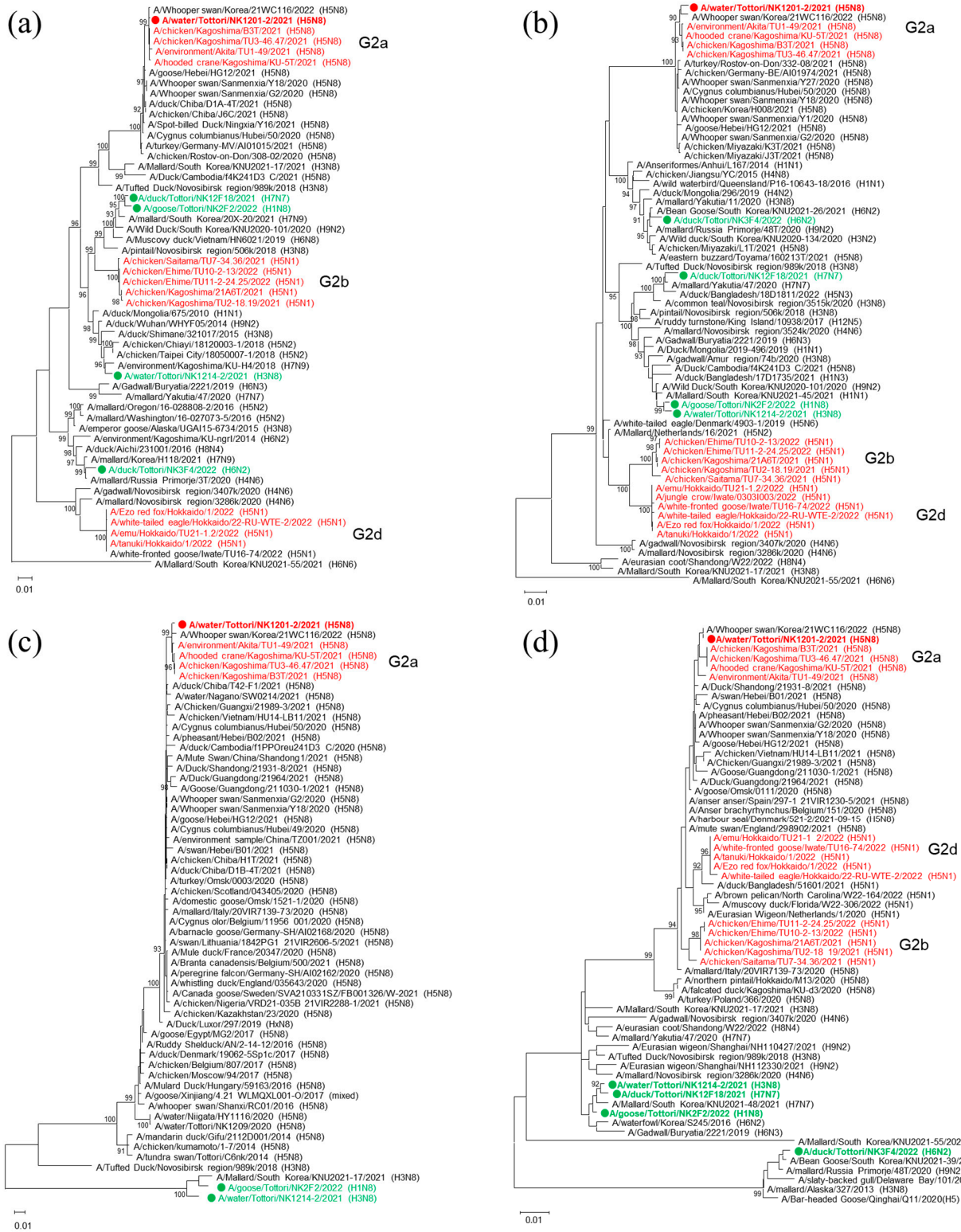


Supplementary Files



(e)

