

Highly Pathogenic Avian Influenza A(H5N1) Virus–Induced Mass Death of Wild Birds, Caspian Sea, Russia, 2022

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In May 2022, we observed a substantial die-off of wild migratory waterbirds on Maliy Zhemchuzhniy Island in the Caspian Sea, Russia. The deaths were caused by highly pathogenic avian influenza A(H5N1) clade 2.3.4.4.b virus. Continued surveillance of influenza viruses in wild bird populations is needed to predict virus spread over long distances.

The coastal and estuarine wetlands of the northern Caspian Sea, which borders southeast Russia, provide support for millions of waterfowl and shorebirds during nesting, molting, migration, and wintering periods (1,2); >300 species of birds are found in this region. The area is crossed by several migration flyways (Figure 1), of which the Black Sea/Mediterranean Flyway is the main migratory route (3). The region plays a critical role in the reproduction of colonial nesting birds, such as pelicans, cormorants, herons, gulls, and terns.

Maliy Zhemchuzhniy Island is located in the northern part of the Caspian Sea (Figure 1). Monitoring data on waterbirds has shown the high ecologic

importance of this area, not only during the nesting period but also during bird migration. The island has had >150 species of birds registered since 2016. A breeding colony of Caspian gulls is located on the island, along with colonies of Great black-headed gulls and Caspian terns, which are all listed in the Red Data Book of Russia. We investigated mass deaths of wild migratory waterbirds on Maliy Zhemchuzhniy Island that occurred in May 2022. The study was approved by the Committee on Biomedical Ethics at the Federal Research Center of Fundamental and Translational Medicine in Novosibirsk, Russia (protocol nos. 2013-23, 2019-3, and 2021-10).

The Study

On April 28, 2022, near the end of the egg incubation period, we had counted a total of 26,769 Great black-headed gull nests, 7,340 Caspian gull nests, and 5,267 Caspian tern nests on Maliy Zhemchuzhniy Island. In May, 1 week later, we detected mass deaths of waterbirds on the island comprising 25,157 Great black-headed gulls, 3,507 Caspian gulls, 5,641 Caspian terns, and 14 Dalmatian pelicans (Appendix 1 Figure 1, <https://wwwnc.cdc.gov/EID/article/29/12/23-0330-App1.pdf>). Nearly all gull and tern chicks died during the nesting period. The mass death event began during hatching of Great black-headed gulls. We only found the corpses of chicks (with down but without feathers) that were similar in age. We assume that not all of the chicks actually died from disease; death of adult birds likely led to the deaths of chicks in their nests. The Caspian terns were still incubating eggs at that time; consequently, the death of adult terns led to the death of egg clutches in their nests. We

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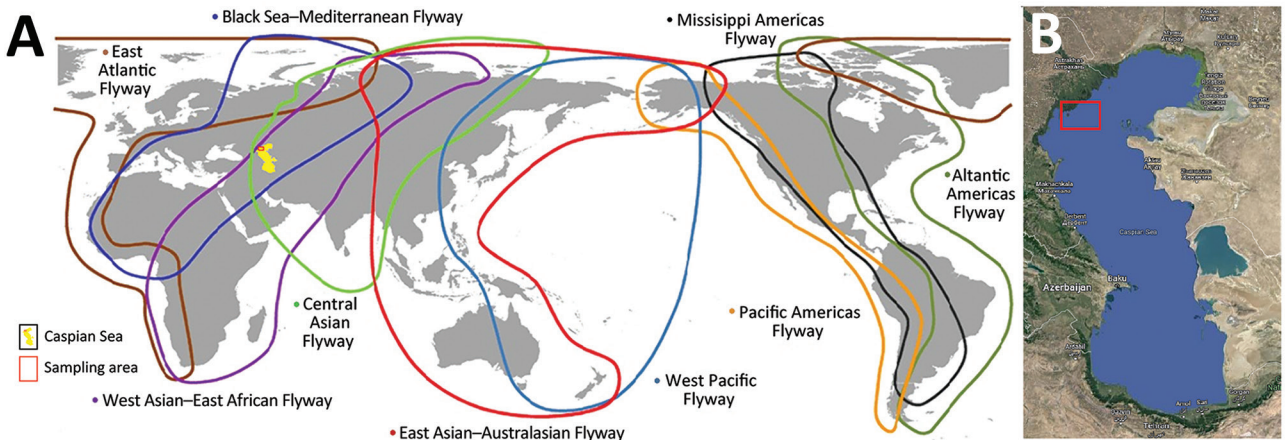


