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Genomic diversity and reassortment of highly pathogenic avian influenza A/H5N1 virus (clade 2.3.4.4b) in Brazil: Evidence of multiple introductions and intra-epidemic reassortment in 2025

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ABSTRACT

Highly pathogenic avian influenza (HPAI) A/H5N1, clade 2.3.4.4b, has spread globally, with the first outbreak in a commercial poultry farm in Brazil reported in 2025. This study aimed to genetically characterize A/H5N1 samples from multiple Brazilian regions using whole-genome next-generation sequencing (NGS) to investigate viral diversity and reassortment events. Phylogenetic analyses revealed that Brazilian isolates segregated into two distinct clades: one showing high similarity to viruses detected in Argentina in 2025 suggesting adaptation of new reassortants along a regional dissemination route in South America; and another clustering with North American isolates, indicating an independent introduction. Genotyping confirmed the emergence of previously unreported genomic rearrangements in South America, underscoring the complexity of viral evolution in the region. These findings highlight intra-epidemic genomic diversity and reinforce the need for continuous, in-depth molecular surveillance to monitor the dynamics and potential impacts of A/H5N1 on animal and public health.

1. Introduction

Highly pathogenicity avian influenza HPAI A/H5N1 poses a significant threat to poultry health and global public health, causing high mortality in wild birds, domestic birds, and marine mammals. Since its first detection in 1996 in China, the gs/Gd lineage (A/goose/Guangdong/1/96) has undergone extensive genomic diversification, giving rise to multiple clades. Clade 2.3.4.4b, currently predominant, is responsible for recent outbreaks across several continents, including South America.

One of the mechanisms amplifying this genomic diversity is reassortment - a genetic process unique to viruses with segmented genomes, such as influenza viruses. It occurs when two or more viral variants co-infect the same host cell. During viral replication, RNA segments from both variants can be rearranged and randomly packaged into progeny virions. Reassortment is a key mechanism in influenza virus evolution and host adaptation, often facilitating emergence in new wildlife communities and contributing to variants (Dugan et al., 2008). This mechanism can contribute to the emergence of variants with panzootic or pandemic potential (WHO, 2023).

Genomic sequencing of the highly pathogenic avian influenza HPAI A/H5N1 virus, re-emerging in Argentina in February 2025, revealed the emergence of novel triple-reassortant viruses. These isolates contain gene segments derived from Eurasian H5N1, alongside low pathogenic viruses from South and North American lineages. These diversifications of the clade 2.3.4.4b H5N1 lineage in the Americas, emphasizing the critical importance of sustained genomic surveillance to assess and mitigate zoonotic and avian health risks (Vanstreels et al., 2025).

In Brazil, the first HPAI A/H5N1 case was recorded in wild birds in May 2023 (Reischak et al., 2023). In 2023 and 2024, A/H5N1 viruses

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were detected in subsistence poultry, wild birds, and mammals in Brazil; these belonged to clade 2.3.4.4b and genotype B3.2, showing high genetic similarity to isolates from Chile, Uruguay, and Argentina and suggesting a Pacific migratory route (Rivetti Jr et al., 2024).

Exactly two years after the first detection in wild birds, in May 2025 the first outbreak of HPAI A/H5N1 in a breeding poultry establishment was confirmed in southern Brazil (Montenegro, Rio Grande do Sul). Following mortality notification by the integrator company on May 11, 2025, the Official Veterinary Service (OVS) quarantined the property on May 12, 2025 and forwarded samples to the Federal Agricultural Defense Laboratory in São Paulo (LFDA-SP). Laboratory confirmation occurred on May 15, 2025, three days after notification and investigation. On that date, mortality in broiler house 01 was total (8550/8550 birds), and in broiler house 02 reached 84 % (7100/8458 birds). The 1358 remaining birds were culled on May 16, 2025, and sanitation began immediately with the disposal of carcasses, risk materials (nests, litter), and fertile eggs at an incubator in Soledade/RS linked to the outbreak. Disinfection of the farm began on May 17, 2025 under OVS supervision. The rapid OVS response, including laboratory diagnosis four days after notification, was crucial to containing the outbreak within 39 days and preventing spread to other commercial farms (Cardenas et al., 2025).

Associated with this first occurrence in a commercial establishment, A/H5N1 was also confirmed in wild birds in two separate events in Rio Grande do Sul. The first occurred at the above-mentioned breeding farm in Montenegro, while the second involved captive wild birds in a zoo in Sapucaia do Sul, approximately 32.5 km in a straight line from the commercial outbreak. In the zoo event, more than 100 bird deaths were recorded, including species of ducks and swans. After this first record in a commercial farm in Brazil, further detections of HPAI A/H5N1 occurred in wild birds and in domestic chickens kept outside commercial production systems - commonly known as backyard flocks - in different Brazilian states.

This study aimed to genetically characterize HPAI A/H5N1 samples detected from domestic chickens from a production system in Montenegro (RS), as well as from free-living birds and backyard chicken from various Brazilian states. All samples were diagnosed and analyzed by Brazil's Official Veterinary Service at Federal Agricultural Defense Laboratories of the Secretariat of Animal and Plant Health and Inspection of the Brazilian Ministry of Agriculture and Livestock.

