species in the *Charadriiformes* order. Positive cases were also detected in vultures (*Cathares aura n* = 20, *Coragyps atratus n* = 5), swans (*Cygnus melancoryphus n* = 31, *Coscoroba swan n* = 4), and Humboldt penguins (*n* = 4) (Supplementary Data). Positivity rates were highest in these avian orders: *Pelecaniformes* (45.79%), *Podicipediformes* (37.50%), *Cathartiformes* (31.65%), *Suliformes* (29.77%).

One hundred and thirty-six positive cases were found in poultry, primarily from backyard farms (including chickens, turkeys, domestic ducks, and geese), with only 14 positive cases from commercial poultry farms. Importantly, 38 positive cases were detected in marine mammals, including 32 South American sea lions *(Otaria flavescens*), two endangered marine otters (*Lontra felina)*, two Burmeister's porpoises (*Phocoena spinipinnis*) and two Chilean dolphins (*Cephalorhynchus eutropia*) (Supplementary Data). High positivity rates were observed in South American sea lions (28.07%, 32/114). From December 2022 to May 2023, 4.016 sea lion stranding cases were reported, in contrast to the annual average strandings of 205 cases previously documented by SERNAPESCA, indicating a 1960% increase in stranded animals (Fig. 1; Supplementary Fig. 1)[14]. All sampled marine mammals presented unspecific signs of disease such as weakness, anorexia, and weight loss[20]. In some animals, additional specific signs were observed, such as acute dyspnea, tachypnea, profuse nasal secretion, sialorrhea, and abdominal breathing (respiratory syndrome), as well as neurological signs such as tremors, ataxia, paralysis of limbs, disorientation, and nystagmus (neurological syndrome)[14]. Similarly, during this period, an 2.24 increase in the number of strandings of Chilean dolphins, Burmeister's porpoises, Fin whale (*Balaenoptera physalus*), and estimated 23.46 higher incidence of Humboldt penguins (*Spheniscus humboldti*) strandings[21] associated with increased mortality events were also documented between Arica and Coquimbo Regions (SERNAPESCA personal communication, Fig. 1).

Single introduction of HPAIV H5N1 clade 2.3.4.4b from North America into Peru and Chile

Whole (*n* = 159) and partial genome sequences (*n* = 18) were obtained from 316 positive samples (Ct values < 37) collected in Chile from wild

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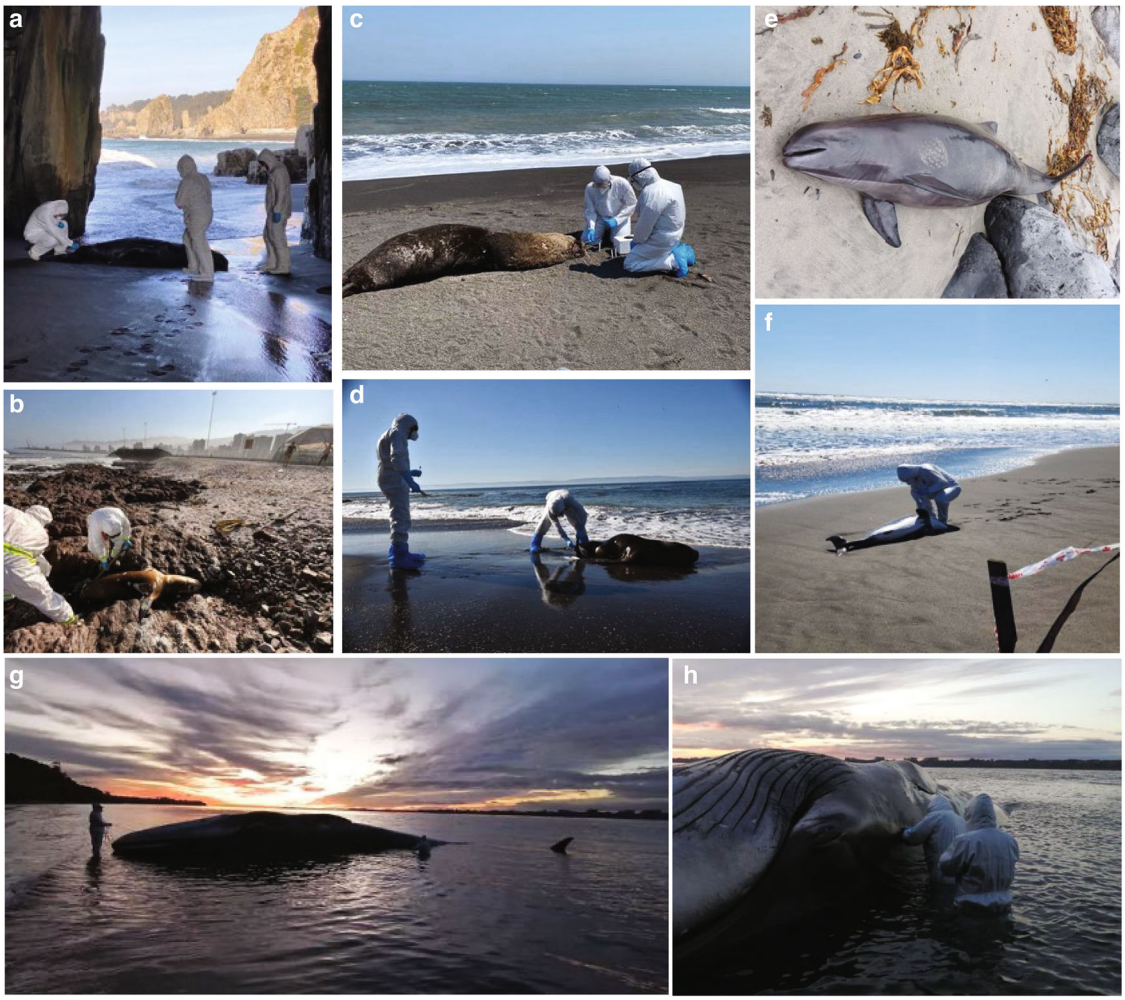

Fig. 1 | Unprecedented mortality of marine mammals associated with the HPAIV H5N1 outbreak in Chile. Documented mortality events of South American sea lions (**a**) Ñuble Region, (March 19), (**b**) Arica Region (March 1), (**c**) Atacama Region, January 10 (**d**) Tarapaca Region, March 07, (**e**) Burmeister´s porpoise, Atacama Region (March 29) (**f**) Chilean dolphin, Maule Region (March, 18) and (**g**, **h**) Fin whale, Biobio Region (April 24), in the Pacific coasts of Chile during the H5N1 HPAIV outbreak in Chile during January - April 2023.

birds ($n = 182$), poultry ($n = 82$), and marine mammals ($n = 52$), and one human case (Supplementary Data). The sequenced viruses were collected from 14 Chilean regions over 18 weeks, allowing for detailed phylogenetic analysis using Bayesian approaches to quantify viral gene flow. Phylogenetic analysis revealed that all H5N1 viruses collected from Chile had 4:4 reassortant genomes (B3.2 genotype) similar to the H5N1 viruses observed during the same time period in Peru[22] (annotated trees for individual segments are available, at the GitHub repository at https://github.com/mostmarmot/ChileHPAIV) and at the beginning of the outbreak in Chile[13]. The reassortant B3.2 viruses retained four segments (PA, HA, NA, and MP) from the original Eurasian AIV lineage and acquired four new segments (PB2, PB1, NP, NS) from the Americas AIV lineage. The viruses from Chile and Peru clustered together into a single monophyletic cluster inferred for each genome segment (Fig. 2, Supplementary Fig. 2), which represents a single introduction from North America into Peru/Chile, estimated to have occurred between August 14 – October 4, 2022 (95% HPD, Fig. 2). The Peru/Chile H5N1 introduction subsequently spread to Argentina, Brazil, Ecuador, Uruguay, and South Atlantic islands in the Antarctic region (Fig. 3). In contrast, Colombia and Venezuela had independent introductions of other H5N1 genotypes from North America that did not appear to spread onward to other countries in South America (Fig. 2; see http://bit.ly/3Jye2FN for an animated visualization inferred from the MCC tree). Continuous phylogeographic reconstructions inferred that the H5N1 epidemic in Chile was driven primarily by short-range north-to-south movements (Fig. 4a). The period of most rapid spatial dispersal occurred in late 2022 following the arrival of the virus in Peru and early expansion in an immunologically naïve population (Fig. 4b)[8].