Figure 1. Major bird migration flyways (A) and sampling area of birds (B) in study of highly pathogenic avian influenza A(H5N1) virus–induced mass death of wild birds, Caspian Sea, Russia, 2022. Map of migration routes was provided online by the East Asian–Australasian Flyway Partnership (<https://www.eaaflyway.net/the-flyway>). Yellow shading in panel A indicates the location of the Caspian Sea; red rectangles in both panels indicate sampling location of dead birds on Maliy Zhemchuzhnyi Island.

did not observe live chicks on the island during the remaining 2022 nesting season.

In May 2022, we collected 10 samples from deceased Caspian terns on the island. All samples tested positive for H5Nx avian influenza virus (AIV) by real-time PCR. We characterized 5 isolates by using complete genome sequencing, phylogenetic analysis, and intravenous pathogenicity index testing (Table). We identified all 5 isolates as highly pathogenic avian influenza (HPAI) viruses, according to the amino acid sequence of the hemagglutinin (HA) polybasic proteolytic cleavage site (PLREKRRKR/G) (Appendix 1 Figure 2) and according to intravenous pathogenicity index values of 2.92–2.93 (reference values were for chickens) (Appendix). We determined the HPAI neuraminidase (NA) subtype was N1 by using sequence analysis.

The A/goose/Guangdong/1/96 (Gs/GD) strain, which was isolated in 1996 from a domestic goose, is considered the ancestor of AIV carrying the highly pathogenic H5 HA subtype (4,5). The Gs/GD lineage of HPAI H5N1 viruses evolved into several sublineages that subsequently reassorted with low pathogenicity avian influenza (LPAI) viruses, leading to the formation of H5Nx reassortant variants (6,7). Viruses of the Gs/GD lineage ini-

tially circulated in southern China. However, during 2004–2005, they began spreading throughout Asia, Europe, the Middle East, and Africa (8). An increased number of available sequences led to the creation of the H5 virus classification and identification of 10 clades (4,8–13). Subsequently, clade 2 was split into 5 subclades, each containing numerous genetic subgroups. Clade 2.3.4.4 is dominant and has been divided into several genetic subgroups, including 2.3.4.4.b, which, in turn, diverged into 2 sublineage branches, B1 and B2 (14). According to phylogenetic analysis of HA segments, we found the Caspian Sea strains (the AIV strains isolated from dead birds in the Caspian Sea region) belonged to HPAI H5N1 clade 2.3.4.4.b (Figure 2). Furthermore, we found the HPAI H5N1 virus isolates from Maliy Zhemchuzhnyi Island belonged to the B2 sublineage because isoleucine was present at position 548 in HA (14).

The polymerase basic (PB) 1, polymerase acidic (PA), HA, nucleoprotein (NP), NA, and matrix (M) protein gene segments of the Caspian Sea strains were phylogenetically related to H5N1 viruses previously identified in wild birds in Israel in January 2022. However, all 8 gene segments were similar to those of strains from Romania isolated in February

Table. Sequenced viruses isolated from Caspian terns on Maliy Zhemchuzhnyi Island in study of highly pathogenic avian influenza A(H5N1) virus–induced mass death of wild birds, Caspian Sea, Russia, 2022*

Isolate	Location	Sample type	Collection date	IVPI	GISAID no.
A/Caspian_tern/Astrakhan/30/2022	45°02' N, 48°19' E	Intestine	2022 May 15	2.92	EPI_ISL_16020401
A/Caspian_tern/Astrakhan/32/2022	45°02' N, 48°19' E	Intestine	2022 May 15	2.92	EPI_ISL_16020402
A/Caspian_tern/Astrakhan/34/2022	45°02' N, 48°19' E	Intestine	2022 May 15	2.93	EPI_ISL_16020403
A/Caspian_tern/Astrakhan/36/2022	45°02' N, 48°19' E	Liver	2022 May 15	2.93	EPI_ISL_16020404
A/Caspian_tern/Astrakhan/38/2022	45°02' N, 48°19' E	Liver	2022 May 15	2.92	EPI_ISL_16020405

*Nucleotide sequences were deposited in the GISAID database (<https://www.gisaid.org>). IVPI, intravenous pathogenicity index.