2. Materials and methods

2.1. Sample collection and selection

Samples were obtained from the central nervous system, oral, cloacal, oropharyngeal and trachea swabs, and a pool of lung and trachea from animals as part of the passive surveillance for avian influenza. These samples were obtained from animals and collected by the State Official Veterinary Service at each occurrence. After notification of a suspected avian influenza case, an Official Veterinarian visited the property within 12 h to investigate suspected cases in domestic and wild birds. Collected samples were sent to the Federal Agricultural Defense Laboratory of São Paulo (LFDA-SP), a World Organization for Animal Health (WOAH) reference laboratory and a Food and Agriculture Organization (FAO) reference center for animal influenza in the region.

All tests complied with ISO/IEC 17025:2017 general requirements for the competence of testing and calibration laboratories and with Chapter 3.3.4 of the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2024).

2.2. HPAI H5N1 confirmation—PCRs and partial sequencing

Samples were initially tested for A/H5 by three RT-qPCR protocols (VetMAX Gold AIV, Thermo Fisher; Naguib et al., 2017; NVSL/APHIS/USDA, 2024), followed by Sanger sequencing (Sanger et al., 1977). Additionally, A/H5-positive samples underwent RT-qPCR neuraminidase subtyping (NVSL/APHIS/USDA, 2023) and were subsequently shipped to the Federal Agricultural Defense Laboratory of Minas Gerais (LFDA-MG) in molecular transport medium (MTM; Bioboavista, Brazil) for NGS on the Illumina platform (MiSeq ROU).

2.3. Whole-genome sequencing

Sample selection for NGS considered the geographical distribution of occurrences across Brazil to maximize coverage. Samples with Ct < 25 and those representing different bird species were prioritized for sequencing.

Sequencing was performed by sequencing-by-synthesis on the Illumina MiSeq™ platform using the Nextera XT DNA Library Prep Kit (Illumina, Inc., Cat. No. FC-131-1024).

Total RNA was extracted from inactivated samples using the TRIzol® Reagent protocol (Chomczynski and Sacchi, 1987) at a 0.4:1 ratio. After centrifugation, the aqueous phase was recovered and purified with chloroform. RNA purification and concentration were performed using the RNA Clean & Concentrator-5 kit with DNase I (Zymo Research, Cat. No. R1013). Extraction efficiency was monitored by spiking MS2 bacteriophage (single-stranded RNA; Qiagen®) into the sample prior to processing. Subsequent quantification of MS2 RNA in the extract enabled assessment of recovery and process integrity, ensuring that material loss did not compromise analysis.

Extracted RNA underwent One-Step RT-PCR for cDNA synthesis and subsequent amplification of the eight viral genome segments. Reactions used the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High-Fidelity DNA Polymerase (Thermo Fisher Scientific, 2017; Cat. No. 12574026) and universal degenerate primers for influenza A (Zhou et al., 2009): AIV F (5′-CTGGATACGCCAGCRAAAGCAGG-3′) and AIV R (5′-GACCTGATGCGGAGTAGAAACAAGG-3′). Cycling conditions were optimized to amplify all segments with high replication fidelity.

The resulting cDNA was then used for library construction with the Nextera XT DNA Library Prep Kit. Library construction involved tagmentation to fragment cDNA and adapter ligation. Subsequent PCR cycles used adapters to amplify DNA and add dual index adapter sequences, enabling multiplexing. Amplicons were pooled and purified with AMPure XP (Beckman Coulter, Indianapolis, IN, USA). Libraries were quality-controlled with D1000 ScreenTape (Agilent 5067–5582) on a TapeStation System and sequenced with the MiSeq Reagent Kit v2 (paired-end, 300 cycles). PhiX (PhiX174 DNA) was added to all runs to monitor library preparation and sequencing performance.

Bioinformatics analyses used an in-house pipeline integrating open-source tools. Steps included read filtering and trimming with Cutadapt v5.0 (Martin, 2011); read quality assessment with FastQC v0.12.1 (Andrews, 2010); removal of contaminants and host (cell-line) genome with BWA, SAMtools, and BEDTools (Li and Durbin, 2009; Li et al., 2009; Quinlan and Hall, 2010); and de novo assembly with SPAdes v1.1.4 (Bankevich et al., 2012). Genomic visualization/exploration used IGV (Robinson et al., 2011); genome annotation used the NCBI Influenza Virus Sequence Annotation Tool and Geneious 2025.2.2 (Kearse et al., 2012).

2.4. Phylogeny, reassortants and genotyping

The Flusurver genotyping tool (https://flusurver.bii.a-star.edu.sg/), enabled by GISAID data (Elbe and Buckland-Merrett, 2017) was used to confirm the classification clade.

H5 clade 2.3.4.4b hemagglutinin (HA) nucleotide sequences available in GISAID EpiFlu database and collected since January 1, 2020 were downloaded (accessed September 23, 2025). Incomplete, poor-quality, or duplicate sequences were excluded, resulting in 20,158 sequences. The dataset was aligned using MAFFT v7.520 with default parameters (Katoh and Standley, 2013), and a maximum-likelihood phylogenetic tree was generated with FastTree (Price et al., 2009).