Frequent interspecies transmission of H5N1 in Chile

H5N1 genomes were obtained from 13 taxonomic orders in Chile, providing information about how the virus transmitted over time and space among species with different behaviors and habitats (Fig. 3). The data set included six orders of aquatic wild birds (*Anseriformes, Charadriiformes, Pelecaniformes, Podicipediformes, Sphenisciformes*, and *Suliformes*), three orders of terrestrial wild birds (*Cathartiformes, Falconiformes*, and *Psittaciformes*), two orders of domestic birds (Anseriformes, Galliformes), two orders of marine mammals (*Carnivora* and *Cetacea*), and one order of terrestrial mammal (Primate, in this case human). Anseriformes are known to be a key reservoir for LPAIVs and tend to be oversampled, but most of our sequenced viruses came from pelicans, boobies, cormorants, gulls, and other seabirds. The MCC tree (Fig. 3) using concatenated genomes shows a high degree of mixing of H5N1 viruses between different orders. The "Markov jump" counts allow to quantify the rates of virus gene flow across the entire South American clade ($n = 293$ viruses) and provide at least four observations about the H5N1 ecology in South America (Fig. 5). First, there was extensive

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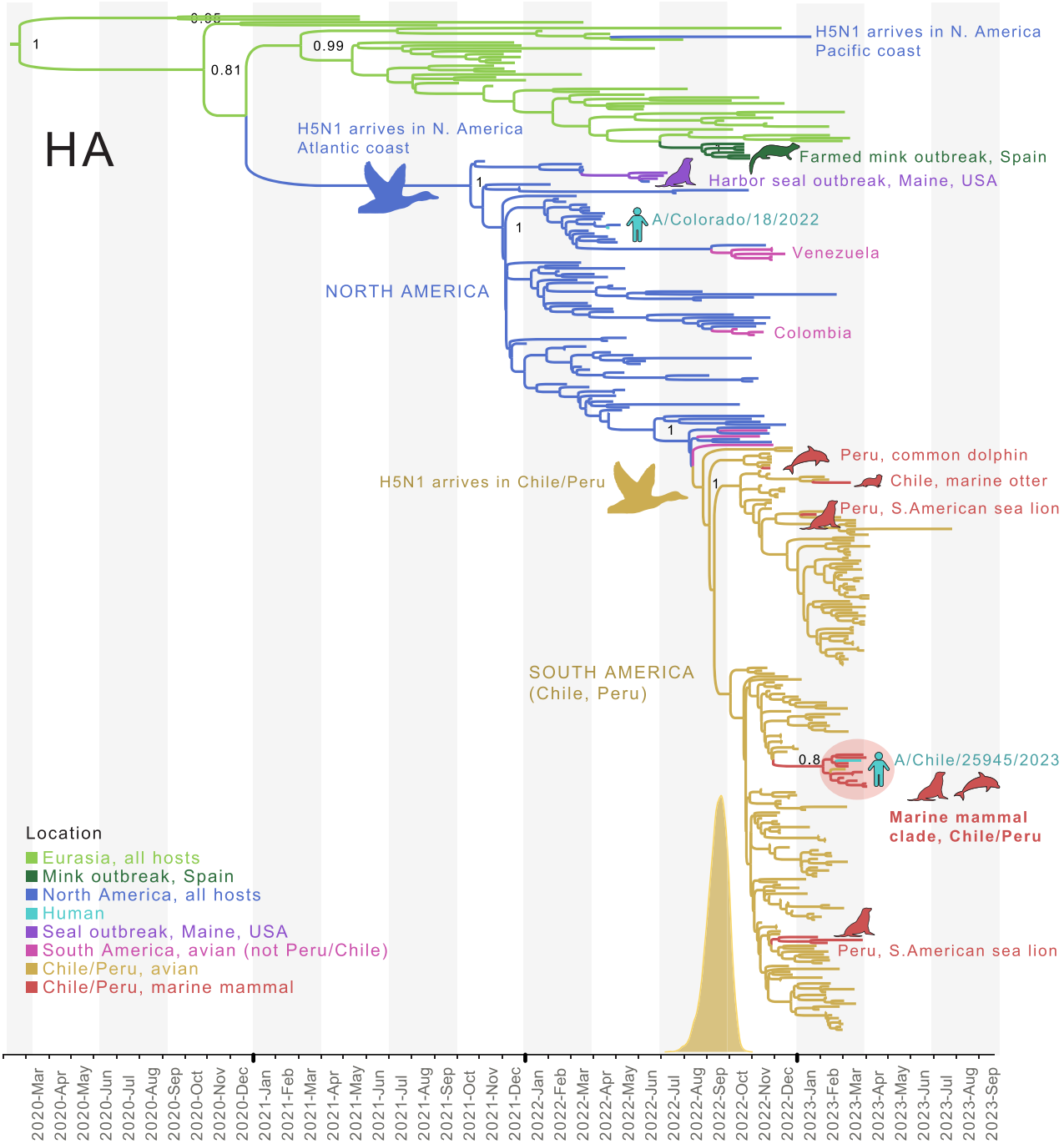

Fig. 2 | Introduction of H5N1 viruses into Chile. Time-scaled maximum clade credibility (MCC) tree inferred for the HA segment. The data set includes 177 H5N1 viruses sequenced for this study from marine mammals, poultry, wild birds, and one human case, and 204 background H5N1 viruses collected globally from all species during 2021-2023, downloaded from GISAID on August 25, 2023. Posterior probabilities are provided for key nodes. Branches shaded by location and host species. Posterior density plot, shaded yellow, of the inferred timing (95% HPD) of the introduction of H5N1 into Chile and Peru during the Southern Hemisphere spring (Northern Hemisphere autumn) of 2022. Thick marks in x-axis indicate the separation in the years plotted.

viral transmission back and forth between various wild aquatic birds and Galliformes (poultry). Second, there was extensive viral transmission back and forth between different seabirds, particularly *Charadriiformes* (including gulls and terns), *Pelecaniformes* (including pelicans), and *Suliformes* (including cormorants). Among these avian orders, there is no clear reservoir species. Third, *Charadriiformes* appear to be the most likely source of the H5N1 virus that caused the outbreak in marine mammals. Fourth, there are relatively few H5N1 viruses available in South America from Anseriformes, which are considered the natural reservoir for LPAIV, but which perform an unclear role in the ecology of H5N1 clade 2.3.4.4b in the Americas. Most of the sequenced viruses from Anseriformes from Chile and other South American countries were from domestic ducks and geese, with high rates of spillover from domestic poultry.

H5N1 outbreak in marine mammals in Chile

All H5N1 viruses collected from marine mammals in Chile during March–April 2023 descend from a single avian-to-mammal introduction ("marine mammal clade," posterior probability = 1.0, Fig. 3 and Supplementary Fig. 3). The MCC tree indicates that the avian-to-

