Supplemental file 1

Adaptation of Pandemic H2N2 Influenza A Viruses in Humans

Running title: Genetic analysis of H2N2 influenza A viruses

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Supplemental Text 1 : Full Methods Summary

Virus Sequencing. The complete genomes of the influenza viruses were sequenced using a high-throughput Next-generation sequencing pipeline at the JCVI that includes the 454/Roche GS-FLX and the Illumina HiSeqTM 2000. Viral RNA was isolated using the ZR 96 Viral RNA kit (Zymo Research Corporation, Irvine, CA, USA). The influenza A genomic RNA segments were simultaneously amplified from 3 μL of purified RNA using a multi-segment RT-PCR strategy (1, 2). The influenza M-RTPCR amplicons were barcoded and amplified using an optimized SISPA protocol (3-5). Subsequently, the SISPA amplicons were purified, pooled and size selected (~800 bp or ~200 bp) and the pools were used for both Roche 454 (Roche Diagnostics Corporation, Indianapolis, IN) and Illumina (Illumina, Inc., San Diego, CA, USA) library construction (~50) viruses per library. Samples were sequenced on the 454/Roche-FLX and Illumina HISeq2000 platforms. Libraries were prepared for sequencing on the 454/Roche GS-FLX platform using Titanium chemistry or for sequencing on the Illumina HiSeqTM 2000. The sequence reads were sorted by barcode, trimmed, searched by TBLASTX against custom nucleotide databases of full-length influenza A segments downloaded from GenBank to filter out both chimeric influenza sequences and non-influenza sequences amplified during the random hexamer-primed amplification. The reads binned by segment, and Illumina reads were de novo assembled using CLC Bio's clc_novo_assemble program. The resulting contigs were searched against the corresponding custom full-length Influenza segment nucleotide database to find the closest reference sequence for each segment. Both 454/Roche GS-FLX and Illumina HiSeqTM 2000 reads were then mapped to the selected reference influenza A virus segments using the

clc_ref_assemble_long program. At loci where both GS-FLX and Illumina sequence data agreed on a variation (as compared to the reference sequence), the reference sequence was updated to reflect the difference. A final mapping of all next-generation sequences to the updated reference sequences was then performed. Any regions of the viral genomes that were poorly covered or ambiguous after Next Generation sequencing were amplified and sequenced using standard Sanger sequencing approach. The consensus sequences were deposited in GenBank (Table S1).

Sequence Selection for Phylogenetic Analysis. We downloaded complete influenza A gene segments of different subtypes and of different host origin from NCBI Influenza database on 28 November 2013. 11285 sequences were downloaded, supplemented by full genomes of 175 isolates newly sequenced for this project, adding to a total of 12685 sequences used. Nucleotide sequences of the coding regions of each of the segments were aligned using MAFFT (6) and were further manually adjusted and assembled to only include coding regions of each gene segment.

Phylogenetic Analysis. Maximum likelihood trees for the large dataset were inferred based on nucleotide alignments using the program FastTree 2.1.5 under the generalized time reversible (GTR) model with gamma-distributed rates among sites (GTR+Γ) (7). To conduct a temporal phylogenetic analysis, sequences were also downloaded with associated meta-data of date of isolation. If the exact day and month of the year was not available, sequences were given a mid-year root. However, since we were interested in the adaptation involved in the host species jump, greater temporal resolution was required for the first year of pandemic transmission. Hence, dates of isolation were extrapolated (to month of transmission) from available epidemiological and scientific reports of the period (8-10). Temporal phylogenies and

rates of evolution were inferred with the program BEASTv1.8.0 using a relaxed clock model that allows rates to vary among lineages within a Bayesian Markov chain Monte Carlo (MCMC) framework (11). This method was used to sample phylogenies and dates of divergences between viruses from their joint posterior distribution, in which the sequences are constrained by their known date of sampling. For the analyses using Bayesian MCMC sampling, in all cases chain lengths of at least 100 million steps were used and a 10-20% 'burn-in' was removed. At least two independent runs of each were performed and compared or combined to ensure adequate sampling.

Reassortment Analysis. Reassortment within the H2N2 pandemic clade (n=49) was evaluated by comparing the patristic distances of trees estimated independently for each gene segment. A distance matrix was calculated from the posterior distributions of a sample of 500 trees obtained from a MCMC analysis with a GTR+G substitution model as described above. The resulting distance matrix was visualized with a multi-dimensional scaling plot to characterize the phylogenetic relationships between the gene segments using the R statistical software package (12).

Selection Analysis. To determine the degree of natural selection, the ratio of nonsynonymous (*dN*) and synonymous (*dS*) substitutions per codon (*dN*/*dS* ratio) were estimated for the entire tree (global model) using single-likelihood ancestor counting (SLAC) method (13), implemented through the Datamonkey webserver (14). The selection pressures acting on the internal and external branches of the tree were measured using the 2-ratio model of the CODEML program in the PAML suite (15). Selection on specific sites was analyzed using two methodologies and sites were considered relevant if both methods detected the same positively selected codons. The first method used was the mixed effects model of evolution (MEME), which is a

useful tool in the detection of both fixed and episodic diversifying selection between branches (16). A *p* value of 0.05 was used as a statistical cutoff for a positively selected site in this method. The second method used to detect specific sites undergoing selective pressure was employed through the Tdg09 program (17), which allows for the definition of host-specific linages to determine sites that contribute to the host-shift event. Sites with a false discovery rate (fdr) less than 0.05 was used reported as significant in this method.

Uracil and CpG Content. The nucleotide content of pandemic H2N2 gene segments were assessed to determine adaptation levels of adaptation in a human/mammalian hosts. To estimate the observed to expected CpG ratios, a custom perl script was executed using the following formula: 100*('CG'/(G*C)*(A+U+G+C)), where A, U, G, C represents the counts of the respective nucleotides and 'CG' the number of adjacent C and G representing the CpG pattern (18). Uracil content values were measured using PAUP* 4.0b10 (19), as it has been postulated that the uracil content of the Influenza A virus genomes tends to increase steadily in mammalian hosts (20, 21). Uracil content values and CpG ratios were visualized and plotted using the R statistical software package (12).

Positions of Positively Selected Sites in HA Structure. The positions identified as significant positively selected sites were plotted on the crystal structure of the A/Singapore/1/1957(H2N2) virus HA [PDB accession code 2WR7] using MacPyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC) for visualization of the trimer.