Phylogenetic analyses were subsequently performed using the Nextstrain Augur pipeline (Hadfield et al., 2018), with results visualized according to the region of sampling.

In the analysis of the reassortants, full-length sequences of all segments of the HPAI A/H5N1 viruses were aligned using MAFFT v7.520 with default parameters. Phylogenetic analyses were performed by maximum likelihood in IQ-TREE (Nguyen et al., 2015), with automatic model selection and statistical support estimated by 1000 bootstrap replicates **o**f Ultrafast Bootstrap (UFBoot). Resulting trees were visualized and edited in iTOL (Interactive Tree of Life) (Letunic and Bork, 2024).

A/Hubei/1/2010 (GISAID EPI ISL 96896) was used as the outgroup in all segment-specific phylogenies, as it represents a candidate lineage for the HPAI A/H5N1 vaccine within clade $2.3.2.1a$, thus enabling accurate tree rooting.

Viral genotyping used GenoFLU, an open-source bioinformatic platform developed by USDA-VS for genotyping avian influenza viruses. (https://github.com/USDA-VS/GenoFLU). This tool was designed for North American genotyping.

For reassortment inference across all segment sequences, large-scale phylogenetic analysis was facilitated by PARNAS (Markin et al., 2023; https://github.com/flu-crew/parnas), which reduces initial tree sampling by selecting a representative subset of taxa while preserving genetic diversity up to a pre-specified sequence-divergence threshold. Using a radius of 0.005 (0.5 % sequence of divergence), the tool selected the minimum number of representatives needed to cover all taxa in the original tree. The HA phylogeny used the H5 clade 2.3.4.4b hemagglutinin (HA) nucleotide sequences available in GISAID EpiFlu database and collected since January 1, 2020 were downloaded (accessed September 23, 2025). Incomplete, poor-quality, or duplicate sequences were excluded, resulting in 20,158 sequences. These sequences of Eurasian, North American, and South American sequences were reduced to 682 representatives at the 0.5 % threshold, enabling a more tractable, computationally efficient tree without loss of critical information for reassortment detection.

3. Results

3.1. HPAI H5N1 confirmation by PCRs and Sanger sequencing

Between January and October 2025, a total of 1287 investigations were conducted. Of these, 274 involved sample collection, resulting in 19 outbreaks positive for HPAI H5N1. These 19 outbreaks positive were distributed as follows: one case in poultry, nine cases in non-commercial birds, and nine cases in wild birds. Fifteen samples from the central nervous system were selected from these 19 positive occurrences for whole-genome sequencing (Tables 1 and 2). This selection prioritized samples exhibiting lower Ct values (indicating higher viral load) and aimed to encompass the broadest possible diversity of species and affected Brazilian regions. Information regarding these occurrences during this period is available on the dashboard of the Brazilian Ministry of Agriculture and Livestock (MAPA, 2025. https://mapa-indicadores.agricultura.gov.br/publico/extensions/SRN_v2_EN/SRN_v2_EN.html).

Samples were confirmed as A/H5-positive by all three RT-qPCR assays and were subjected to neuraminidase subtyping followed by partial HA (segment 4) Sanger sequencing. Partial HA sequencing confirmed a polybasic cleavage site motif (PLREKRKKR/GLF or PLREKRRKR/GLF), consistent with the high-pathogenicity profile (Slomka et al., 2007).

3.2. Dataset quality and genomic data

Selected samples yielded mean coverage >1000 × , Q30 > 90 %, and passing filter read rates >85 % across runs, providing robust data for genomic analyses (Goodwin et al., 2016). PhiX metrics and MS2 detection confirmed run adequacy and library readiness, supporting sequencing performance, including cluster generation efficiency and read quality.

After cleaning and trimming, results indicated consistent quality across samples with no evidence of contamination or systematic bias, validating the dataset for downstream phylogenomics and comparative analyses. Nucleotides from each genomic segment were only considered for phylogenetic analyses when coverage was ≥300 × . Alignment checks and selection of high-quality, above-threshold bases were performed by visual inspection, and positions below threshold were called ambiguous (N) to ensure reliability and minimize base-calling errors.

3.3. Emergence of two distinct HA clades

Analysis using segments 4 - HA and 6 - NA confirmed that Brazilian sequences from 2025 clustered within clade 2.3.4.4b. Youk et al. (2022) described the introduction and spread of this clade in North America, especially from 2021 onward, with Eurasian-North American genotype B3 introduction. Since 2022, this clade-2.3.4.4b virus - with predominance of genotype 3.2 - has been detected in South America (PAHO/WHO, 2025).