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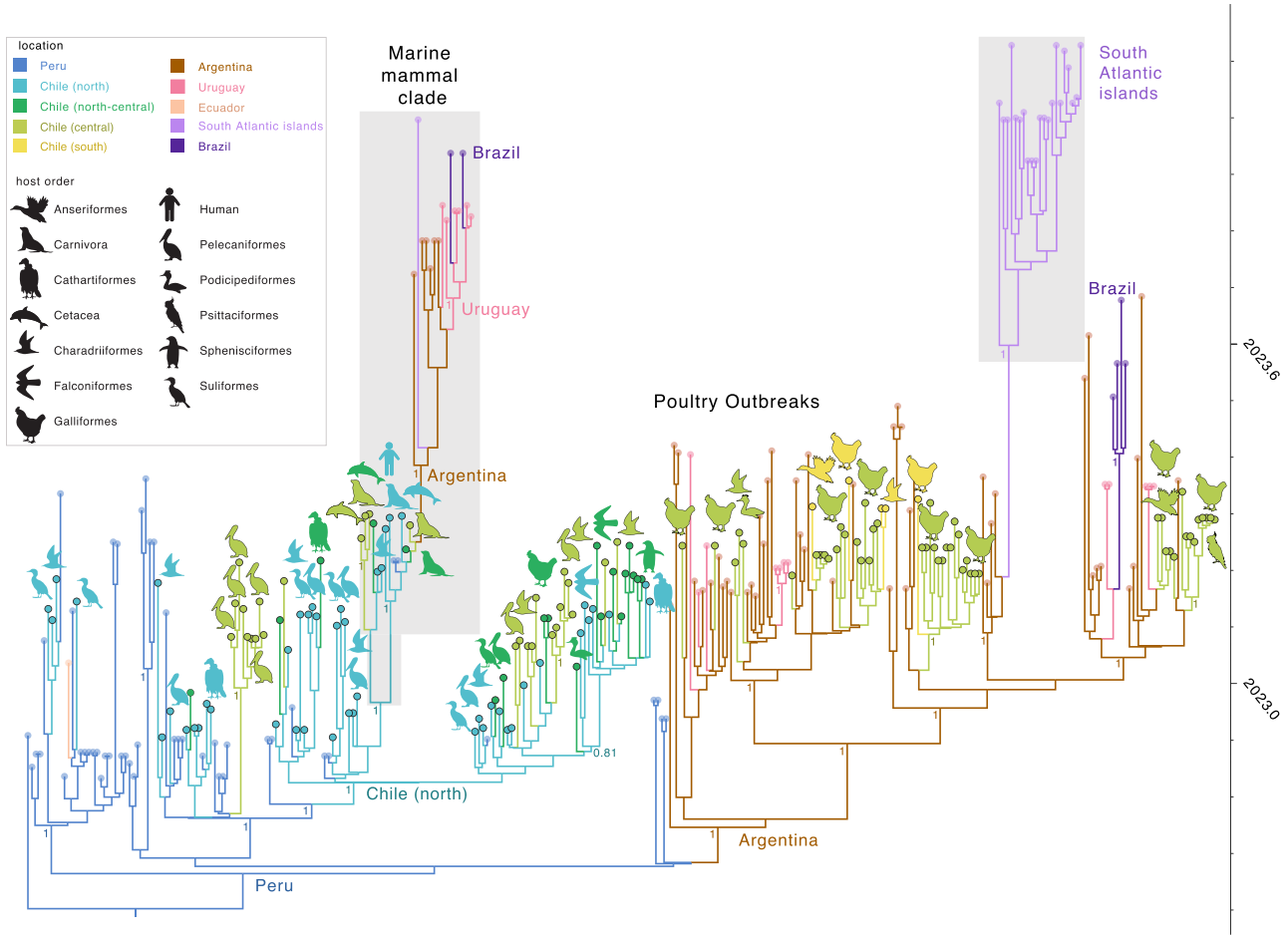

Fig. 3 | Interspecies transmission and spread of the Peru/Chile H5N1 virus. Timescale MCC tree inferred from concatenated genomes. Dataset includes 293 H5N1 viruses from the entire South American clade including 177 viruses sequenced for this study (see Fig. 2). Tree showed the single introduction from Peru (blue) and Chile (light blue and green) and the subsequently spread to Argentina (brown), Brazil (purple) and the South Atlantic islands in the Sub Antarctic Region (light purple). Shaded areas denote specific clusters within the tree.

mammal introduction occurred in northern Chile in late 2022, several months before the earliest marine mammal samples were collected in Peru and Chile. It is therefore possible that an avian-to-mammal spillover event occurred in Peru or another country with less intensive sample collection than Chile. Remarkably, the marine mammal clade that was first detected in Chile and Peru in March 2023, also includes H5N1 viruses sampled from marine mammals in Argentina, Brazil, and Uruguay during August–October 2023, suggesting long-term sustained transmission of H5N1 in South American marine mammals among multiple countries, spanning several thousands of kilometers of coastline. In northern Chile, the virus appears to have spilled back from marine mammals to one shorebird (A/sanderling/Arica y Parinacota/240265/2023) and one human (A/Chile/25945/2003) (Figs. 3 and 5).

Poultry outbreaks in central and southern Chile

H5N1 outbreaks occurred in poultry in central and southern Chile in March–April 2023, during the same months that H5N1 was causing outbreaks in marine mammals in northern Chile. However, the poultry outbreaks are positioned in a different section of the phylogenetic tree, closer to viruses collected from poultry in Argentina and Uruguay (right section of MCC tree, Fig. 3 and Supplementary Fig. 3). Therefore, the outbreaks in poultry and marine mammals do not appear to be epidemiologically linked. Some wild bird viruses from central Chile (e.g., A/black skimmer/Maule/240379/2023) and southern Chile (e.g., A/brown-hooded gull/Los Rios/247093-1/2023) are positioned near the poultry viruses, consistent with frequent transmission in both directions between poultry and wild birds. The close phylogenetic relationships between poultry viruses from different South American countries (e.g., Chile, Argentina, Brazil) in the right section of the tree does not necessarily mean that long-distance international H5N1 transmission is occurring via live poultry trade. This could be an artifact of sampling bias, as most wild bird sampling in Chile is conducted in coastal regions, and few wild bird samples are available from the inland regions where Chilean poultry are farmed. Moreover, only few H5N1 sequences are available from wild birds in the major poultry producers of Brazil and Argentina, which prevent further phylogeographic resolution of these analyses.

High frequency of PB2 mammal-adapted mutations in Chile

If mammal-to-mammal transmission truly occurred in South American marine mammals, then known mammalian adaptations in the polymerase should emerge and be conserved among all marine mammal viruses. Indeed, this was evidenced in all 43 viruses in the marine mammal clade, including all five countries, which have the D701N mutation in PB2 previously associated with AIV adaptation to mammalian hosts (Figs. 5 and 6)[23–26]. Additionally, 37 viruses in this clade have both the D701N and Q591K substitution (Figs. 6 and 7). The D701N mutation has occurred sporadically among H5N1 viruses in nature but is rarely fixed and most D701N clusters are small (<5 viruses) (Fig. 6). The Q591K mutation is even rarer, and double Q591K/D701N mutated viruses have never been observed outside the South

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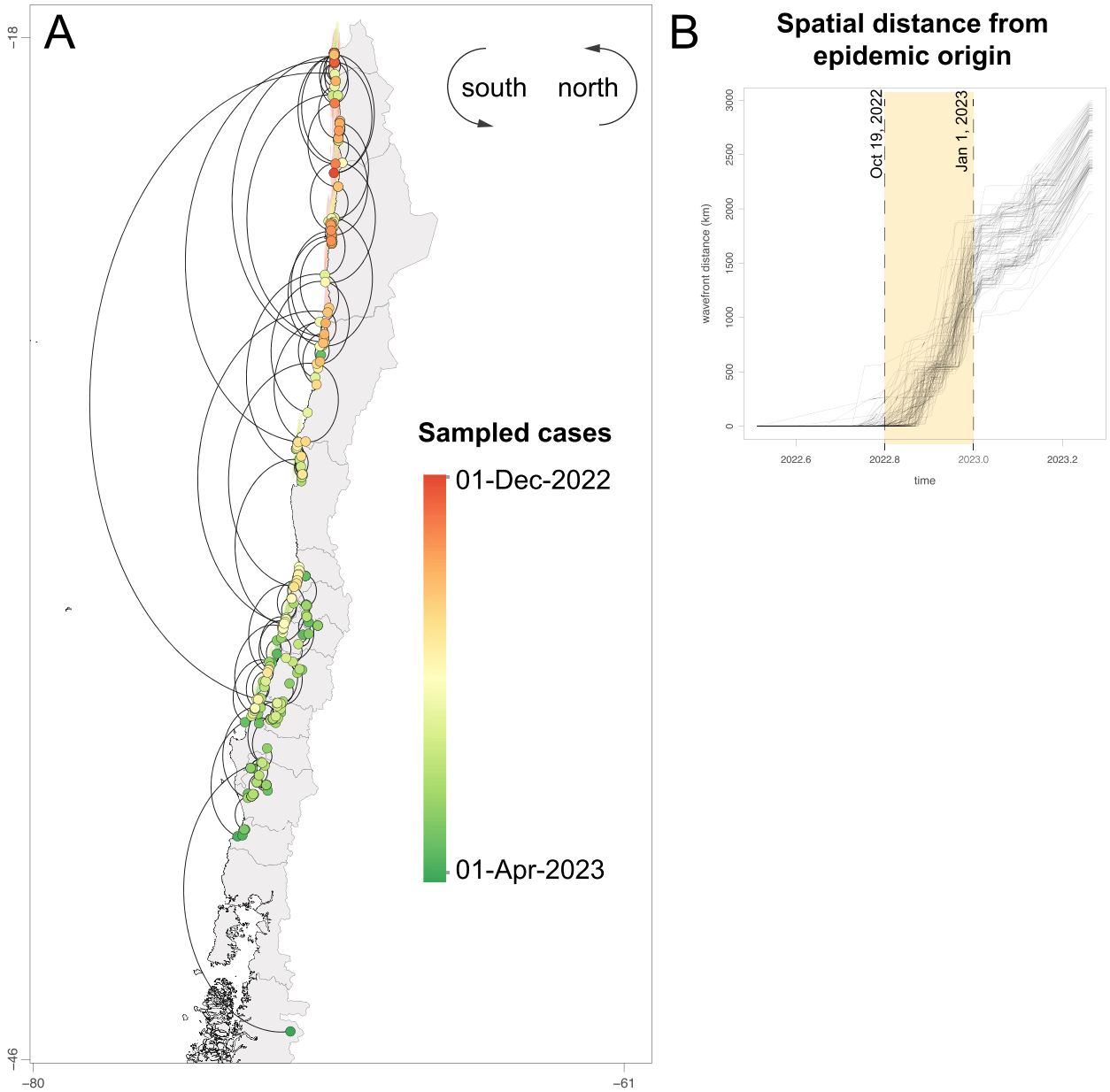

Fig. 4 | Spatial-temporal diffusion of the H5N1 2.3.4.4b lineage in Chile. **A** Continuous phylogeographic reconstruction of the spatial spread of the virus inferred from 143 the HA sequences, using a continuous phylogeographic approach implemented using BEAST and visualized using the SERAPHIM package in R. **B** The timing of the wavefront and dispersal of the H5N1 outbreak in Chile during 2022-2023 visualized using SERAPHIM.