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Supplementary Figure S1: Maximum likelihood (ML) trees of the PB2 (A), PB1 (B), PA (C), HA (D), NP (E), NA (F), M (G), and NS (H) segments used in this study. ML trees inferred using FastTree v2.1.5 under the generalized time reversible (GTR) nucleotide substitution model for all 8 segments of Influenza A isolates. Human H2N2 isolates represented in blue; human H3N2 isolates represented in orange; human H1N1 isolates represented in brown; all swine isolates represented in green; and all avian isolates represented in black. Isolates used for further temporal phylogenetic and selection analyses indicated by large bracket lines. Images can be zoomed in to visualize details.

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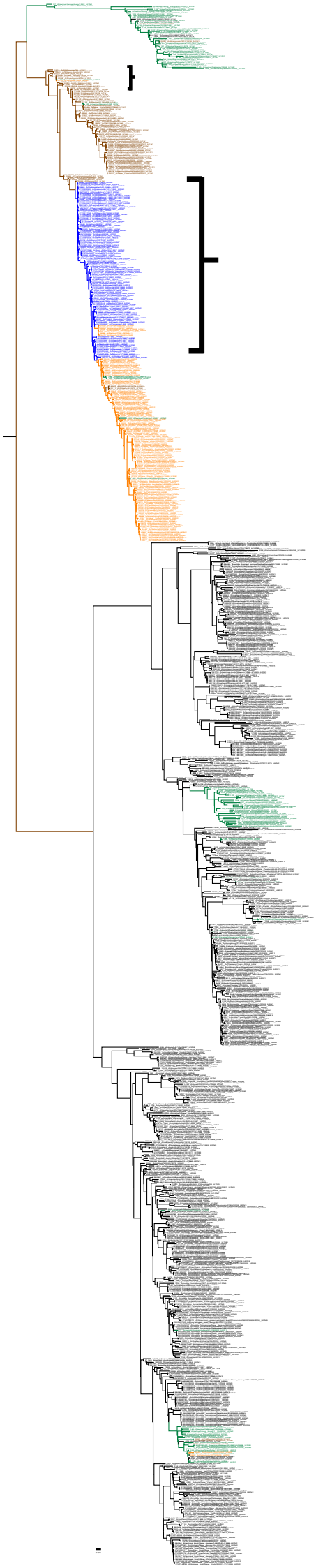

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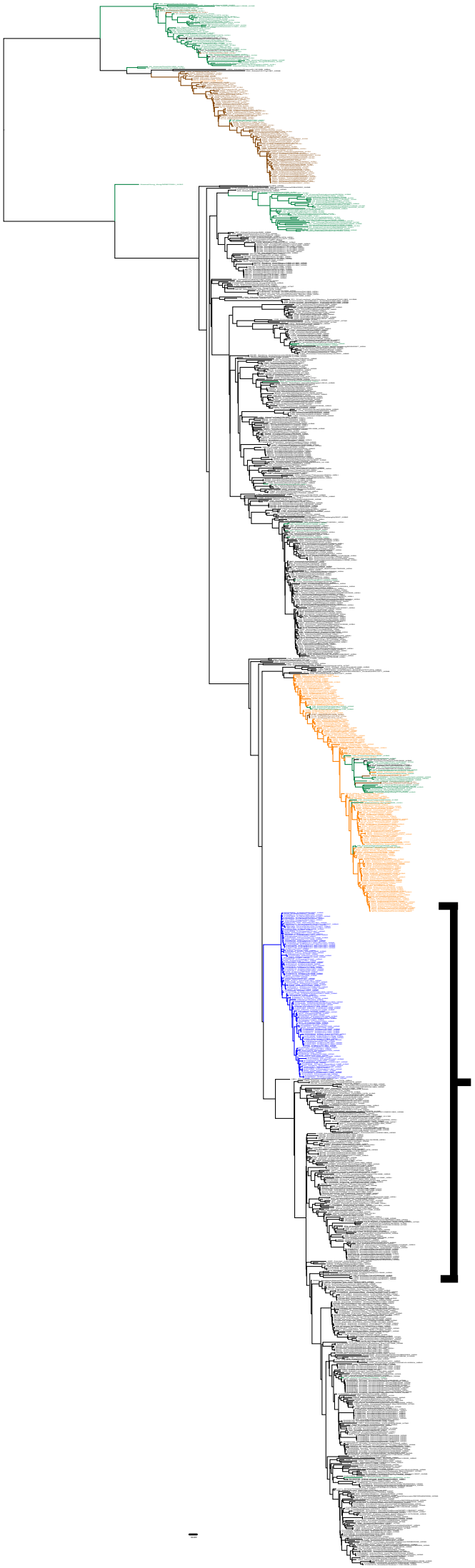

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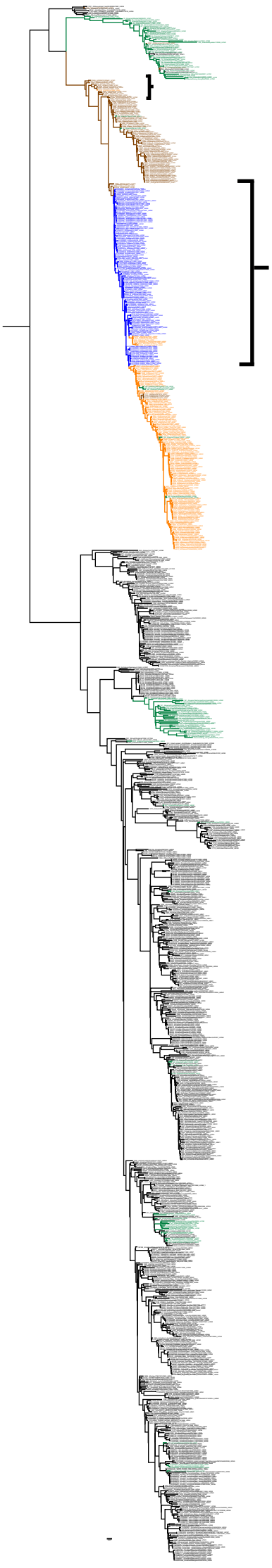

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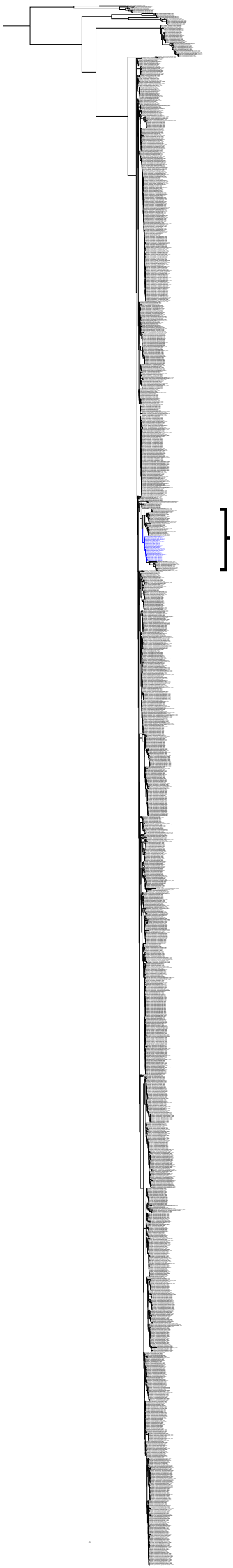
NP (n=1262)