Brazilian 2025 isolates segregated into two distinct HA clades (Fig. 1). The first included sequences from production poultry (Montenegro municipality) and wild birds (Sapucaia do Sul) in Rio Grande do Sul in both farmed and wild birds (Black swan - *Cygnus atratus*, Black-necked swan - *Cygnus melancoryphus*, Chicken - *Gallus gallus*, and Ovenbird - *Furnarius rufus*) showing high identity to viruses detected in Argentina (Vanstreels et al., 2025) and Bolivia in 2025. This grouping supports a hypothesis of regional epidemiological connectivity in the Southern Cone, mediated by migratory birds. In addition to geographical proximity to the zoo where wild birds were affected, A/H5N1 was confirmed in a wild Ovenbird (*Furnarius rufus*) in the vicinity of the poultry establishment during sanitation.

The second clade comprised Brazilian sequences from the Northeast, Midwest, and Southeast (Espírito Santo, Ceará, Federal District, Goiás, Rio de Janeiro, Mato Grosso, Minas Gerais, and São Paulo states), which

Table 1
Genotyping of Brazilian Influenza A/H5N1 sequences from 2025 showing reassortment previously described in Argentina in 2025, according to Vanstreels et al. (2025).

Samples ID	State	City	Animal	Genotyping									Genomic annotation	
				PB2	PB1	PA	HA	NP	NA	MP	NS	Genotype	Description	Nº genbank
-	-	-	-	am2.1	am1.	ea1	ea1	am1.4.1	ea1	ea1	am1.1	B3.2	-	-
2025-0571-0001	Rio Grande do Sul	Sapucaia do Sul	Black Swan	Not assigned	Not assigned	Not assigned	ea1	am1.1	ea1	ea1	Not assigned	Not assigned	A/Cygnus atratus/SapucaiaSulBR/1246-N5-2025/2025(H5N1)	PV659823-PV659830
2025-0571-0002	Rio Grande do Sul	Sapucaia do Sul	Black-necked swan	Not assigned	Not assigned	Not assigned	ea1	am1.1	ea1	ea1	Not assigned	Not assigned	A/Cygnus melancoryphus/SapucaiaSulBR/1246-N7-2025/2025(H5N1)	PV660431-PV660438
2025-0572-0001	Rio Grande do Sul	Montenegro	Chicken [1]	Not assigned	Not assigned	Not assigned	ea1	am1.1	ea1	ea1	Not assigned	Not assigned	A/Gallus gallus/MontenegroBR/1245-N3-2025/2025(H5N1)	PV660439-PV660446
2025-0643-0001	Rio Grande do Sul	Montenegro	Ovenbird	Not assigned	Not assigned	Not assigned	ea1	am1.1	ea1	ea1	Not assigned	Not assigned	A/Furnarius rufus/MontenegroBR/1355-N-2025/2025(H5N1)	PV796400-PV796407

Chicken 1: origin from productive poultry establishment; Highlights indicate not assigned, according to the origin by the GenoFLU tool, indicating recombinants.

Table 2
Genotyping of Brazilian Influenza A/H5N1 sequences from 2025 showing reassortment not yet reported in South America, with phylogenetic relatedness to North American (USA) dairy cattle sequences.

Sample ID	State	City	Animal	Genotyping									Genomic annotation	
				PB2	PB1	PA	HA	NP	NA	MP	NS	Genotype	Description	Nº genbank
-	-	-	-	am2.1	am1.	ea1	ea1	am1.4.1	ea1	ea1	am1.1	B3.2	-	-
612-001	Minas Gerais	Mateus Leme	Black Swan	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Cygnus atratus/ MateusLemeBR/1367-N-2025/2025/(H5N1)	PV740254-PV740261
613-001	Minas Gerais	Mateus Leme	Black Swan	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Cygnus atratus/ MateusLemeBR/1369-T-2025/2025/(H5N1)	PV740310-PV740317
645-001	Mato Grosso	Campinápolis	Chicken	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Gallus gallus/ CampinapolisBR/1529-N2-2025/2025(H5N1)	PV796574-PV796581
644-001	Distrito Federal	Brasília	White-faced whistling duck	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Dendrocygna viduata/BrasiliaBR/1480-N1-2025/2025(H5N1)	PV849977-PV849984
644-002	Distrito Federal	Brasília	Emu	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Dromaius novaehollandiae/BrasiliaBR/1706-N-2025/2025(H5N1)	PV851412-PV851419
678-001	Goiás	Santo Antônio da Barra	Chicken	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Gallus gallus/SantoAntoniodaBarraBR/1611-N3-2025/2025(H5N1)	PV851442-PV851449
804-001	Espírito Santo	São Mateus	White-faced whistling duck	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Dendrocygna viduata/SaoMateusBR/1834-N-2025/2025(H5N1)	PX121555-PX121562
806-001	São Paulo	São Paulo	Chicken	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Gallus gallus/SaoPauloBR/2000-N2-2025/2025(H5N1)	PX119929-PX119936
807-001	Ceará	Quixeramobim	Chicken	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Gallus gallus/QuixeramobimBR/2086-N-2025/2025(H5N1)	PX120162-PX120169
808-001	Rio de Janeiro	Rio de Janeiro	Guinea fowl	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Numida meleagris/RiodeJaneiroBR/2148-N-2025/2025(H5N1)	PX121547-PX121554
809-001	São Paulo	Monte Azul Paulista	Chicken	Not assigned	Not assigned	ea1	ea1	Not assigned	ea1	ea1	am1.1	Not assigned	A/Gallus gallus/MonteAzulPaulistaBR/2168-N-2025/2025(H5N1)	PX120418-PX120425

Highlights cracked means not assigned, according to the origin of the GenoFLU tool, indicating recombinants.