American marine mammal clade. Viruses with the single D701N mutation and the double Q591K/D701N mutation appear to co-circulate over time and space during March-April 2023 in the north, north-central, and central regions of Chile. However, the single D701N mutants were not detected in South America after April 1, 2023, whereas the double Q591K/D701N mutants spread across Argentina, Brazil, and Uruguay during the second half of 2023[27].

Unique viral genomic signatures characterize the marine mammal cluster

Additional synonymous and non-synonymous mutations were found only in the marine mammal clade. These included substitutions T215K and M473I in PB2, S515A and L548F in PB1, A20T, M86I, T162I, L336M, I459V and M548I in PA, I119T in NP, D210G in NA and I266T in NS; and several nucleotide changes that were unique and defined this cluster for each viral segment (Fig. 7a and Supplementary Fig. 4 and Supplementary Data). The human case (A/Chile/25945/2003) shared 6 amino acid changes across the genome with marine mammal viruses: Q591K and D701N in PB2; A20T, M86I, and M548I in PA, and I226T in NS1 (Fig. 7). Many of these mutations were also seen in the marine mammal viruses in Argentina, Brazil, and Uruguay, providing further evidence in support of onward transmission of a mammalian adapted virus in South American marine mammals.

To further investigate whether the additional virus mutations in the PB2 D701N cluster were fixed and transmitted between marine mammals, or whether they arose de novo independently during virus replication as a result of a spillover event (see hypothetical scenarios shown in Supplementary Fig. 5), we focused on studying the within-host diversity. This was done by performing minor single nucleotide variant (SNV) analyses on the raw data of all the animal and the human virus sequences obtained from Chile in this study (9 marine mammals, 1 bird, and 1 human). Mutations arising de novo are expected to have

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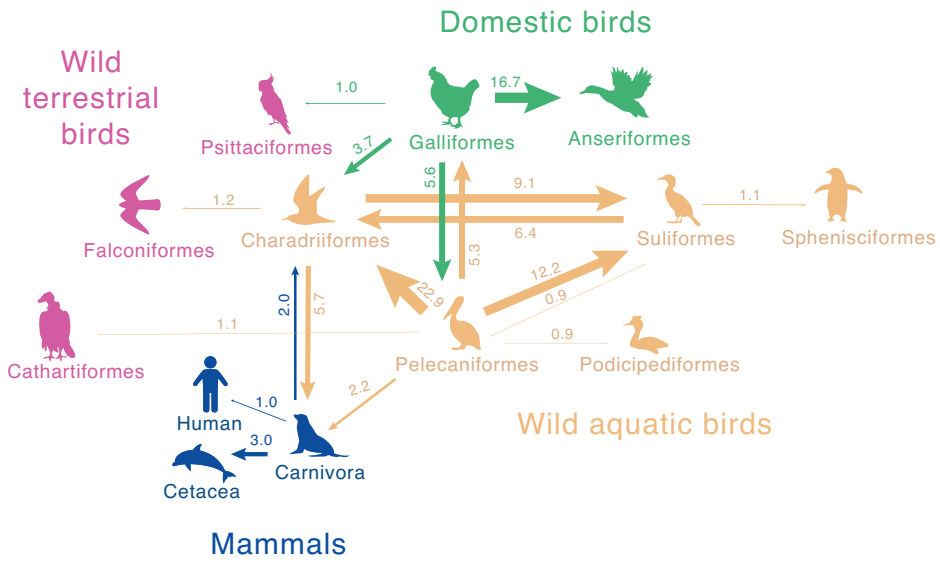

Fig. 5 | H5N1 ecology of transmission events in the H5N1 outbreak in South America. Estimated interspecies transmission events between the 13 affected animal orders in South America. The data set included six orders of aquatic wild birds (yellow), 3 terrestrial wild birds orders (pink), 2 orders of domestic birds (green) and 3 orders of mammals, including the human case, (blue) during the H5N1 outbreak, based on "Markov jump" counts. The width of the arrow is proportional to the estimated number of jumps in that direction as shown by the numbers next to the arrows.

low or variable frequencies in the different isolates, whereas fixed mutations will be represented in the raw data consistently at high allele frequencies. With the exception of two viruses (A/South American sea lion/Tarapaca/240524-2/2023 and A/South American sea lion/Valparaiso/244738-1/2023), SNVs of the D701N cluster were consistently found at high allele frequencies across the viral genomes, specifically on those viruses containing the D701N/Q591K mutations (Fig. 7b, and Supplementary Data). The rest of the PB2 D701N mutations were present at allele frequencies ranging from 72-100% for the animal viral sequences (9 marine mammals and 1 bird) and at 99% for the human virus. Allele frequencies for Q591K were between 58-100% and 100% for the animal and human viral sequences, respectively (Fig. 7b, and Supplementary Data). Analyses of the additional 13 non-synonymous mutations located across the genome had allele frequencies of 74–100%. Similarly, 9 synonymous SNVs located within the eight viral genes were also unique to the marine mammal cluster at high allele frequencies (Fig. 7b, Supplementary Data). These data indicate that the 701N viruses contain unique fixed genomic signatures present only within this cluster, providing further support that onwards mammal-to-mammal transmission of these genotype occurred spanning hundreds of kilometers in the Pacific coastline of Chile that subsequently continued through the Atlantic coastline of Argentina, Uruguay and Brazil.

Discussion

The emergence, spread and evolution of HPAIV H5N1 in South America since October 2022 has had a devastating impact on animal health and biodiversity. The Chilean authorities, between December 2022 and the first half of 2023, reported >62,300 wild birds and 17,294 penguins and marine mammal deaths associated with the outbreak. These included 14,987 South American sea lions, 10,971 Peruvian boobies, 8233 gulls (*Larus sp.*), 4.861 Peruvian pelicans, 2,235 Humboldt penguins, 34 marine otters, 22 Burmeister's porpoises and 16 Chilean dolphins (https://www.sernapesca.cl/informacion-utilidad/registro-de-varamientos/, and https://www.sag.gob.cl/ia). By sequencing 177 H5N1 isolates, our study traces the origins of Chile's massive multi-host epizootic, which appears to be closely connected to Peru's HPAIV outbreak that was detected 1 month before Chile's outbreak and caused similar mass die-offs in seabirds and marine mammals[22]. Our Bayesian phylodynamic analysis revealed the rapid north-to-south dissemination of HPAIV from Peru to Chile via wild bird movements during late 2022. This seeded the massive multi-host epizootic observed in Chile that caused severe disease in seabirds, birds of prey, commercial and backyard poultry, multiple species of marine mammals, as well as one human case. Importantly, wild birds appear to be the sole source of the poultry outbreaks as well as the marine mammal outbreak in Peru and Chile, and no connection was observed between the outbreaks in poultry and marine mammals (Figs. 3–5 and Supplementary Fig. 4). The clustering pattern of marine mammal viruses on the tree and the shared set of uncommon mutations supports a scenario in which the marine mammal outbreak in Peru and Chile was seeded by a single virus introduction from wild birds, and was the genesis of the subsequent mammal-to-mammal transmission spanning thousands of kilometers of Chile and Peru's Pacific coastline during an 18 week period (Supplementary Fig. 5).