NA (n=3342)



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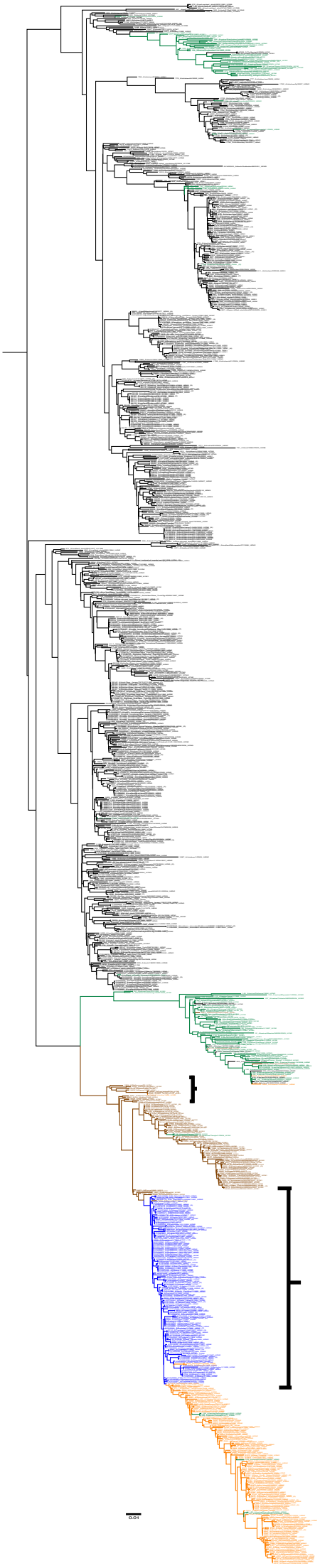

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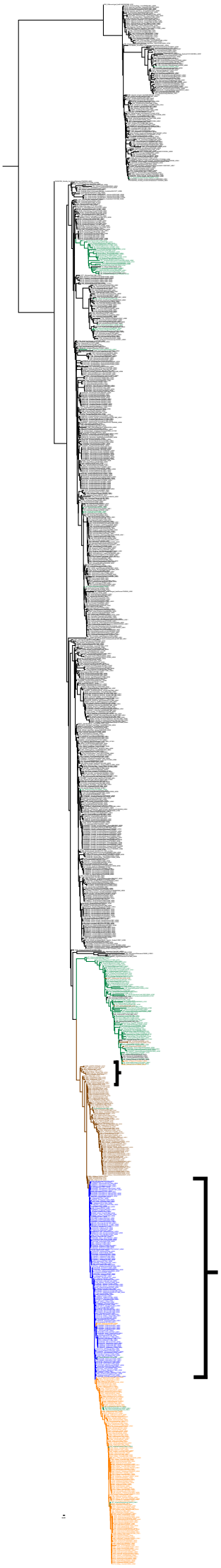

Figure S2: Temporal Phylogenies of human H2N2 (blue) virus gene segments and their associated avian H2N2 (brown) or human H1N1 (black) sister clades. Swine sequences denoted in green. Temporal phylogenies inferred using an uncorrelated log-normal distribution (ucld) clock model and a Gaussian Markov random field (GMRF) Bayesian skyride coalescent tree prior. Blue horizontal bars at nodes represent the 95% HPDs of TMRCAs