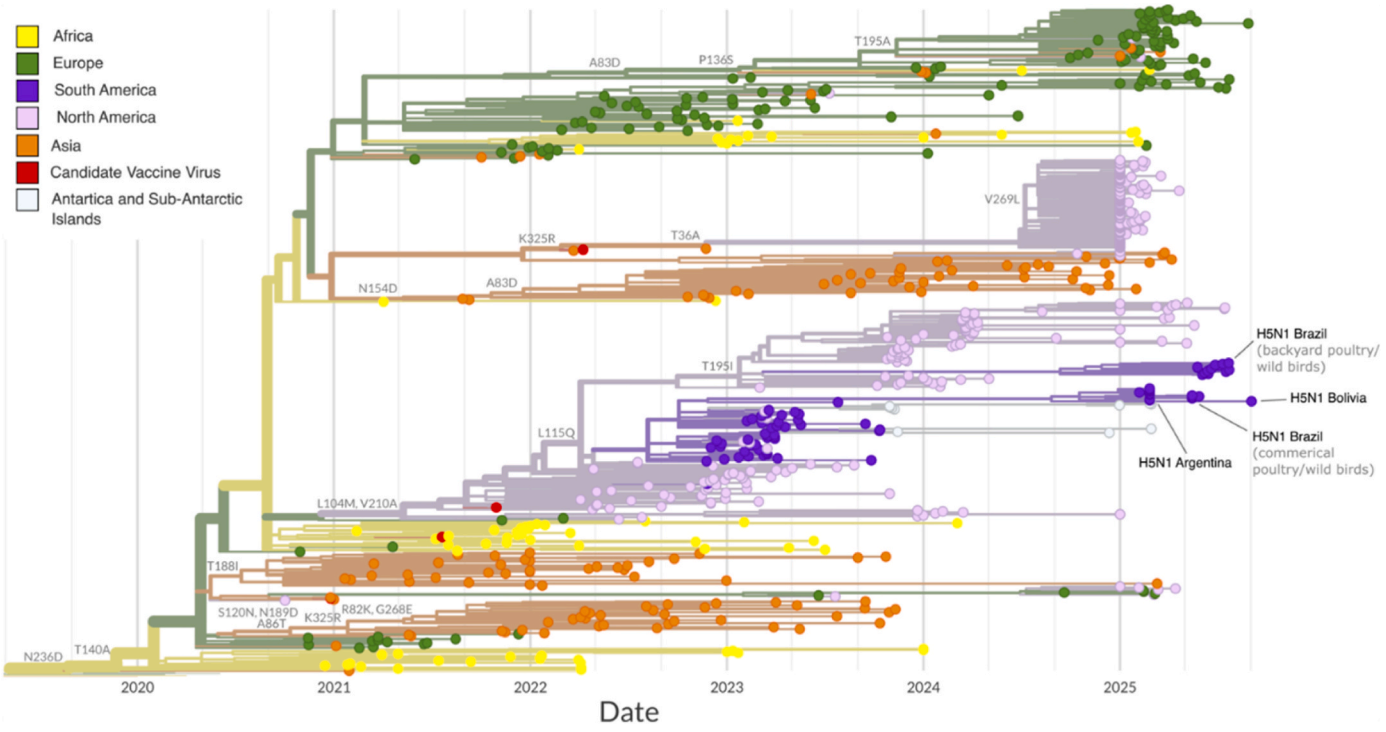

Fig. 1. – H5 clade 2.3.4.4b hemagglutinin (HA) nucleotide sequences available in GISAID EpiFlu database and collected since January 1, 2020 were downloaded (accessed September 23, 2025). Maximum-likelihood phylogenetic tree was generated with FastTree (Price et al., 2009). Phylogenetic analyses were subsequently performed using the Nextstrain Augur pipeline (Hadfield et al., 2018), with results visualized according to the region of sampling.

clustered predominantly with North American isolates from dairy cattle in California, USA - suggesting an independent introduction unrelated to the events recorded in southern Brazil. This clustering with North American sequences may reflect multiple viral introductions into Brazil, underscoring the complexity of H5N1 dispersal dynamics on the continent and continued HA segment circulation since introduction in 2022.

These findings highlight intra-epidemic genomic diversity in H5N1 HA sequences in Brazil in 2025, with implications for molecular

surveillance, containment strategies, and assessment of zoonotic risk associated with different circulating lineages.

3.4. Complex genomic reassortment and genotype variation

Genotyping of H5N1 genomic segments detected in Brazil in 2025 using GenoFLU (USDA-VS, 2025) produced partial classifications, preventing assignment of a definitive genotype to the analyzed samples. By contrast, Brazilian sequences from the 2023–2024 outbreaks were consistently classified as genotype B3.2 (Rivetti Jr et al., 2024) and are summarized in Table 1. This discrepancy suggests the possible emergence of variants with distinct genomic architectures in 2025, emphasizing the importance of continuous genomic surveillance to monitor diversity and evolution of HPAI A/H5N1 viruses in the region and the need for a genotyping system that is global and representative of diversity in South America.