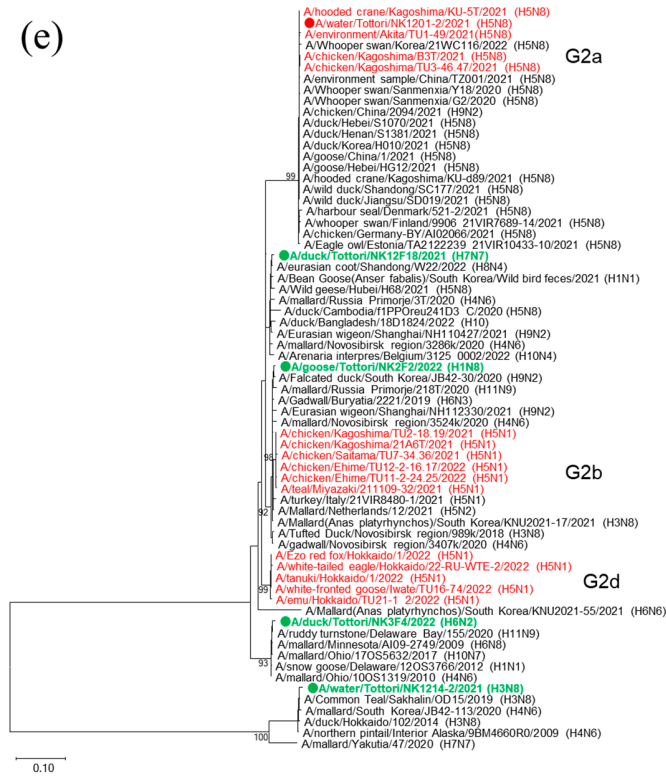


Figure S1. Phylogenetic trees based on coding region of five gene segments of NK1201 HPAIV and LPAIVs isolated at Nikko wintering site. PB2, 1-2280 bp (a); PA, 1-2151 bp (b); N8 NA, 1-1413 bp (c); M, 1-982 bp (d) and NS, 1-838 bp (e) trees were constructed using the maximum likelihood method with the general time-reversible model and 1,000 bootstrap replicates. G2a, G2b, and G2d indicate the classification of H5N8 and H5N1 HPAIVs isolated in the 2021-2022 winter season in Japan based on their HA gene sequences [19]. Bootstrap values >90% are shown at the nodes. Red: H5N8 HPAIVs isolated during 2021-2022 in Japan; Green: LPAIV isolates from wintering site for migratory birds in Tottori Prefecture. Filled circle indicate isolates in this study.

Table S1: Nucleotide and amino acid similarities of NK1201 genes to most closely related influenza viruses