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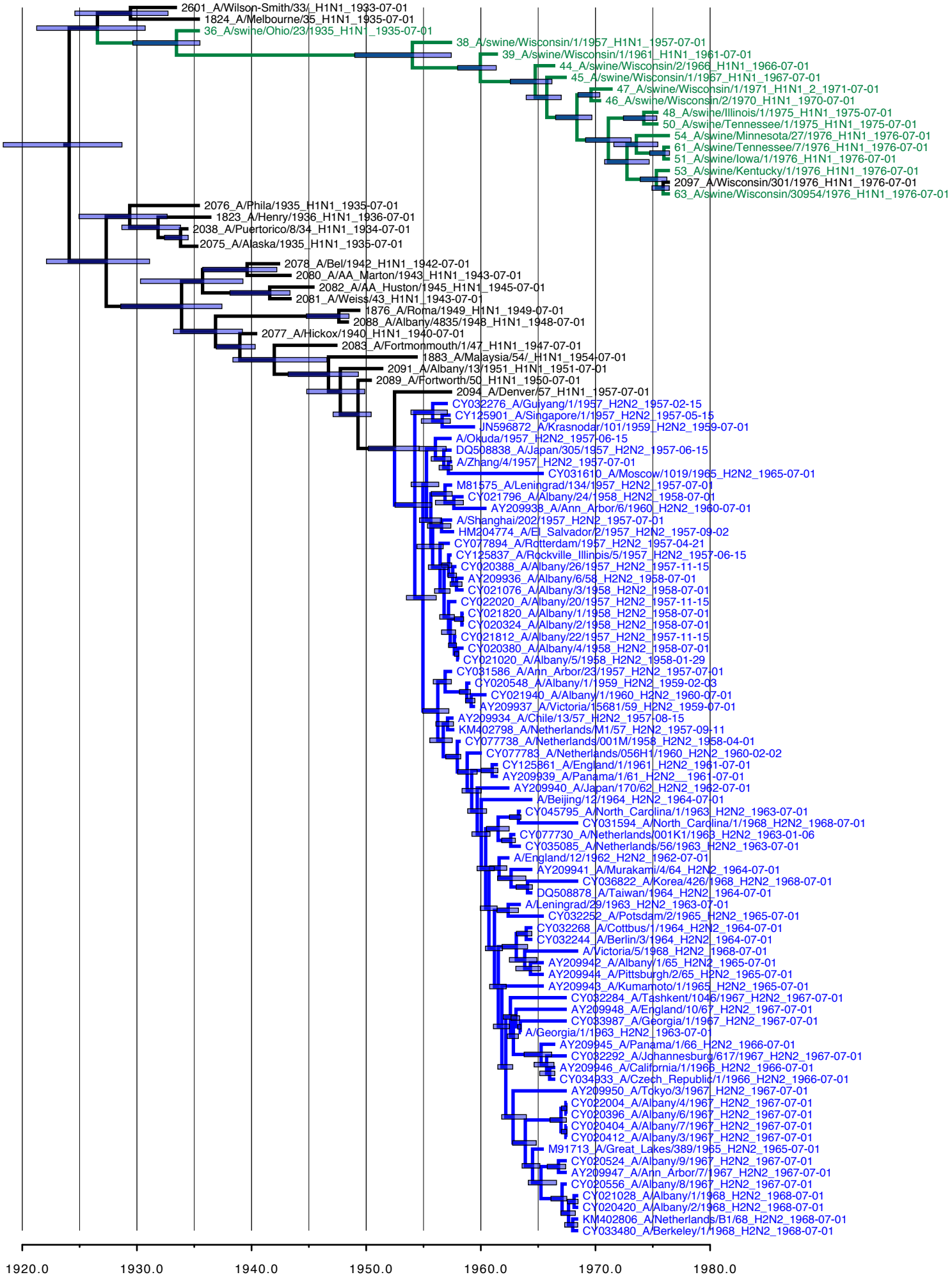
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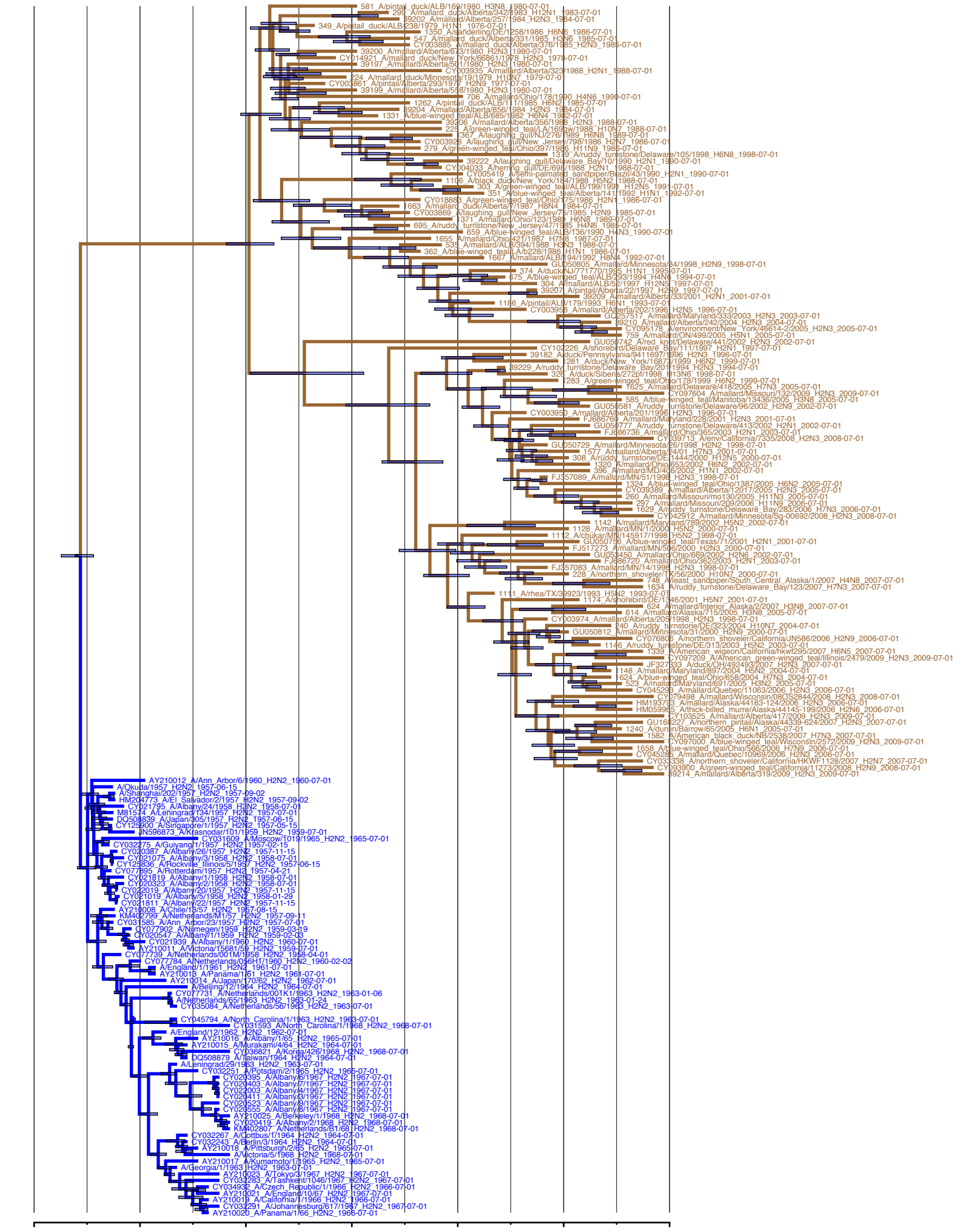