2022 (Appendix 1 Figures 3–9). The phylogenetic relationships of PB2 and nonstructural (NS) gene segments between AIV strains from Israel and Romania remains unclear, because no sequences were available for the segments from Israel in the GISAID EpiFlu database (<https://www.gisaid.org>).

Conclusions

On the basis of virus phylogeny and chronology of virus detection, we hypothesize that the HPAI virus found in the Caspian Sea region was present in birds migrating to their wintering sites during autumn 2021 and was detected in Israel during the winter months of 2022. During spring migration in 2022, the virus strain spread from the Middle East

to nesting areas, leading to wild bird deaths on Maliy Zhemchuzhnyi Island. Because of the lack of some genetic data on closely related viruses, it remains unclear whether the Caspian Sea strains were transmitted through Europe (Romania) from Israel or directly from Israel.

The HPAI H5N1 viruses detected during the mass death of birds on Maliy Zhemchuzhnyi Island evolved from sequential reassortment of multiple genetic variants of LPAI and HPAI viruses (Appendix 1 Figures 3–9). The new variants probably acquired M and HA gene segments from viruses (Egyptian-like) detected in Siberia and Kazakhstan in 2020 (15). PB2, PB1, PA, NP, and NA gene segments from HPAI viruses likely emerged as a result of reassortment with