Mammal-to-mammal transmission of avian influenza viruses is suspected to have occurred in marine mammals on multiple occasions. Notable examples include a multi-country outbreak of avian H10N7 viruses affecting harbor seals in the North Sea (2014–2015)[28], and an H5N1 an outbreak in marine mammals in New England, USA (2022)[29]. However, definite evidence has been difficult to establish due to the insufficient contemporary background sequence data from wild birds in these regions. One strength of our sampling strategy during the Chilean 2022-2023 HPAIV outbreak is the breadth and intensity of sampling across diverse wild bird species, including 31 avian orders, which helps contextualize the marine mammal viruses. At this level of wild bird sampling, one would expect more bird viruses interspersed with marine mammal viruses from Chile, including standard bird viruses that do not have the D701N or Q591K mutations (Supplementary Fig. 5). The position of one bird virus (A/sanderling/Arica y Parinacota/240265/2023) within the marine mammal clade was surprising, but the fact that this virus contains the D701N mutation, as well as other mutations observed in marine mammals and no other birds in Chile or Peru, is consistent with marine mammal-to-bird transmission, which is plausible given that shorebirds and sea lions share coastal habitat. The mutations found

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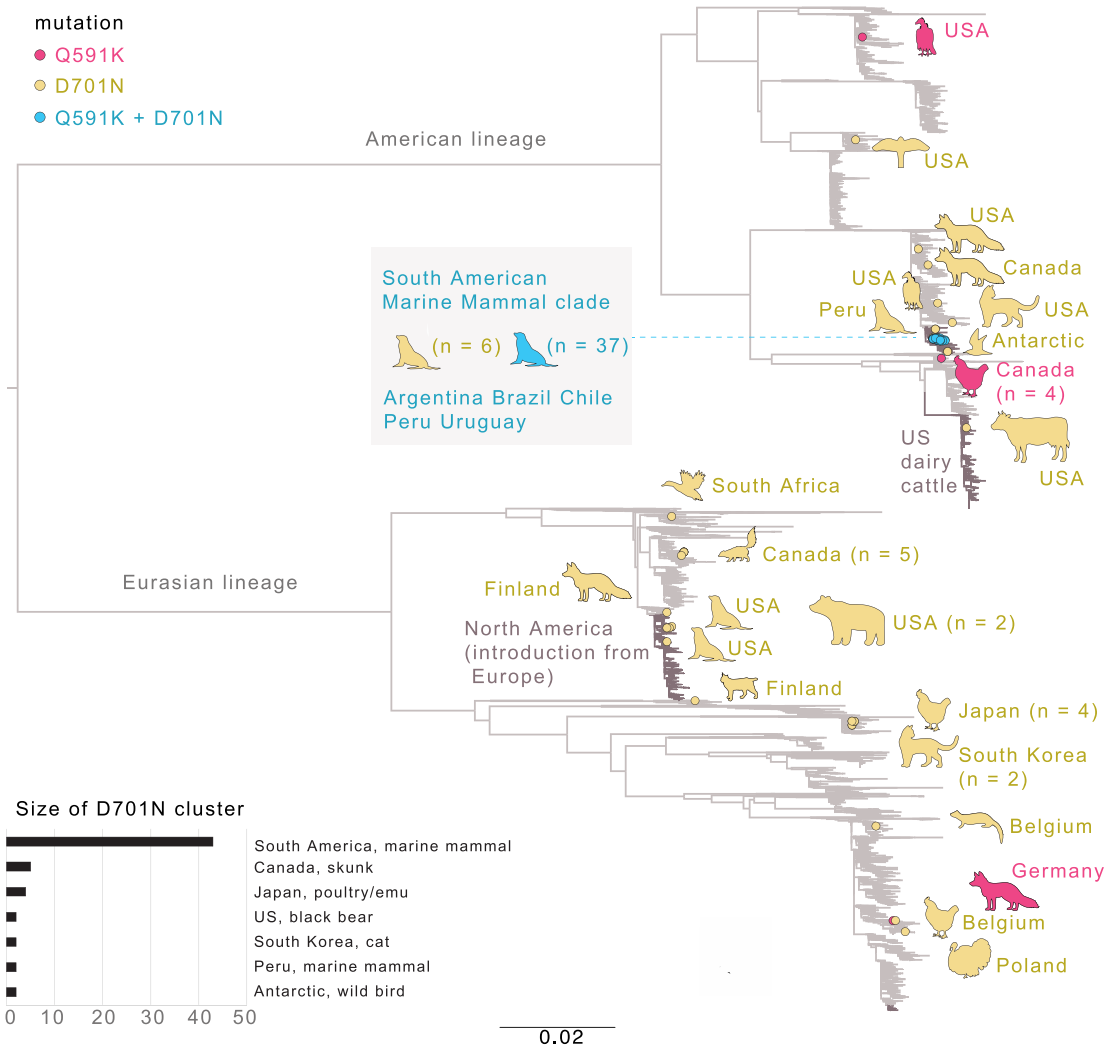

Fig. 6 | Global observations of H5N1 D701N and Q591K mutations in the PB2 segment. ML tree inferred for H5N1 viruses collected from Chile and globally from all host species during January 1, 2021 to September 9, 2024 ($n = 12,663$ PB2 sequences). Tree is midpoint rooted and branch lengths are drawn to scale. Branches are shaded by avian lineage American and Eurasian. The original introduction of H5N1 from Eurasian into North America in 2021 is labeled. Viruses with D701N (yellow), Q591K (pink) and both D701N/Q591K (light blue) are indicated. Number of marine mammals carrying the mutation are described in front of each cartoon. Inset summarizes the number of viruses carrying the D701N mutation identified globally.

in marine mammals were also seen in the human case A/Chile/25945/2023, raising the possibility that marine mammals were the source of the one human H5N1 infection observed in Chile. Discerning the direction of transmission in this context is difficult, but the possibility of transmission between marine mammals and spillover to humans should be considered, warranting intensified monitoring of the fast-changing evolutionary trajectory of H5N1 along South America's biodiverse coastlines.

Prior surveillance studies have detected endemic low-pathogenicity AIVs circulating in Chile's wild aquatic birds that belong to the dominant lineages in the Americas and Eurasia, as well as a South America-specific lineage that has only been found in Chile, Argentina, and occasionally in Peru[30–32]. While HPAIV H5N1 has reassorted frequently with the dominant American lineage in North American birds, producing multiple new genotypes, to date there has been no reassortment between HPAIV and the South American lineage in Chile or other sampled South American countries. This lack of reassortment could be explained by factors such as the severity of the HPAIV H5N1 infection causing rapid death, a low prevalence of other AIV subtypes infecting the same avian population (e.g., displacement of other endemic strains), or a host-related reduced viral-mixing capacity[33]. Current data indicates that multiple independent introductions of HPAIV H5N1 have occurred from North America into other parts of South America (Venezuela, Ecuador, Colombia), which are distinct from the introduction identified here in Chile/Peru[7,13]; however, further spread of those lineages has not been reported to date.

Adaptation of the HPAIV H5N1 viruses to mammals is a significant pandemic concern due to its potential for zoonotic transmission. The D701N mutation has been shown to enhance viral replication and pathogenicity in mammalian hosts, including humans[34–36]. This mutation can enhance the nuclear import of the viral ribonucleoprotein (vRNP) by binding to human importin-α1[37,38]. The Q591K mutation has also been implicated in increased replication and transmission of the virus in mammals[39–42], and it has been identified as a compensatory mutation in the absence of the well-recognized 627 K mammalian adaptation mutation[43]. The positive charge associated with both the PB2-627K or 591 K is thought to disrupt an interaction with an inhibitory host protein[44]. Of note, in stark contrast to previous outbreaks in Southeast Asia, Europe, and North America, the 627 K has not been detected so far in the South American viruses[1] (Fig. 7a).