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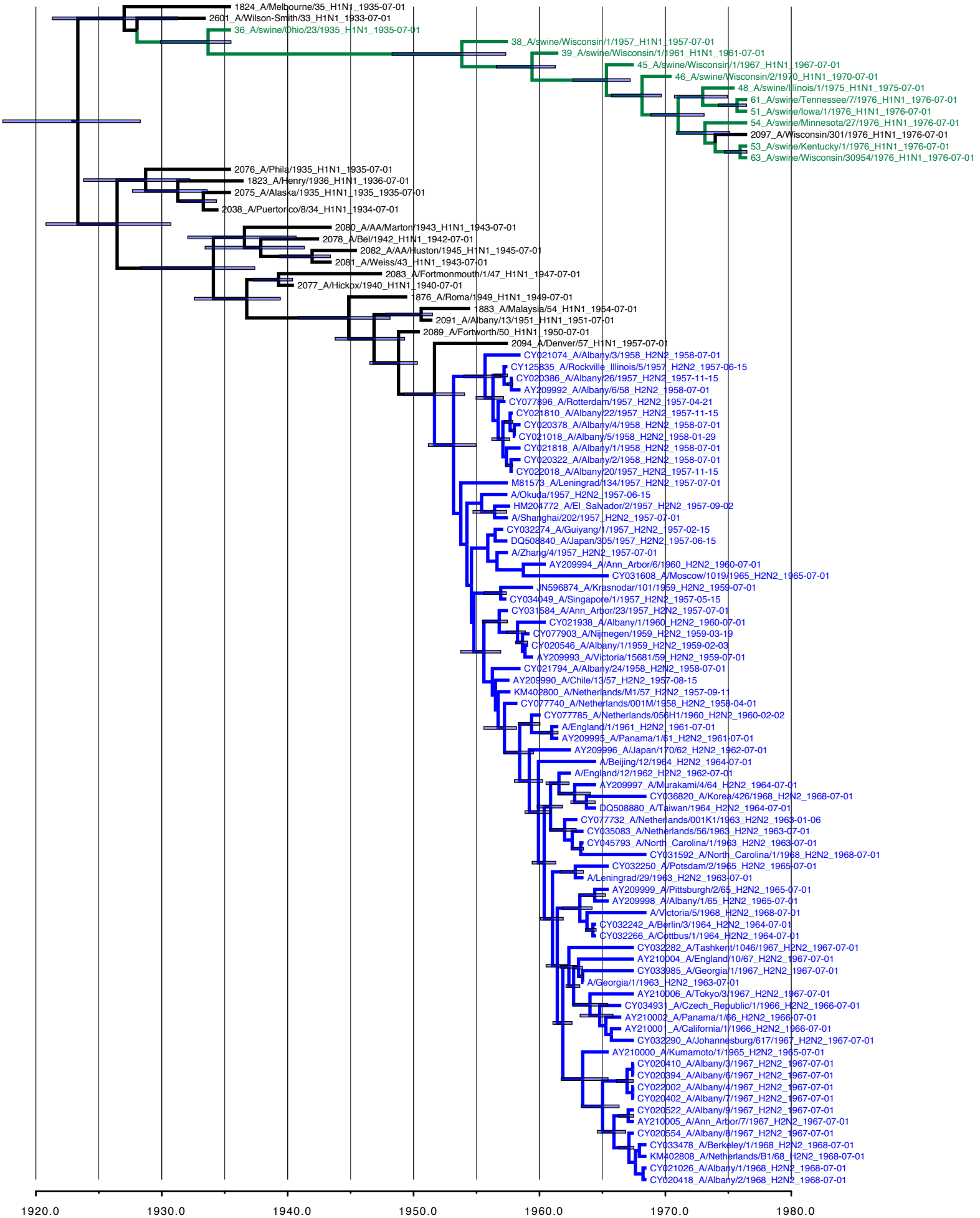
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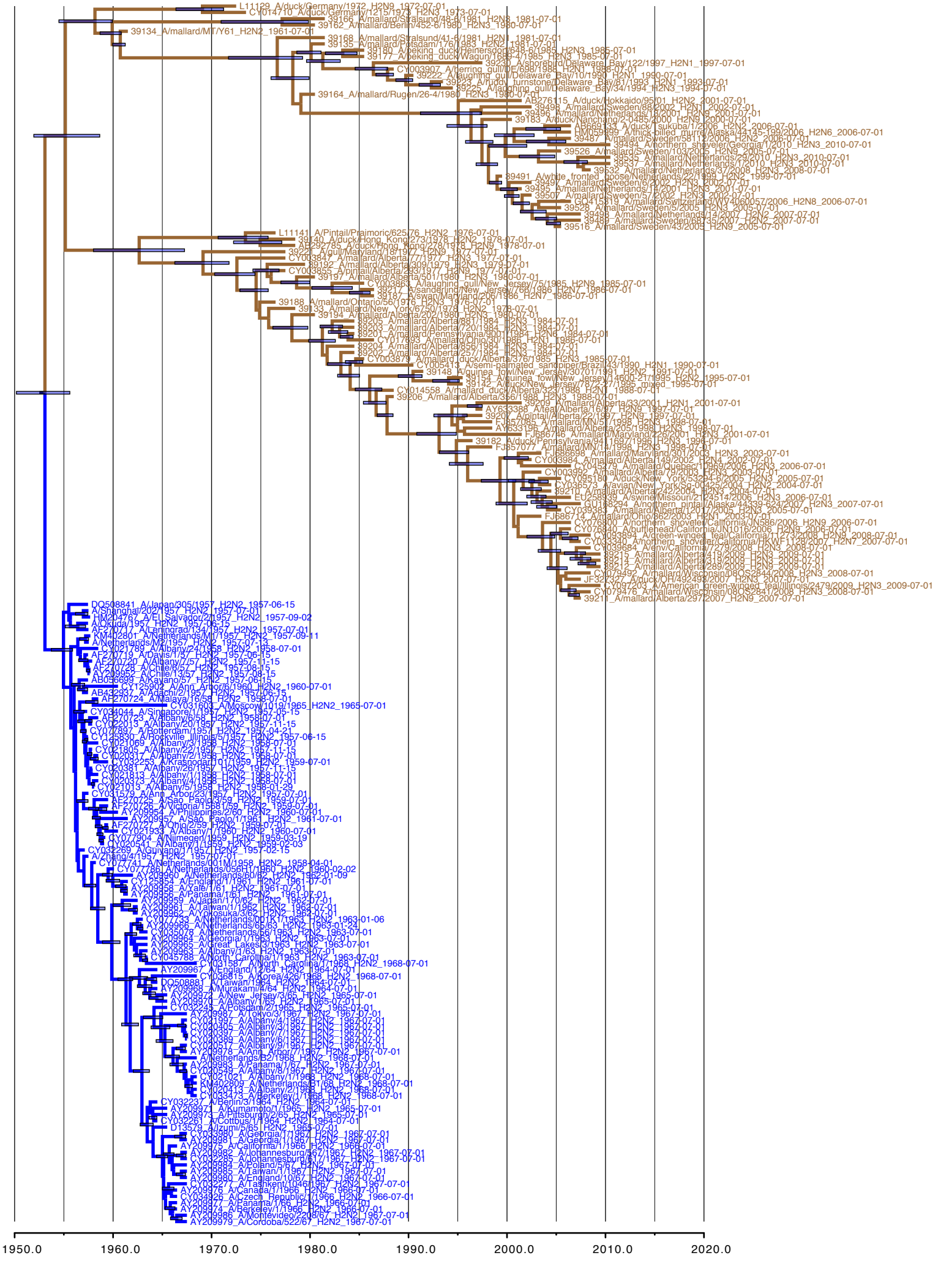
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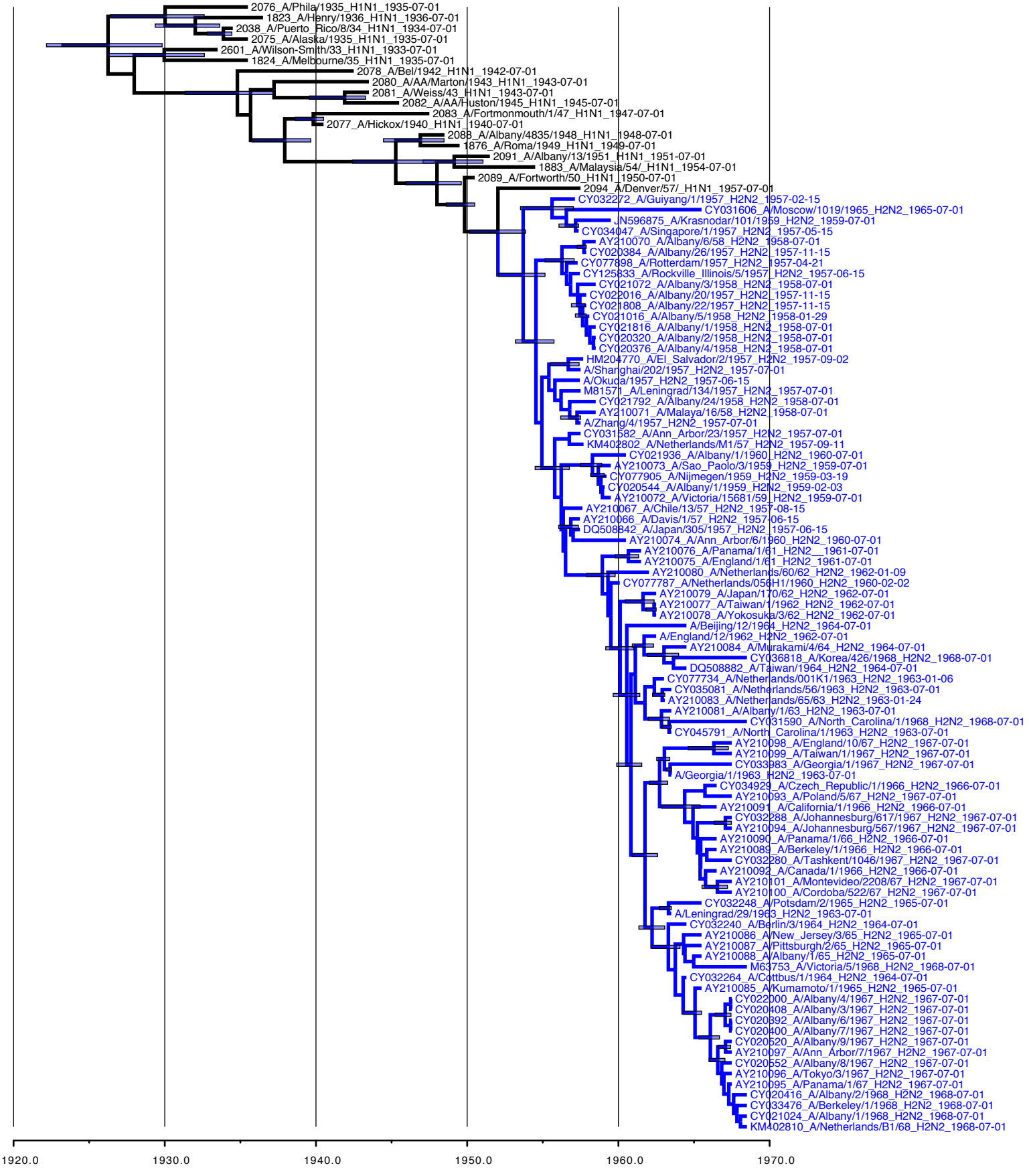
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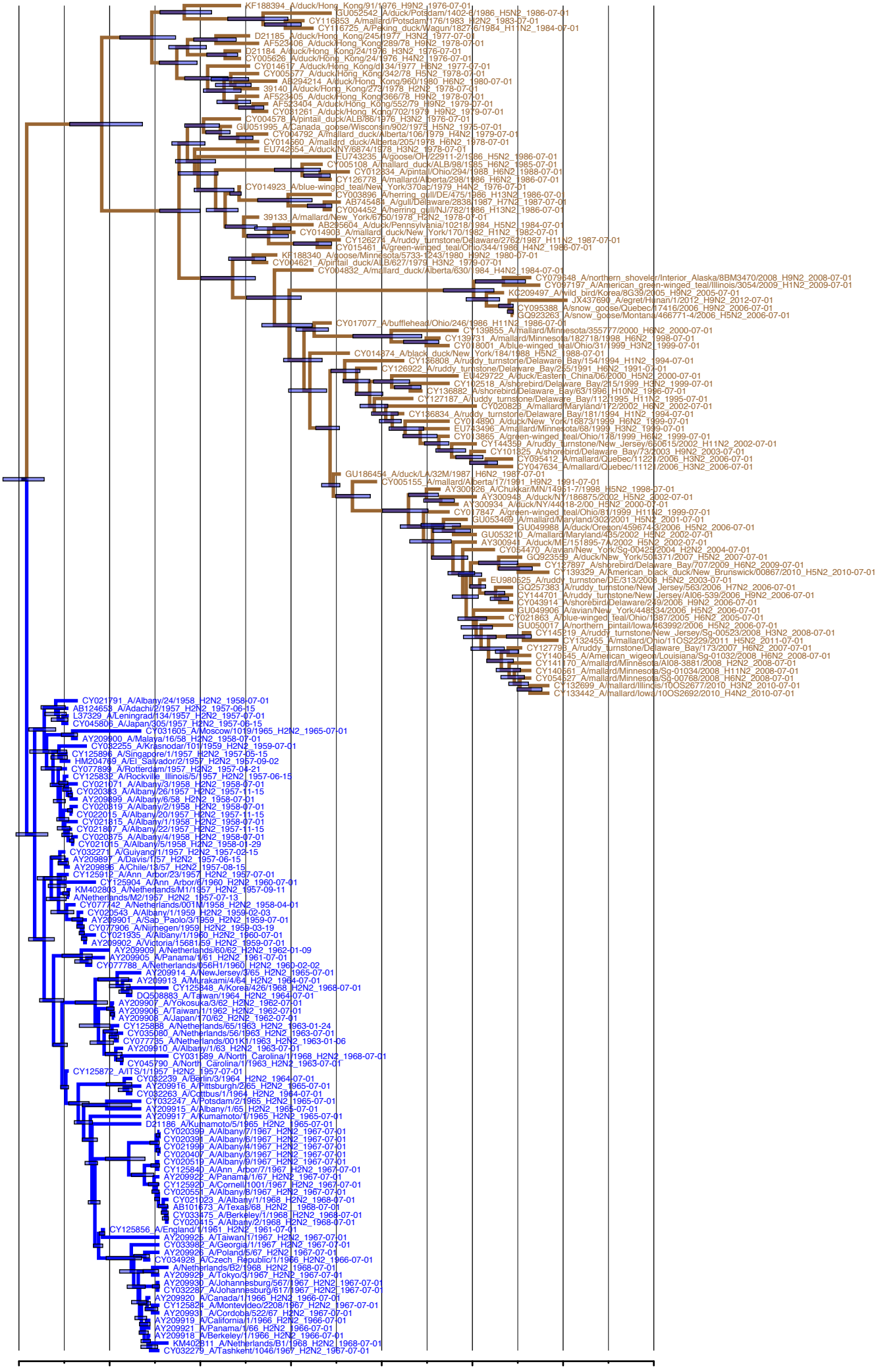