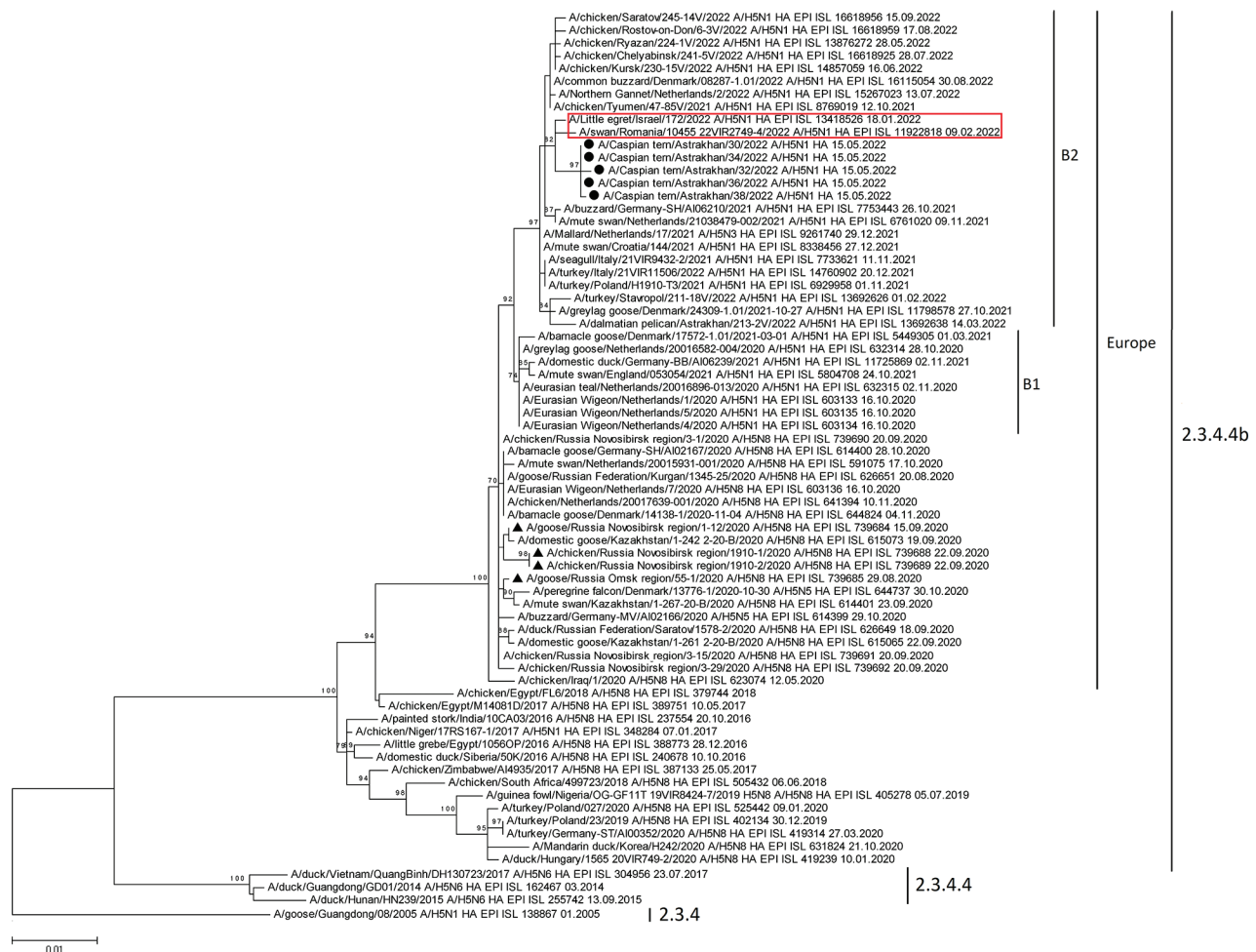


Figure 2. Phylogenetic analysis of viruses isolated from 5 dead Caspian terns in study of highly pathogenic avian influenza A(H5N1) virus–induced mass death of wild birds, Caspian Sea, Russia, 2022. Maximum-likelihood phylogenetic tree was constructed for hemagglutinin gene segments. Black circles indicate highly pathogenic avian influenza (HPAI) A(H5N1) virus strains isolated from the Caspian Sea region; black triangles indicate Egyptian-like HPAI virus strains from Russia isolated in 2020; red box indicates HPAI strains from Israel and Romania that were closely related to viruses from the Caspian Sea. Viruses belonging to clade 2.3.4.4b and B1 or B2 sublineages and those with hemagglutinin genes found in Europe are indicated. Sequences were obtained from the GISAID EpiFlu database (<https://www.gisaid.org>); identification numbers are provided. Scale bar indicates nucleotide substitutions per site.

LPAI viruses during 2020–2021; NS segments likely emerged from LPAI viruses detected during 2021–2022. NS sequences closely related to those of strains isolated in the Caspian Sea regions and Romania were found in LPAI viruses predominantly circulating in Asia during 2019–2021 (Appendix 1 Figure 9). HPAI viruses with such NS sequences have been identified only in Romania and the Caspian Sea.

Gene segments of HPAI H5N1 viruses from the Caspian Sea were closely related to virus segments found in different parts of Eurasia. Specifically, PB1, PA, HA, NA, and M protein gene segments were predominantly related to those in Europe, whereas related NP and NS segments were more prevalent in Asia. In addition, the PA segment from the Caspian Sea strains was also identified in Africa, and PB2 was related to PB2 of viruses detected in the Far East (Japan, Korea, and China), Siberia (Novosibirsk region), Bangladesh, and Europe (Italy, Slovenia, and Czech Republic). Three major flyways pass through the Caspian Sea region: the Black Sea/Mediterranean Flyway, the West Asian–East African Flyway, and the Central Asian Flyway. However, we found that gene segments of HPAI viruses from the Caspian Sea were related to variants identified in the Far East, indicating widespread distribution and exchange of influenza virus genes well beyond the major flyways. Therefore, continued surveillance and monitoring of AIVs (primarily HPAI viruses) in wild bird populations will be needed worldwide to track and predict the spread of these viruses over long distances.

Acknowledgments

We thank those persons who provided sequence information to the GISAID's EpiFlu database (Appendix 2 Table, <https://wwwnc.cdc.gov/EID/article/29/12/23-0330-App2.xlsx>).

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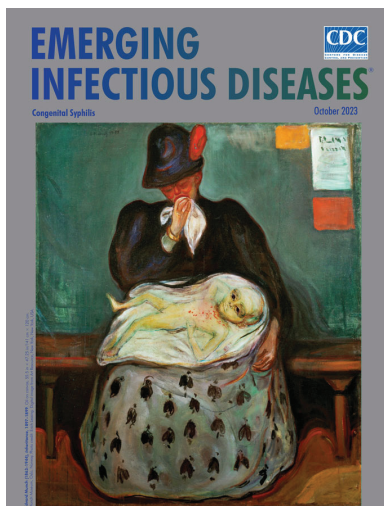
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Appendix 1

Additional Materials and Methods

Sampling Area

The Caspian Sea region is a crucial area for avian influenza virus (AIV) surveillance in Eurasia, because it is situated at the convergence of 3 major bird migratory routes: the Central Asian Flyway, East Africa/West Asia Flyway, and Black Sea/Mediterranean Flyway (Figure 1, main text). Thus, the main migratory pathways of birds from Europe, Africa, and Asia intersect here (1–3). Because of the abundance of saltwater bays, lagoons, river deltas, and lake systems, the western coast of the Caspian Sea provides a highly favorable route for the seasonal migrations of numerous bird species in Eurasia (4,5). The ecologic characteristics of those unique habitats enable wild waterfowl and shorebirds to utilize numerous wetlands in the Caspian Sea region for both wintering and nesting. Moreover, this region also provides potential stopover sites for birds that migrate further into Europe or Africa.