| Genes | Closely related viruses | Collection date | Nucleotide identity (%) | Amino acid identity (%) |
|--------------------------|--|--------------------------|-------------------------|-------------------------|
| Polymerase basic 2 (PB2) | A/chicken/Kagoshima/TU3-46.47/2021 (H5N8) | 2021-11-15 | 2277/2280 (99.87) | 99.60 |
| | A/chicken/Kagoshima/B3T/2021 (H5N8) | 2021-11-14 | 2277/2280 (99.87) | 99.60 |
| | A/Whooper swan/Korea/21WC116/2022 (H5N8) | 2022-01-23 | 2276/2280 (99.82) | 99.60 |
| | A/hooded crane/Kagoshima/KU-5T/2021 (H5N8) | 2021-11-19 | 2275/2280 (99.78) | 99.60 |
| | A/environment/Akita/TU1-49/2021 (H5N8) | 2021-11-10 | 2273/2280 (99.69) | 99.60 |
| | A/mallard/Miyazaki/4501C605/2021 (H5N8) | 2021-01-24 | 2271/2280 (99.69) | 99.60 |
| Polymerase Acidic (PA) | A/hooded crane/Kagoshima/KU-5T/2021 (H5N8) | 2021-11-19 | 2139/2151 (99.44) | 99.86 |
| | A/chicken/Kagoshima/TU3-46.47/2021 (H5N8) | 2021-11-15 | 2139/2151 (99.44) | 99.86 |
| | A/environment/Akita/TU1-49/2021 (H5N8) | 2021-11-10 | 2139/2151 (99.44) | 99.86 |
| | A/chicken/Kagoshima/B3T/2021 (H5N8) | 2021-11-14 | 2139/2151 (99.44) | 99.86 |
| | A/Whooper swan/Sanmenxia/Y18/2020 (H5N8) | 2020-11-13 | 2138/2151 (99.40) | 99.86 |
| | A/Whooper swan/Sanmenxia/G2/2020 (H5N8) | 2020-11-04 | 2138/2151 (99.40) | 99.86 |
| | A/Whooper swan/Sanmenxia/Y1/2020 (H5N8) | 2020-10-30 | 2138/2151 (99.40) | 99.86 |
| | A/chicken/Korea/H008/2021 (H5N8) | 2021-01-04 | 2138/2151 (99.40) | 99.86 |
| | A/Whooper swan/Sanmenxia/Y27/2020 (H5N8) | 2020-11-16 | 2137/2151 (99.35) | 99.86 |
| | A/Whooper swan/Korea/21WC116/2022 (H5N8) | 2022-01-23 | 2136/2151 (99.30) | 99.86 |
| Hemagglutinin (HA) | A/Whooper swan/Korea/21WC116/2022 (H5N8) | 2022-01-23 | 1694/1704 (99.41) | 99.29 |
| | A/environment/Akita/TU1-49/2021 (H5N8) | 2021-11-10 | 1691/1704 (99.24) | 98.94 |
| | A/Whooper swan/Sanmenxia/H735/2020 (H5N8) | 2020-11-11 | 1691/1704 (99.24) | 98.94 |
| | A/goose/Hebei/HG12/2021 (H5N8) | 2021-01 (Day unknown) | 1691/1704 (99.24) | 98.94 |
| | A/Duck/Shandong/21232-5/2021 (H5N8) | 2021-03 (Day unknown) | 1691/1704 (99.24) | 98.94 |
| | A/Goose/Shandong/21153-3/2021 (H5N8) | 2021-02-01 | 1691/1704 (99.24) | 98.94 |
| | A/chicken/Akita/7C/2021 (H5N8) | 2021-11-09 | 1691/1704 (99.24) | 98.94 |
| | A/chicken/Korea/H544/2020 (H5N8) | 2020-12-29 | 1691/1704 (99.24) | 98.94 |
| | A/chicken/Kagoshima/TU3-46.47/2021 (H5N8) | 2021-11-15 | 1690/1704 (99.18) | 99.12 |
| | A/chicken/Kagoshima/B3T/2021 (H5N8) | 2021-11-14 | 1690/1704 (99.18) | 99.12 |
| | A/hooded crane/Kagoshima/KU-5T/2021 (H5N8) | 2021-11-19 | 1689/1704 (99.12) | 99.12 |
| Neuraminidase (NA) | A/environment/Akita/TU1-49/2021 (H5N8) | 2021-11-10 | 1406/1413 (99.50) | 99.15 |
| | A/chicken/Kagoshima/TU3-46.47/2021 (H5N8) | 2021-11-15 | 1404/1413 (99.36) | 99.36 |
| | A/chicken/Kagoshima/B3T/2021 (H5N8) | 2021-11-14 | 1404/1413 (99.36) | 99.36 |
| | A/hooded crane/Kagoshima/KU-5T/2021 (H5N8) | 2021-11-19 | 1404/1413 (99.36) | 99.36 |
| | A/Whooper swan/Korea/21WC116/2022 (H5N8) | 2022-01-23 | 1403/1413 (99.29) | 98.94 |
| | A/Whooper swan/Sanmenxia/Y21/2020 (H5N8) | 2020-11-14 | 1401/1413 (99.15) | 98.94 |

Table S1: Nucleotide and amino acid similarities of NK1201 genes to most closely related influenza viruses (continued)