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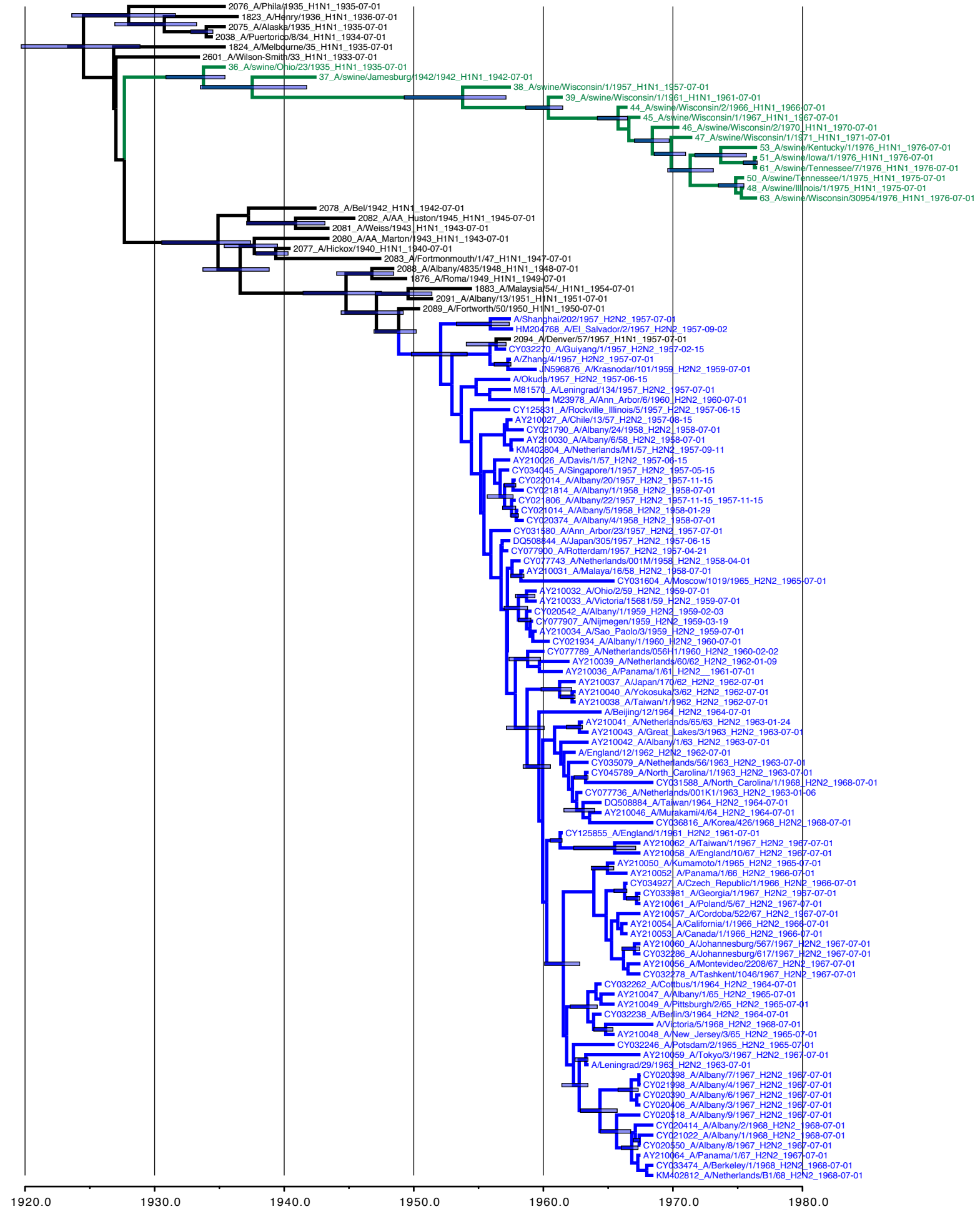
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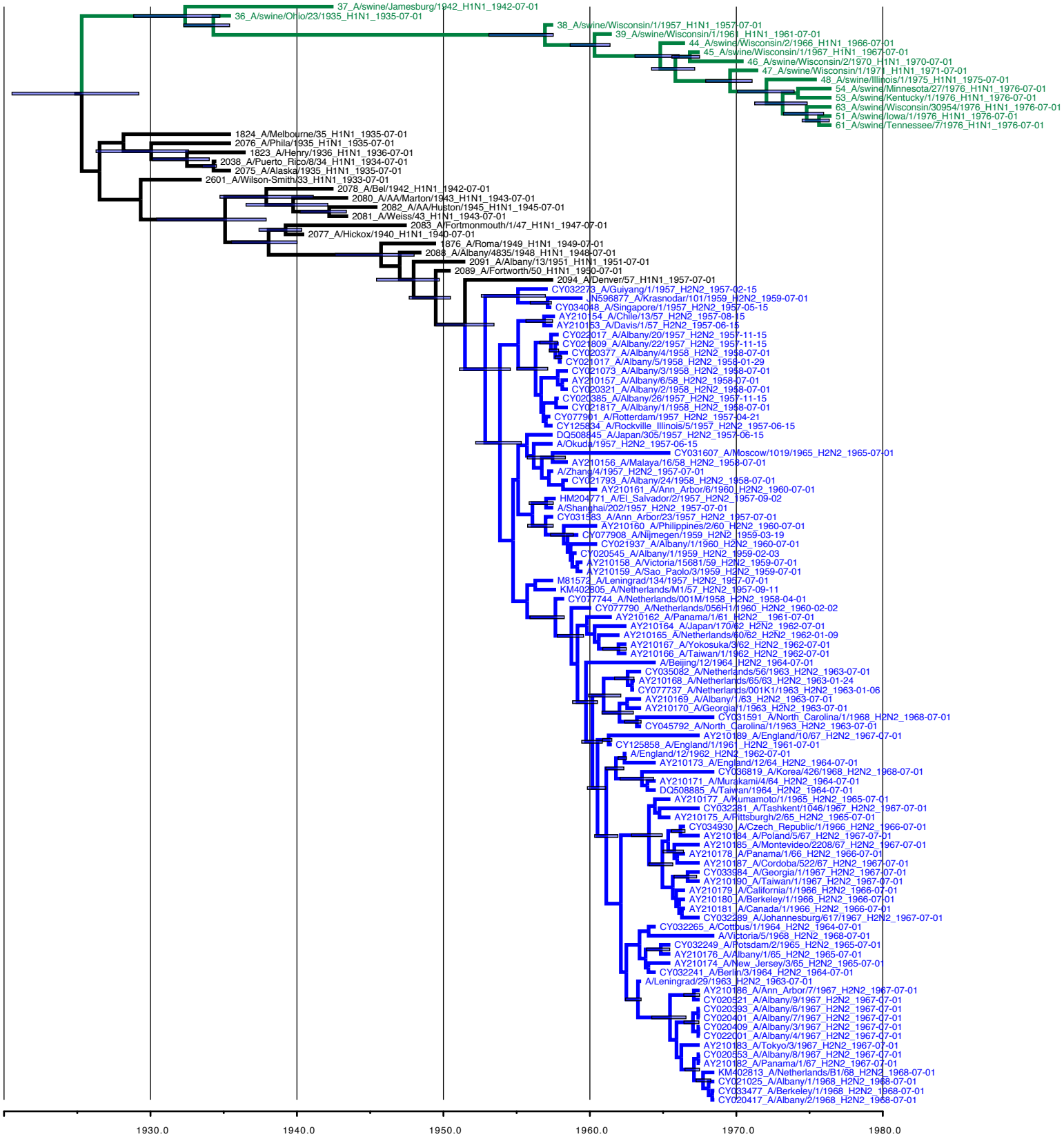
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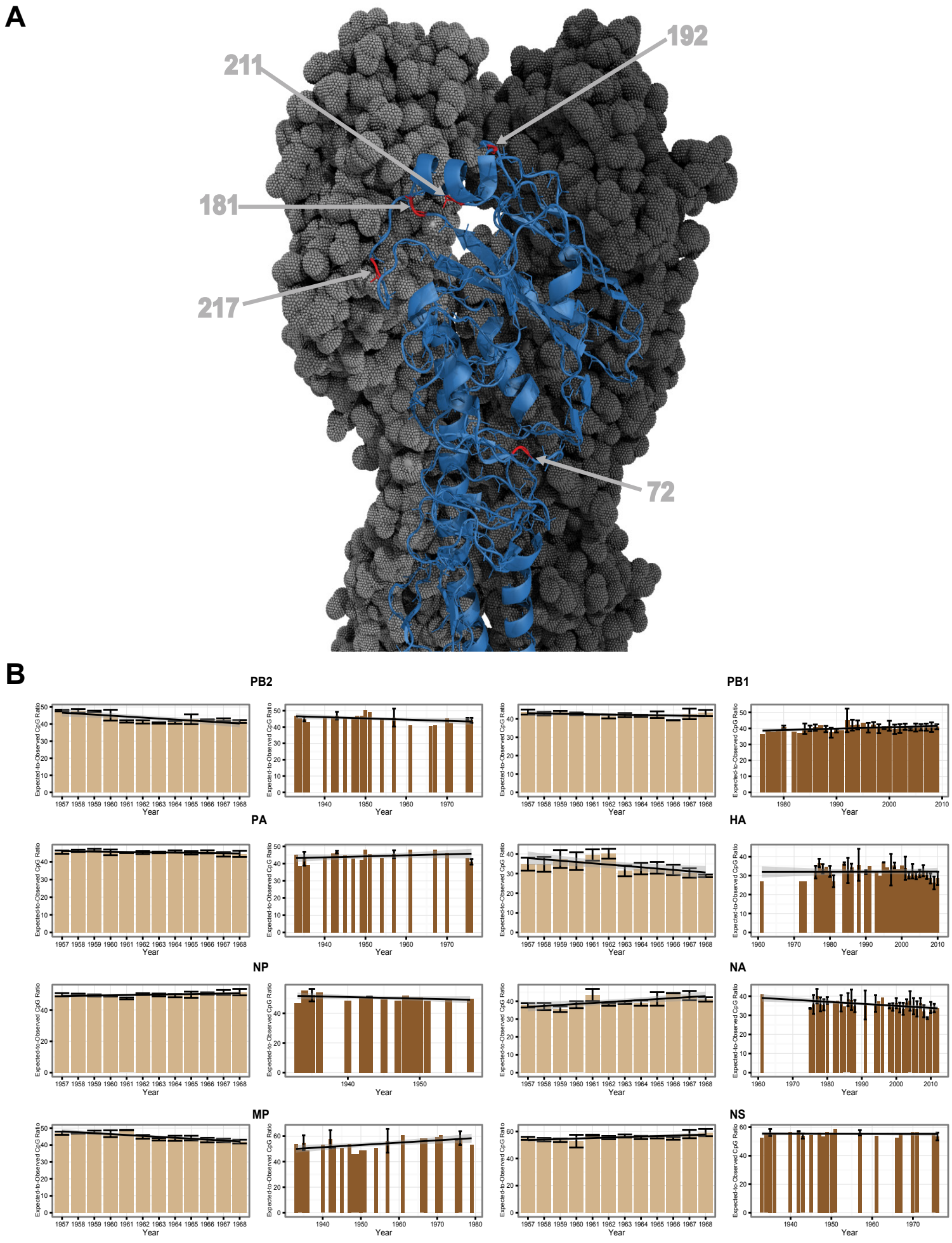
1940.0
1950.0
1960.0
1970.0
1980.0

Figure S3: A. The positions identified as significant positively selected sites plotted on the crystal structure of the A/Singapore/1/1957(H2N2) virus HA trimer [PDB accession code 2WR7] using MacPyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC). The positions that are predicted to be significant in adaptation to human hosts in red. **B**. Observed to Expected CpG ratios in each segment of the human H2N2 isolates (light brown bars) and the respective background/sister clade (dark brown) plotted by year. Error bars represent the standard deviation (SD) by year. Best-fit regression lines (black) shown with 95% confidence interval (grey).


A
211
192
181
217
72
B
PB2
PB1
PA
HA
NP
NA
MP
NS
Expected-to-Observed CpG Ratio
Year

Supplementary Table 1. List of GenBank accession numbers used in this study

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