Reconstructive analysis of the flight routes of wild birds in general, and gulls (Laridae) in particular, was performed as previously described (5) according to data from the Bird Ringing Center of Russia (<https://sev-in.ru/en/bird-ringing-and-scientificinformation-cente>) (Appendix 1 Figures 10). As a result, it has been shown that flight routes passing through the Caspian Sea region cover not only the territory of Eurasia but also parts of Africa. They also pass through countries in Europe and the Middle East. Those results enables us to assume that the influenza virus spreads through avian spring migration to the Caspian Sea, either directly from Israel and the Levant or through Europe (likely via Romania, as indicated by phylogenetic analysis). In

addition, analysis of flight routes and migration corridors (notable for their diverse destinations) indicates the significance of the Caspian region in the spread of avian influenza virus and early detection of new variants.

Maliy Zhemchuzhniy Island is located in the northern part of the Caspian Sea near the maritime boundary of the Republic of Kalmykia and the Astrakhan region (latitude 45°02' N, longitude 48°19' E). This flat, low-lying island, composed of sand and clam shells, was formed during a regression of the Caspian Sea in the 1930s on the site of an underwater shoal. It is constantly exposed to wind and waves, which cause changes in its configuration, and areas are either completely or partially lacking terrestrial vegetation. During 2016–2022, the size of the island remained relatively constant. In 2021, the island was estimated to be 26.33 ha and had a length of ≈ 2 km and maximum width of ≈ 0.3 km.

Samples

In May 2022, we collected 10 samples from Caspian terns on the island during a mass die-off of wild birds. In all of those samples, the matrix protein gene segment of AIV A and the hemagglutinin gene segment of the H5Nx subtype were detected by using multiplex real-time PCR (AmpliSens Influenza virus A H5N1-FRT PCR kit; InterLabService Ltd, <https://en.interlabservice.ru>). All analyzed viruses were isolated from 10-day-old chicken embryonated eggs by using chicken embryo inoculation. All viruses caused the death of chicken embryos within 2 days. Isolates were shown to be H5 positive by using real-time PCR.

Genome Sequencing and Phylogenetic Analysis

Next-generation sequencing of complete genomes was performed by using the Illumina MiSeq platform (<https://www.illumina.com>) and associated reagent kits according to the manufacturer's methodology. RNA was extracted by using the QIAamp Viral RNA Mini Kit (<https://www.qiagen.com>). Whole-genome amplification was performed by using a modified protocol (6). DNA libraries were prepared by using a Nextera DNA Flex Library Prep kit (Illumina) and sequenced by using MiSeq Reagent Kit v3 (600-cycle) (Illumina). Consensus sequences were generated by using Bowtie software (<https://www.bowtiepro.com>).

Nucleotide sequences were deposited in the GISAID database under accession nos. EPI_ISL_16020401–405. Multiple alignments were performed by using MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle>); editing, which included translating the nucleotide

sequences into amino acid sequences, was performed by using BioEdit (<https://bioedit.software.informer.com>) and UGENE (<http://www.ugene.net>) software. Initial maximum-likelihood phylogenies for each of the gene segments were generated with RAxML (7) by using the general time-reversible nucleotide substitution model. Final dendrograms were generated and visualized by using MEGA5 (8). Bootstrap support values were generated by using 1,000 rapid bootstrap replicates.

Intravenous Pathogenicity Index

For determining intravenous pathogenicity index (IVPI) values for 9 viruses, 0.1 mL of a 1:10 dilution of infectious allantoic fluid was intravenously inoculated into ten 6-week-old specific pathogen-free chickens. The IVPI was calculated according to the OIE standard protocol (https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf). Virus isolates with an IVPI value >1.2 were considered highly pathogenic avian influenza A viruses. The challenge study and all experiments with live viruses were conducted in a Biosafety Level 3 facility.