The concerning possibility of long-distance mammalian transmission of H5N1 in marine mammals in Chile warrants additional

https://doi.org/10.1038/s41467-025-57338-z

a

	PB2			PB1		PA					NS
Arica/240265	•	M473I	•	D701N	•	•	•	•	•	•	•	I226T
Arica/240270-1	•	M473I	•	D701N	•	L548F	•	•	•	•	•	I226T
Atacama/245355	•	M473I	•	D701N	•	L548F	•	•	•	•	•	I226T
Valparaiso/243136-1	T215K	•	•	D701N	M40I	L548F	•	•	L336M	I459V	•	I226T
Maule/246026	T215K	•	•	D701N	M40I	L548F	•	•	L336M	I459V	•	I226T
Nuble/248244-1	T215K	•	•	D701N	M40I	L548F	•	•	L336M	I459V	•	I226T
Peru/TAC-INS-011	•	•	•	D701N	•	L548F	•	M86I	•	•	•	I226T
Tarapaca/240524-2	•	•	Q591K	D701N	?	?	•	M86I	•	•	M548I	I226T
Peru/AQP-SER00R	•	•	Q591K	D701N	•	L548F	•	M86I	•	•	M548I	?
Antofagasta/246506-1	•	•	Q591K	D701N	•	L548F	•	M86I	•	•	M548I	I226T
Bio Bio/246296-1	•	•	Q591K	D701N	•	L548F	A20T	M86I	•	•	M548I	I226T
Atacama/242444-1	•	•	Q591K	D701N	•	L548F	A20T	M86I	•	•	M548I	I226T
A/Chile/25945/2023	•	•	Q591K	D701N	•	•	A20T	M86I	•	•	M548I	I226T
Wild bird/poultry Chile/Peru	•	•	•	•	•	•	•	•	•	•	•	•

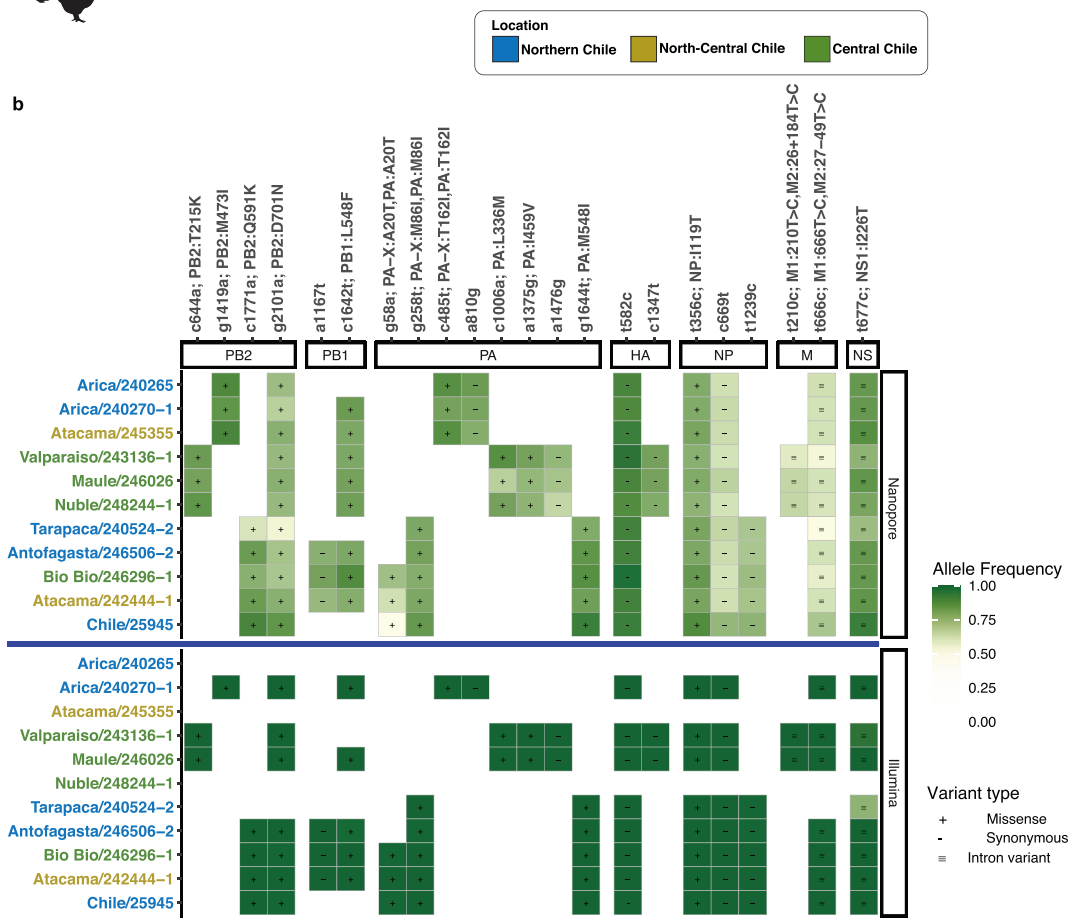

Fig. 7 | Amino acid changes and variant analyses in the D701N cluster. a Amino acid substitutions are listed for the 13 H5N1 viruses in the D701N cluster (Fig. 2), including 11 sequenced for this study and 2 from Peru. These mutations were not observed in wild birds or poultry sequenced in this study, except for the sanderling that is part of the D701N cluster. **b** Frequency of single nucleotide variants (SNV), and amino acid substitutions for A/H5N1 genomic positions were calculated for the 701N cluster genome segments employing the raw data obtained by Illumina sequencing (when available) or nanopore sequencing. SNV analyses were also performed for all avian segments (Supplementary Fig. 4 and Supplementary Data). Positions are numbered according to the reference genomes OQ352545-OQ352552. Viral isolates are indicated on the left and the are colored according to their geographical origin: Northern Chile (blue), North-Central Chile (brown) or Central Chile (green).

sequencing of H5N1 viruses in other South American countries, particularly from wild birds. While we cannot exclude other sources of infection in marine mammals, as more sequence data have become available from marine mammals outbreaks in other countries, including from Argentina, Uruguay and Brazil, where massive pinniped and sea bird die-offs were reported in September 2023, it has become apparent that the pinniped outbreaks on the west and east coasts of South America are truly genetically linked (this report and refs. 27,45,46). Furthermore, the mammalian cluster in Chile contains viruses with the D701N or the double Q591K/D701N mutations, which contrasts to the marine mammal viruses seen later during the outbreaks in the Pacific coast of South America, where only the double Q591K/D701N mutant viruses have been observed since the second half of 2023. Whether the double Q591K/D701N mutation and/or the additional non-synonymous and synonymous mutations identified provide an evolutionary advantage to the virus (e.g. increased fitness in mammals) warrants further evaluation. Nonetheless, an underlying question highlighted during this outbreak is whether this novel reassorted genomic constellation of the B3.2 H5N1 South American clade, which contains PB2, PB1, NP, and NS segments from the American AIV lineage, provides an increased capability to generate the 701N and 591K mutations, and potentially other mammalian adaptations (Fig. 7). Our analyses that this novel cluster likely arose first in Peru and Chile, and subsequently become fixed and was transmitted among Sea lions throughout the South American coastline[27]. Additional characterization of AIV sequences from 2023 and subsequent seasons will be crucial to determine whether reassorment events have occurred and assess if the dynamics and diversity of endemic strains have been modulated by the introduction of the B3.2 H5N1 lineage. Hence, continued surveillance and monitoring of the HPAIV outbreak in Chile, the Americas and Antarctica, along with experimental studies using in vitro and animal models to monitor phenotypic changes, are necessary to assess the risk posed by these H5N1 viruses and to inform public health authorities to improve pandemic preparedness and to control and protect human and animal populations during outbreak situations.

Methods

Sample collection

This study includes active and passive surveillance performed by the Chilean Agricultural and Livestock Service (SAG) and the National Fisheries Services (SERNAPESCA) following their outbreak response protocols. The study was also approved by the Institutional Safety Committee at Pontificia Universidad Catolica de Chile (Protocol # 230412005).

Sampling was conducted between December 1, 2022, and April 19, 2023, to trace the HPAIV H5N1 outbreak across the country. Samples were collected from the Chilean territory, spanning between parallels 18 and 54 south latitudes, using protocols recommended by the United States Department of Agriculture National Veterinary Services Laboratories (USDA-NVSL; protocol NVSL-WI-0023). Briefly, oropharyngeal (ORP), tracheal (TRS), and cloacal (CLO) swabs, feces, or tissues were collected from gallinaceous poultry (ORP or TRS), domestic waterfowl and other wild birds (ORP or CLO). TRS, rectal swabs (RCS), and nasal swabs (NAS) or spiracle swabs from marine mammals were collected on cryotubes containing viral transport media (NVSL media #10088 10%, P/S 1% Gentamicin 0.1%, and Fungizone) and were then submitted for diagnostic reverse transcription PCR (RT-PCR) to the reference laboratory. Pools of up to 10 combined cloacal/tracheal (CCT) or CLO swabs were processed from gallinaceous poultry and domestic ducks, which were grouped by the same species, the same premises, and the same sampling route, in accordance with NVSL guidelines. Samples from positive pools were then tested individually to determine the positive sample/s in the pool.

A total of 18,501 samples, obtained from 9745 submission cases, were derived for viral diagnostic. Of them, 70.53% samples were from poultry ($n = 13,195$), 27.32% from wildlife birds ($n = 4901$, including environmental and captive birds), and 1.43% from non-human mammals ($n = 243$). Avian samples were collected from 23 different orders, including highly represented orders such as, *Galliformes* (59.03%), *Anseriformes* (10.52%), *Charadriiformes* (9.14%), to lesser represented orders, such as *Ciconiiformes* and *Tinamiformes* (0.01% each). Also, non-human mammal samples from cases of orders *Cetacea* (Burmeister's porpoise $n = 4$, Chilean dolphin $n = 4$ and humpback whale $n = 1$) and *Carnivora* (South America sea lion $n = 114$, marine otter $n = 4$ and others $n = 13$) were tested (Supplementary Data).