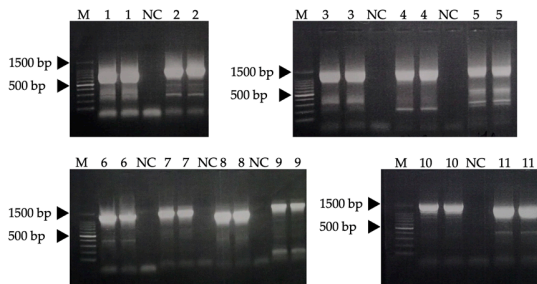
| | | | | |
|----------------------------|---|--------------------------|-----------------|--------------------------|
| Matrix protein (M) | A/Whooper swan/Korea/21WC116/2022 (H5N8) | 2022-01-23 | 981/982 (99.90) | M1: 100 M2: 100 |
| | A/environment/Tokushima/T47-36/2021 (H5N8) | 2021-02-09 | 980/982 (99.80) | M1: 100 M2: 100 |
| | A/hooded crane/Kagoshima/KU-d89/2021 (H5N8) | 2021-01-16 | 980/982 (99.80) | M1: 100 M2: 100 |
| | A/chicken/China/JM01/2020 (H5N8) | 2020-12 (Day unknown) | 980/982 (99.80) | M1: 100 M2: 100 |
| | A/tundra swan/Shanghai/CM20111601/2020 (H5N8) | 2020-11-16 | 980/982 (99.80) | M1: 100 M2: 100 |
| | A/Whooper swan/Sanmenxia/Y25/2020 (H5N8) | 2020-11-18 | 980/982 (99.80) | M1: 100 M2: 100 |
| | A/environment/Akita/TU1-49/2021 (H5N1) | 2021-11-10 | 979/982 (99.69) | M1: 100 M2: 98.97 |
| | A/chicken/Kagoshima/B3T/2021 (H5N8) | 2021-02-08 | 978/982 (99.59) | M1: 100 M2: 100 |
| Nonstructural protein (NS) | A/hooded crane/Kagoshima/KU-5T/2021 (H5N8) | 2021-11-19 | 837/838 (99.88) | NS1: 100 NS2: 100 |
| | A/hooded crane/Kagoshima/KU-d89/2021 (H5N8) | 2021-01-16 | 836/838 (99.76) | NS1: 100 NS2: 100 |
| | A/goose/China/1/2021 (H5N8) | 2021-01-29 | 836/838 (99.76) | NS1: 100 NS2: 100 |
| | A/goose/Hebei/HG12/2021 (A/H5N8) | 2021-01 (Day unknown) | 836/838 (99.76) | NS1: 100 NS2: 100 |
| | A/Whooper swan/Sanmenxia/Y12/2020 (H5N8) | 2020-11-08 | 836/838 (99.76) | NS1: 100 NS2: 100 |
| | A/chicken/China/2094/2021 (H9N2) | 2021-11-12 | 836/838 (99.76) | NS1: 100 NS2: 100 |
| | A/chicken/Kagoshima/B3T/2021 (H5N8) | 2021-11-14 | 836/838 (99.76) | NS1: 100 NS2: 100 |
| | A/wild goose/Shandong/SC196/2021 (H5N8) | 2021-01-23 | 834/838 (99.52) | NS1: 99.54 NS2: 99.17 |
| | A/Whooper swan/Korea/21WC116/2022 (H5N8) | 2022-01-23 | 834/838 (99.52) | NS1: 99.54 NS2: 99.17 |

LPAIV genes amplification

The PCR mixture was prepared in a final volume of 20 µl containing; 10µl of GoTaq® green master mix (a premixed ready-to-use solution containing GoTaq® DNA polymerase supplied in 2X Green GoTaq® reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM MgCl₂), 1 µl of each 10 µM forward and reverse primers, 7.5 µl of DNase/RNase free water and 0.5 µl of cDNA.

PCR condition: 95°C for 2 minutes of denaturation and 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute followed by a final extension at 72°C for 5 minutes.

Gel-electrophoresis result of one of the LPAIV (NK12F18-H7N7)



M: DNA ladder (100 bp); 1 (NS, 890 bp); 2 (M, 1027 bp); 3 (NA, 1413 bp), 4 (NP, 1565 bp); 5 (HA, 1778 bp); 6 (PB1a, 1246 bp); 7 (PB1b, 1431 bp); 8 (PAb, 1159 bp); 9 (PAa, 1487 bp); 10 (PB2a, 1473 bp); 11 (PB2b, 1260 bp); NC, negative control. For each gene, 20 μ l of PCR reaction was divided into 10 μ l and loaded into two wells. The large segments (PB2, PB1, and PA) were divided into two segments for ease of amplification using universal primers designed by Hoffmann *et al.* [27] and primers designed in our laboratory by primer plus 3 program (<https://www.primer3plus.com/index.html>). The full segment was amplified for HA, NP, NA, NS, and M genes using universal primers designed by Hoffmann *et al.* [27].