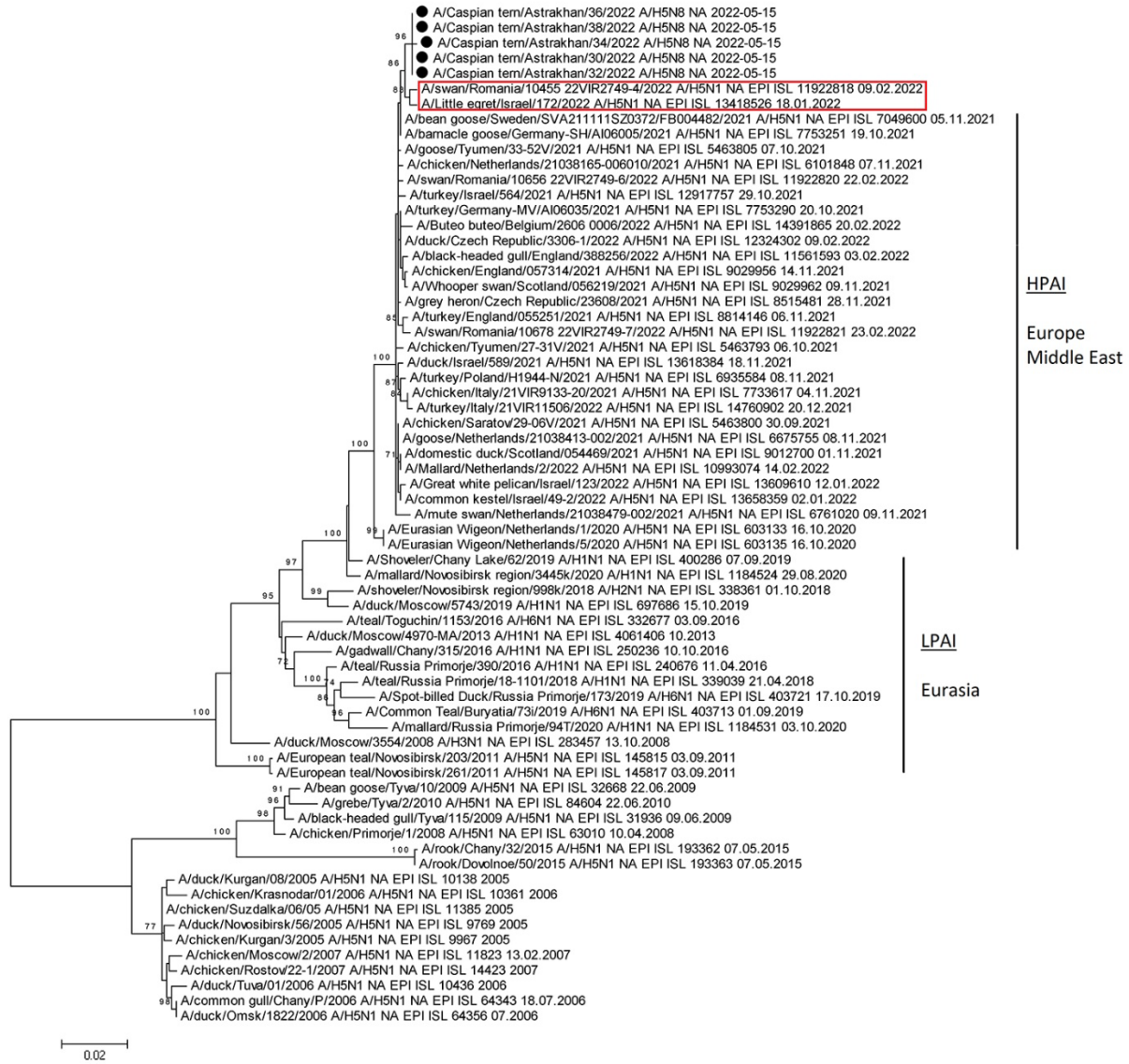
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Appendix 1 Figure 1. Dead waterbirds found on Maliy Zhemchuzhniy Island in the northern Caspian Sea, Russia, May 2022.