Whole-genome analysis of H5N1 samples detected in Brazil between May and August 2025 also revealed two previously unreported types of genomic variation in samples from domestic chickens and wild birds (sequences highlighted in green - Fig. 2). In Rio Grande do Sul, viruses displayed a triple reassortment with a 4:3:1 genotype configuration comprising segments of distinct phylogenetic origins: four segments (PB2, PB1, PA, NP) derived from South American low-pathogenic avian influenza (LPAI); three segments (HA, NA, MP) from Eurasian H5N1, specifically genotype B3.2; and the NP segment related to North American LPAI - a genomic pattern also identified in H5N1 viruses detected in Argentina in February 2025 (Vanstreels et al., 2025) (Table 1).

A second group of sequences, distinct from those from the production-bird focus and wild birds detected in Rio Grande do Sul (sequences highlighted in blue - Fig. 2), showed unresolved provenance for three segments (PB2, PB1, NP) by GenoFLU, while five segments (PA, HA, NA, MP, NS) belonged to Eurasian H5N1, genotype B3.2. Following the inability of the standard GenoFLU tool to assign definitive genotypes to the PB2, PB1, and NP segments, a detailed phylogenetic analysis was performed to clearly elucidate the origin and evolutionary characteristics of these unassigned segments. The PB2 and NP segments showed genetic links with Colombian sequences originating from the black-bellied whistling duck (*Dendrocygna autumnalis*), which were characterized as HPAI A/H5N2, in addition to being highly phylogenetically related to North American LPAI sequences, forming a clade with a bootstrap support value of 80–89 %. The HA segment was classified as North American lineage (am1.1), related to sequences detected in US dairy cattle (Table 2 and Fig. 3).

4. Discussion and conclusions

Ducks and geese (order Anseriformes) play a critical role in avian influenza reassortment. As natural hosts and reservoirs of nearly all known influenza A subtypes, these birds can carry asymptomatic infections, allowing prolonged viral replication and shedding - especially via the fecal-oral route in aquatic environments. Intense reassortment dynamics in these aquatic hosts can generate new combinations of genomic segments, some acquiring the ability to infect other species—including domestic and wild birds and mammals - with pandemic potential (Lee et al., 2023). As proposed by Vanstreels et al. (2025),