Molecular diagnosis of Influenza A virus

IAV diagnosis of animal samples was performed at the SAG Livestock Virology Laboratory and the Biotechnology Laboratory (SAG, Lo Aguirre). First, RNA was extracted from samples by the MagMAX™ Core Viral/Pathogen kit (Thermofisher, AM1830) using the KingFisher ThermoScientific™ KingFisher™ Flex Purification Systems. Then, real-time RT-PCR for AIV diagnosis was done using the VetMAX-Gold AIV Detection Kit (cutoff Ct value = 38 / internal control 25–30 Ct, Applied Biosystems™ Cat No. 4485261) and the Detection Kit Genome amplification (Thermo Fisher Scientific, internal control 25-30 Ct). Positive samples were tested to determine the Influenza A subtype H5 lineage (specific 2.3.4.4 clade) with specific real-time RT-PCR following the standard operating procedures of NVSL-USDA[47–49]. A BAL sample from the human case were tested for influenza A virus by real-time RT-PCR at the local hospital and was then characterized as Influenza A(H5) and A(H5N1) HPAIV by real-time RT-PCR at the Instituto de Salud Pública Chile (ISP).

Whole genome Sequencing

The criteria for sample selection for whole genome sequencing included the selection of the maximum possible geographical, temporal and species representation, from samples with a diagnostic Ct value < 37 to achieve the amplification of all 8 viral segments. Hence, viral RNA was re-extracted from 316 positive original samples derived from SAG to the Laboratory of Molecular Virology, Pontificia Universidad Católica de Chile (LVM-UC), and at the Animal Virology Lab, University of Chile. First, we performed TRIzol™ lysis (Invitrogen ™ 15596018) followed by extraction with E.Z.N.A Viral RNA Kit (R6874, Omega Biotek). Then, the viral genome was amplified using a multi-segment one-step RT-PCR genome amplification with primers, Opti1-F1 5′:GTTACGCGCCAGCAAAAGCAGG-3′, Opti1-F25′:GTTACGCGCCAGCGAAAGCAGG-3′ and Opti1-R1:5′:GTTACGCGCCAGTAGAAA-CAAGG-3′[5]. Two hundred forty-two samples were selected and processed and one hundred seventy-seven (73.14%) of them were successfully amplified and sequenced. Amplicon were purified with SPRISelect Beads (Beckman counter B2338) using a ratio of 0.45x. Next-generation sequencing was then performed with the Oxford Nanopore Technology (ONT) platform using the Native Barcoding Kit (SQK-NBD114.96) for the library preparation at the LVM-UC. The First End-prep was performed using the NEBNext Ultra II End Repair/dA-tailing Module (NEB, E7546), the native barcodes were ligated with the Native Barcoding Expansion 96 (EXP-NBD196) and NEB Blunt/TA Ligase Master Mix (NEB, M0367) selecting a single barcode per sample. The barcoded library was pooled together and purified using SPRISelect beads. An AMII adapter was ligated to the library using the NEBNext Quick Ligation Module (NEB, E6056), purified using SPRISelect beads, and quantified with a Qubit Fluorometer (Invitrogen™). The library was loaded on the sequencer using the ligation sequencing kit (SQK-LSK109) according to ONT instructions for the R.9 flow cells. Sequencing was carried out for 72 h.

Nanopore Reads (FASTQ) were filtered with NanoFilt requiring an average Phred quality score of at least 7 and a read length between 400 to 2600 nucleotides[50]. Genomes were assembled by reference. The reference was chosen by BLAST-searching preliminary assembled contigs constructed with filtered reads which were de novo assembled with Canu[51]. Sequences of the Influenza Virus Resource database of NCBI were used for the BLAST-search. Reads were mapped to the selected reference with minimap2 and used to build a consensus sequence for each segment, which was carried out with the tools medaka, longshot, Samtools y Bcftools[52–55]. Once consensus genomes were obtained, they were checked for quality and annotated by the NCBI Influenza Virus Sequence Annotation Tool (https://www.ncbi.nlm.nih.gov/genomes/FLU/annotation/)[56]. H5 clade classification was carried out with the online tool Subspecies Classification at the BV-BRC portal[57,58]. One hundred fifty-nine complete genomes and eighteen partial genomes were obtained (with at least 6 segments, Supplementary Data). The sequences have been deposited in GenBank and the accession numbers are listed in Supplementary Data.

For the human sample, 8 amplicon fragments corresponding to the AVI H5N1 genome were amplified by RT-PCR One-step using the primers Uni12/Inf-1 GGGGGGAGCAAAAGCAGG, Uni12/Inf-3 GGGGGGAGCGAAAGCAGG, Uni13/Inf-1 CGGGTTATTAGTAGAAACAAGG provided by CDC. The sequencing was performed using 100 ng of DNA utilizing the Nextera DNA Flex Library Prep Kit. We then performed paired-end sequencing (2 × 150 bp) in an Illumina MiSeq, with ~2 million total reads. The genome assembly was made using the iterative refinement meta-assembler (IRMA) approach. Nanopore sequencing was performed using 400 ng DNA per library preparation using the Rapid Barcoding Kit (RBK-004) and the MinION Mk1C sequencer with ~100k total reads. Filtered reads were mapped to the reference IAV A/Thailand/1(KAN-1)/2004(H5N1) (GenBank accession: 266827)[17].

HPAIV H5N1 human case

The clinical data presented corresponds to the only H5N1 influenza virus human case detected in Chile by the Public Institute of Health of the Ministry of Health of Chile through the national surveillance system of severe acute respiratory infections conducted. According to regulations of the Ministry of Health of Chile, data collected during an outbreak response is exempt from undergoing review by an ethics committee. The Public Institute of Health of Chile provided demographic and clinical data and are co-authors of this study. The Ministry of Health of Chile is an active participant in the Disaster Risk Management Committee of the National Service for Disaster Prevention and Response (SENAPRED) to deal with animal health emergencies.

On March, 2023, a 53 year-old male from the Region of Antofagasta in northern Chile began experiencing symptoms, including cough, sore throat, hoarseness, and sought medical treatment at a local hospital 8 days after symptoms progressed. The individual had no declared comorbidities, recent travel history or visit to beaches. Subsequently, on day 9 the patient developed difficulty breathing (dyspnea) and was transferred to a Regional Hospital in Antofagasta, which serves as a severe acute respiratory infection (SARI) Sentinel Site. A nasopharyngeal swab sample was collected as part of routine SARI surveillance and tested negative for SARS-CoV-2 using RT-PCR. On the 10th day, the patient was transferred to the intensive care unit (ICU), where he received mechanical ventilation and antiviral treatment with oseltamivir and antibiotics was initiated. After several weeks in ICU the patient recovered. A BAL sample collected on day 14 since symptom onset tested positive by RT-PCR for a non-subtyped influenza A virus. At ISP, subsequent subtyping by qRT-PCR confirmed avian influenza A(H5) on March 29 and the Ministry of Health of Chile (MINSAL) reported the first case of infection with avian influenza A (H5) virus in Chile to the World Health Organization[59]. The patient samples were forwarded to the WHO Collaborating Center and the CDC for further characterization. On April 5, genomic sequencing conducted by ISP confirmed it as HPAIV H5N1 clade 2.3.4.4b. Sequences were deposited in GenBank Accession numbers PQ295904 - PQ295911.

All three close contacts of the patient identified did not present clinical signs and tested negative for influenza. They completed the monitoring period successfully. Among healthcare workers, a total of nine contacts were identified, all of whom tested negative for influenza and concluded their monitoring period in April. However, during this time, one healthcare worker developed respiratory symptoms. A nasopharyngeal swab test yielded a negative result for influenza and any other respiratory virus. The monitoring period for the contact was extended for an additional 7 days, ending 6 days after initial symptoms. HPAI H5N1 had previously been identified in wild aquatic birds (pelicans and penguins) as well as sea mammals (sea lions) in the Antofagasta Region from December 2022 to February 2023. Initial findings from the epidemiological investigation suggest that the most probable mode of transmission for this human case was through environmental exposure, considering the significant presence of deceased sea mammals and wild birds near the patient's residence, which is within 150 meters of the beach.