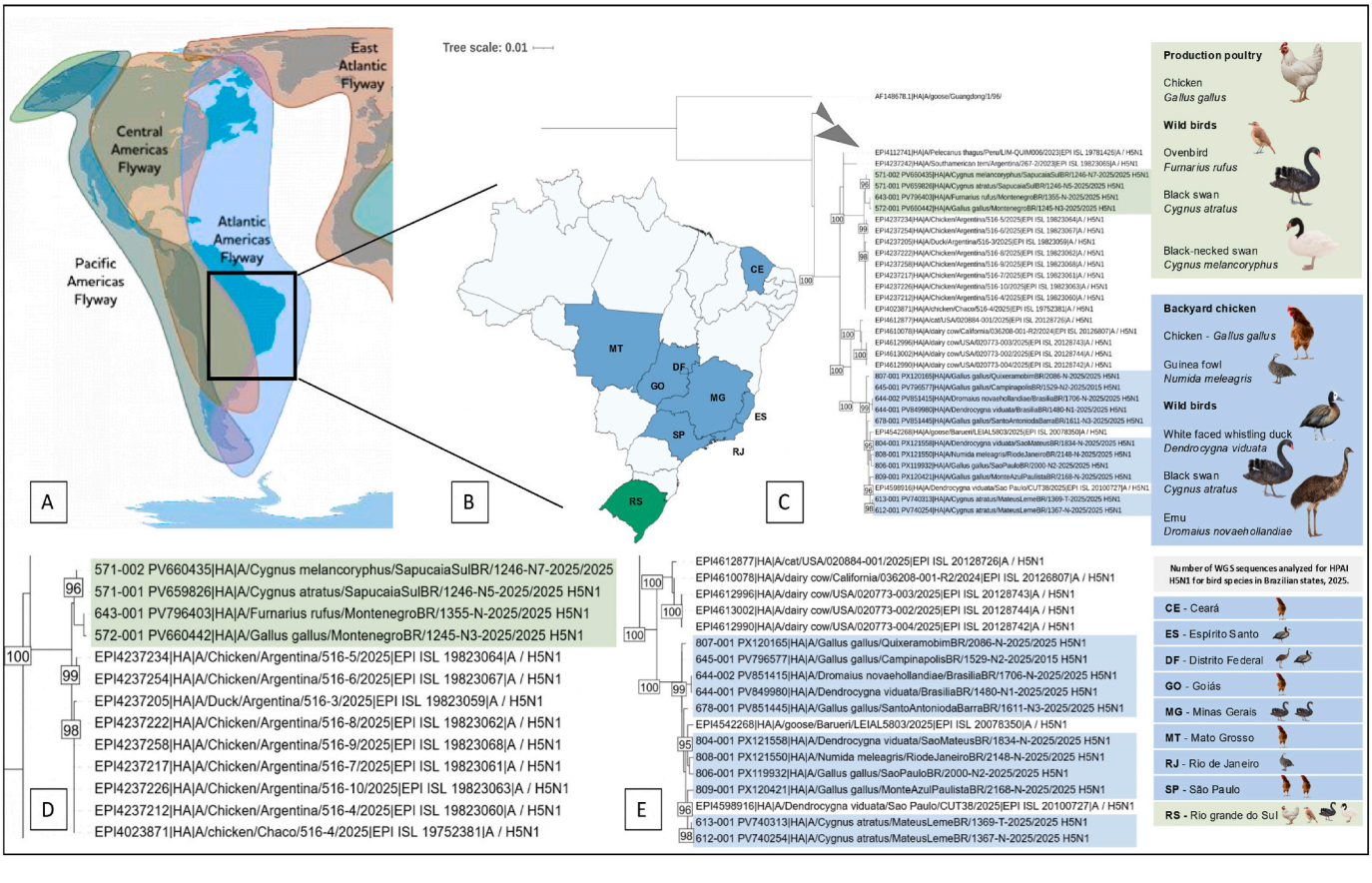

Fig. 2. (C–D–E) Maximum-likelihood phylogenetic tree built from complete HA gene sequences (nucleotide substitution model: K3Pu + F + G4) using IQ-TREE and edited in iTOL (https://itol.embl.de/). Brazilian sequences from 2025 and their locations (B) are highlighted in green for the clade closely related to Argentinian sequences and in blue for the clade closely related to sequences originating from U.S. dairy cattle. Two separate clades are evident (C), indicating possible distinct origins linked to migratory routes of wild birds (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