Phylogenetic analysis

To place the Chilean HPAIV H5N1 sequences in a global context, we downloaded a global background data set of all available AIV sequences for all segments from GenBank and GISAID databases that were submitted January 1, 2021 – September 9, 2024, including avian and mammalian hosts, and aligned them with the Chilean sequences generated in this study, including the human case, using MAFFT[60]. Larger datasets (ranging from 5253 - 5616 sequences) were used for internal gene segments, which included all AIV subtypes. Smaller datasets were used for HA and NA segments since these were limited to one subtype: H5 (n = 1058 sequences) and N1 (2534 sequences). Phylogenetic trees were inferred for each segment separately using maximum likelihood (ML) methods available in IQ-Tree2[61] with a general-time reversible (GTR) model of nucleotide substitution, incorporating gamma-distributed rate variation among sites. To assess the reliability of each node in the phylogenetic trees, a bootstrap resampling process was performed with 1000 replicates. Due to the size of the dataset, we used the high-performance computational capabilities of the Biowulf Linux cluster at the National Institutes of Health (http://biowulf.nih.gov).

After the initial ML trees showed that all Chilean viruses clustered with Peru viruses in a single clade on all eight gene segment trees showing no evidence of reassorment, an additional phylogenetic analysis was performed on just this clade for all eight concatenated segments using a Bayesian phylogeographic approach[61,62]. We used the Markov chain Monte Carlo (MCMC) method available in the BEAST package, v1.10.5[63], again using the NIH Biowulf Linux cluster. An exponential demographic model was used in this outbreak setting, with a (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites and an uncorrelated lognormal relaxed molecular clock. Each tip was assigned a location state and a phylogeographic discrete trait analysis was performed. The MCMC chain was run separately four times for each dataset using the BEAGLE 3[64] library to improve computational performance, until all parameters reached convergence, as assessed visually using Tracer v.1.7.2[65]. At least 10% of the chain was removed as burn-in and runs for the same dataset were combined using LogCombiner v1.10.4. A MCC tree was summarized using TreeAnnotator v.1.10.4 and visualized in FigTree v1.4.4. Animal images were generated with iStockPhoto. The analysis was repeated for six of the eight genome segments (PB2, PA, HA, NP, NA, and MP) for which the majority of viruses in the Chile/Peru clade had sequences available and showed no evidence of reassortment (n = 79). When mammalian adapted PB2 mutations were identified in Chilean samples, an additional phylogenetic analysis was performed to

study the evolutionary relationships of viruses with these PB2 mutations. The analysis used similar ML methods as described above and an updated dataset of all H5N1 PB2 segments submitted to GISAID January 1, 2021 – June 8, 2023, including avian and mammalian hosts ($n = 4120$ H5N1 PB2 sequences). To visualize the spatial dispersal of H5N1 viruses in North America, we performed a continuous phylogeographic analysis, again implemented in BEAST 1.10.5, assigning a geographical coordinate (latitude and longitude) to each tip and using a Cauchy distribution to model among-branch heterogeneity in diffusion velocity[66]. MCMC chains were run for at least 200 million iterations, to reach adequate ESS values as estimated by Tracer 1.7, discarding 10% of sampled trees as burn-in. SERAPHIM was used to visualize continuous dispersal events from the MCC tree[67]. A phylogeographic discrete trait analysis[62] was performed to quantify rates of asymmetric viral gene flow between 13 host orders. A location state (host order) was specified for each viral sequence. The expected number of location state transitions in the ancestral history conditional on the data observed at the tree tips was estimated using 'Markov jump' counts[68,69], which provided a quantitative measure of asymmetry in gene flow between defined populations. A Bayesian stochastic search variable selection (BSSVS) was employed to improve statistical efficiency for all data sets containing more than four location states. All XML and tree files are publicly available in the GitHub repository (https://github.com/mostmarmot/ChileHPAIV). To identify specific genetic variations and mutations that may have implications for the pathogenicity, transmission, and antigenic characteristics of the H5N1 viruses under investigation, we conducted mutation analysis using the CDC H5N1 genetic changes inventory for SNP analysis and incorporated various other previously published mutations of concern[57,70].

Short-read Illumina whole-genome sequencing

The viral genomes sequenced by Illumina short-read sequencing were generated from sequencing one-step RT-PCR amplicons with the Opti1 primers as previously described[5]. The sequencing libraries were prepared with the Nextera XT DNA Sample Preparation Kit (Illumina, FC-131-1096) following the manufacturer's instructions, and sequenced on a MiSeq instrument with the Reagent Kit v2 (500-cycles, MS-102-2003). *De-novo* genome assembly was performed with a custom pipeline as previously described [Mena et al., 2016]. Complete and pass genome sequences were deposited in GenBank under accession numbers PQ300675-PQ300722, PQ300724-PQ300731 and PQ304209 to P304216.

Minor variant analysis

For minor variant analysis the reads were mapped with a custom reference-based assembly pipeline, vRAPID[71] against the reference genome of A/black skimmer/Chile/C61962/2022, GenBank OQ352545-OQ352552. Minor variants and allele frequences were called with bcftools 'mpileup' and 'call' functions with ploidy of 1[53,72]. The vcf output was filtered with 'bcftools filter' to pass reads with quality score of >10, and alleles with read coverage of > = 5 and supported by both read-pairs. The filtered output was used to identify, parse and plot the allele frequencies in R v.4.2.0, with the packages dplyr v. 1.1.2, stringr v.1.5.1 and ggplot2 v3.5.0.9000. Variations were classified according to the allele effect as low (synonymous_variant), moderate (missense_variant), high (frameshift_variant or stop_gained), or modifier (intron_variant or intergenic_region) (Supplementary Table 4).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All the information required to reanalyze the data reported in this study is contained within the manuscript. The viral sequence data generated in this study has been deposited in GenBank under BioProject PRJNA1037139. Specific the accession numbers are listed the Methods section and in the Supplementary Data. We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu™ Database on which this research is based. The list of accession numbers from GISAID, as well as all the XML and tree files are available in GitHub (https://github.com/mostmarmot/ChileHPAIV).

Code availability

This study does not report original code.

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Acknowledgements

We thank the Agricultural and Livestock Service (SAG) and SERNAPESCA personnel for their support and contributions, especially in the sample collection. We are grateful to Eugenia Fuentes Luppichini from the Molecular Virology Lab, Pontificia Universidad Católica de Chile, and Belén Agüero and Vanessa Mendieta from the Animal Virology Lab, Universidad de Chile, for their help in sample processing. Thanks to the Unidad de Bioinformática y Biología Computacional, Pontificia Universidad Católica de Chile for bioinformatics support. We are also grateful to Adriana van DeGuchte and Keith Farrugia for sample processing and library preparation for Illumina sequencing and Zain Khalil for *de-novo* and reference-based genome assembly of Illumina data. We are grateful to the GISAID EpiFlu™ Database, laboratories, and source of original data of influenza A virus (IAV) sequences, especially to the Servicio Nacional de Sanidad Agraria del Perú – SENASA, Peru, source of the closest strains and the Institute of Public Health of Chile. Thanks to Alonso Parra from MINSAL for valuable input about the human case. This study has been partially funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, Centers of Excellence for Influenza Research and Response (CEIRR) under Contracts No. 75N93021C00017, Option 18 A to CPR, VN and RAM, and 75N93021C00014, Option 12 A, to H.v.B., and by the Intramural Research Program of the US National Library of Medicine at the NIH. This work was also supported in part through the computational and data resources and staff expertize provided by Scientific Computing and Data at the Icahn School of Medicine at Mount Sinai and supported by the Clinical and Translational Science Awards (CTSA) grant UL1TR004419 from the National Center for Advancing Translational Sciences (to the Icahn School of Medicine at Mount Sinai). Research reported in this publication was also supported by the Office of Research Infrastructure of the National Institutes of Health under award number S10OD026880 and S10OD030463 (to ISMMS). This study was also funded by the Agencia Nacional de Investigación y Desarrollo de Chile (ANID) FONDECYT grants 11231122 to CPR and 1211517 VN, INACH Regular RT-08-21 and the Pontificia Universidad Católica VRI-UC PBV24019 fund to CPR. The funders had no role in study design, sample collection, data collection and analysis, decision to publish, or preparation of the article. We thank all personnel from the Molecular Genetic and Genomics Subdepartment, Section of Bioinformatics, and Section of Respiratory and Exanthematic Viruses of the Public Health Institute of Chile, for their support and contributions, especially in the sample processing and genomic sequencing of the human sample.

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Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-025-57338-z.

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Peer review information *Nature Communications* thanks Michelle Wille and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. A peer review file is available.

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