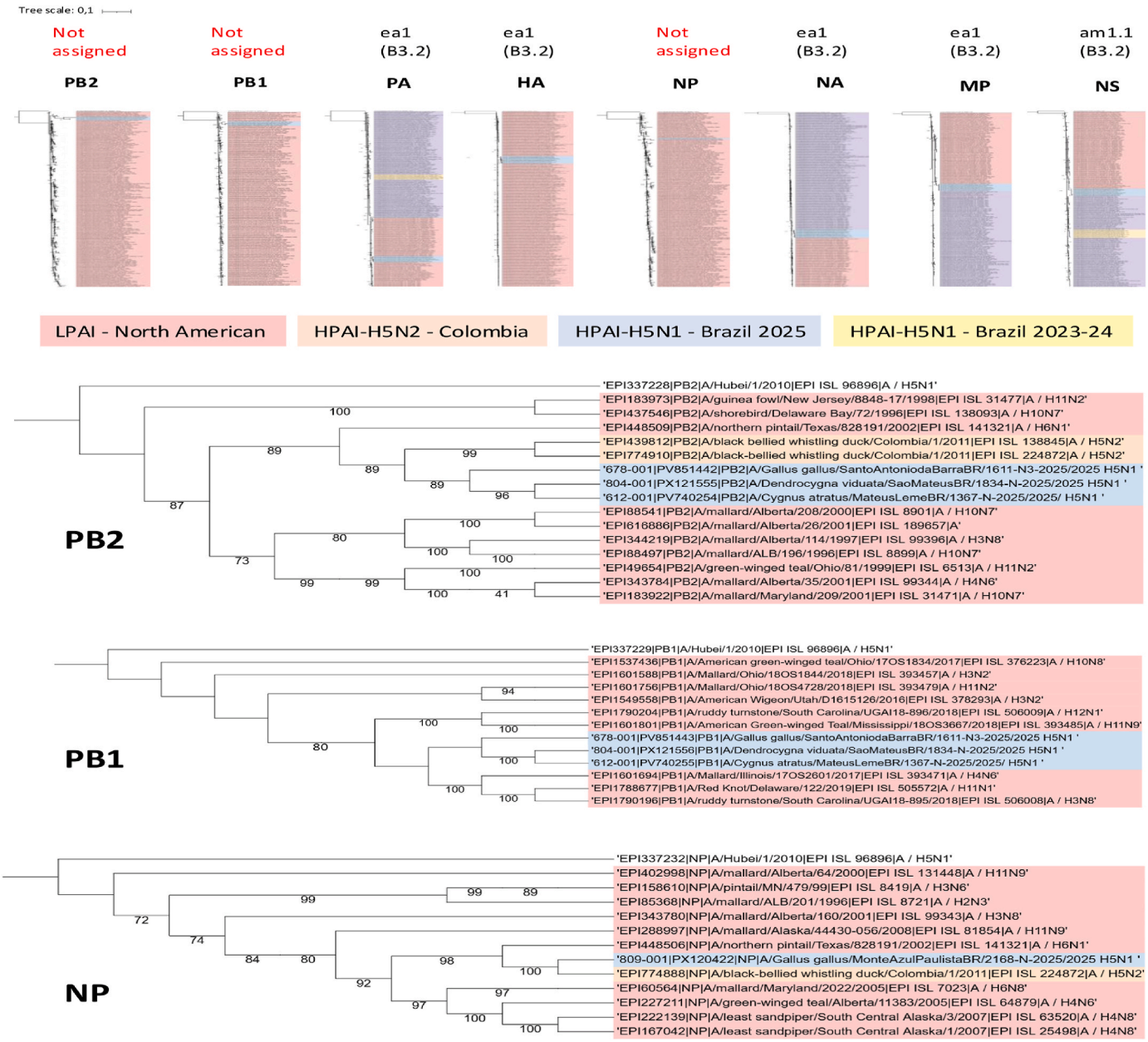

Fig. 3. – Maximum-likelihood phylogenetic trees built from complete genome segments of A/H5N1 viruses detected in Brazil in 2025 (blue), Brazil in 2023–24 (yellow), Colombia (orange), North America (red), and South America (purple), with best-fit models selected by IQ-TREE and trees edited in iTOL (https://itol.embl.de/). The Parnas program (https://github.com/flu-crew/parnas) was used to downsample large phylogenies, selecting an optimal representative subset that preserves the full genetic diversity of the original tree. For Brazilian 2025 sequences the segments PB2, PB1, NP unassigned by GenoFLU, these segments are more closely related to North American samples. PB2 and NP PB2 and NP are also related to sequences from Colombia. Whereas segments (PA, NA, MP, NS) could be assigned and show proximity to South American sequences from 2023 to 25 classified as Eurasian genotype B3.2. An exception is the HA segment, which was genotyped but displays greater proximity to North American sequences, particularly those detected in U.S. dairy cattle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reassortment events between HPAI H5N1 and endemic South American LPAI, the presence of divergent regionally derived genes, and conservation of the Eurasian MP segment - highly preserved among circulating H5N1 - may reflect a selective advantage for MP in the context of viral adaptation. However, unlike the Argentine samples described by those authors, H5N1 viruses detected in Rio Grande do Sul in 2025 did not show a predominance of gastrointestinal clinical signs (backyard chickens). In the single commercial farm affected in the period, neurological signs were the main clinical manifestation, suggesting phenotypic variation among different genomic reassortants in the Southern Cone.

These findings underscore the need for strengthened, continuous molecular surveillance with particular attention to the emergence of new genomic variations that may affect avian influenza epidemiology in the region and, consequently, in Brazil. Such information is essential to update candidate vaccines for human public health, ensuring effectiveness against the most recent and prevalent variants. Likewise, genomic surveillance is crucial to guide animal-health control strategies, providing the technical evidence a country needs to determine if it is the right time to initiate flock vaccination as a preventive measure to contain disease spread and mitigate the risk of human infection.

This study provides comprehensive genomic characterization of

HPAI A/H5N1 viruses detected in Brazil in 2025, emphasizing intra-epidemic genetic diversity among isolates from different regions and sources with multiple incursions into Brazil and genotypic variation (domestic and wild birds). Initial RT-qPCR confirmation, complemented by Sanger sequencing and subsequent whole-genome NGS, not only identified HPAI A/H5N1 but also revealed the complexity of its genomic variations.

Phylogenetic analyses showed that Brazilian isolates belong to clade 2.3.4.4b and group into two distinct clades: one closely related to viruses detected in Argentina in February 2025, and another strongly related to North American isolates, suggesting multiple introduction routes into Brazil. Genotyping evidenced novel genomic rearrangements, including triple reassortants with segments from Eurasian, South American, and North American lineages, reinforcing active viral evolution in the region, with reassortment patterns similar to those reported in Argentina and, specifically in Brazil, involving segments related to US dairy-cattle sequences, genotype B3.13.

The presence of unassignable genomic segments in samples from several regions—particularly in Brazil's Northeast, Midwest, and Southeast—indicates circulation of under-characterized variants with potential impacts on surveillance, diagnosis, and containment strategies. Although the reassortment events described in Argentine (2025) sequences were also observed in Brazil, differences in predominant clinical signs -especially neurological manifestations in a commercial farm - suggest possible phenotypic variation associated with new viral variants that need continues studies.

Overall, our results highlight the complexity of avian influenza ecology and transmission dynamics and underscore the importance of decentralized, continuous genomic surveillance integrating phenotypic, molecular, and ecological data. Early detection and systematic characterization of emerging variants are essential to mitigate impacts on animal health, poultry production, and potential public-health risks.

CRediT authorship contribution statement

Anselmo Vasconcelos Rivetti: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dilmara Reischak:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Lorcan Carnegie:** Writing – review & editing, Software, Formal analysis, Data curation. **Juliana Nabuco Pereira Otaka:** Supervision, Formal analysis, Data curation. **Christian Steffe Domingues:** Supervision, Project administration, Formal analysis. **Fernanda Gomes Cardoso:** Formal analysis. **Ana Luiza Savioli da Silva:** Investigation, Formal analysis. **Soraya Cecília Albieri Camillo:** Formal analysis. **Aristóteles Goés-Neto:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software. **Marcelo Fernandes Camargos:** Writing – review & editing, Visualization, Supervision, Project administration, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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