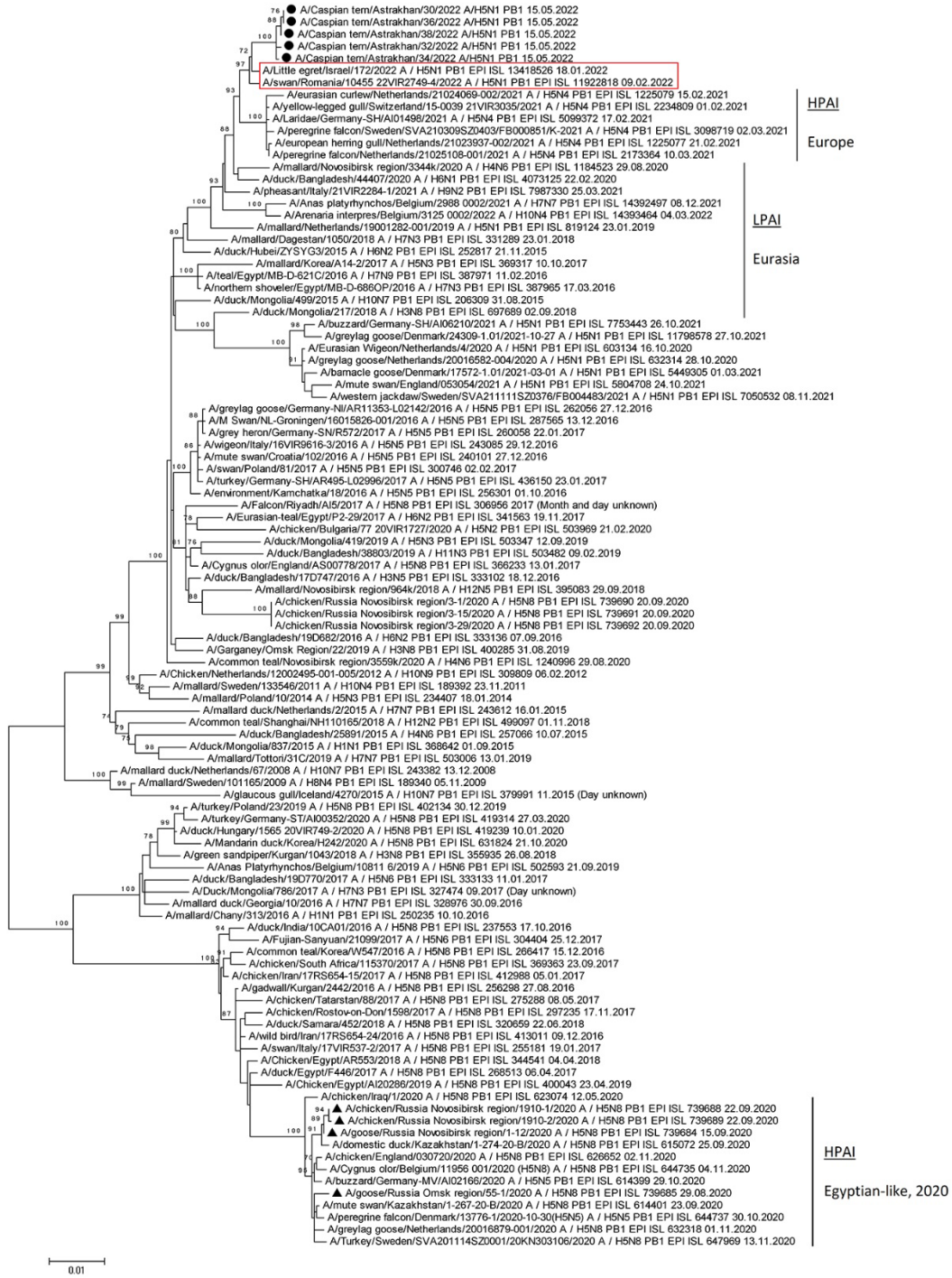


Appendix 1 Figure 3. Phylogenetic analysis of neuraminidase gene segments of avian influenza viruses. Trees were constructed by using the maximum-likelihood method. Closed circles indicate the highly pathogenic avian influenza H5N1 virus strains isolated from dead Caspian terns on Maliy Zhemchuzhnyi Island, Caspian Sea, Russia in May 2022. Red box indicates virus strains from Israel and Romania closely related to the Caspian Sea strains. Sequences were obtained from the GISAID EpiFlu database (<https://www.gisaid.org>). Scale bar indicates nucleotide substitutions per site. HPAI, high pathogenicity avian influenza; LPAI, low pathogenicity avian influenza.



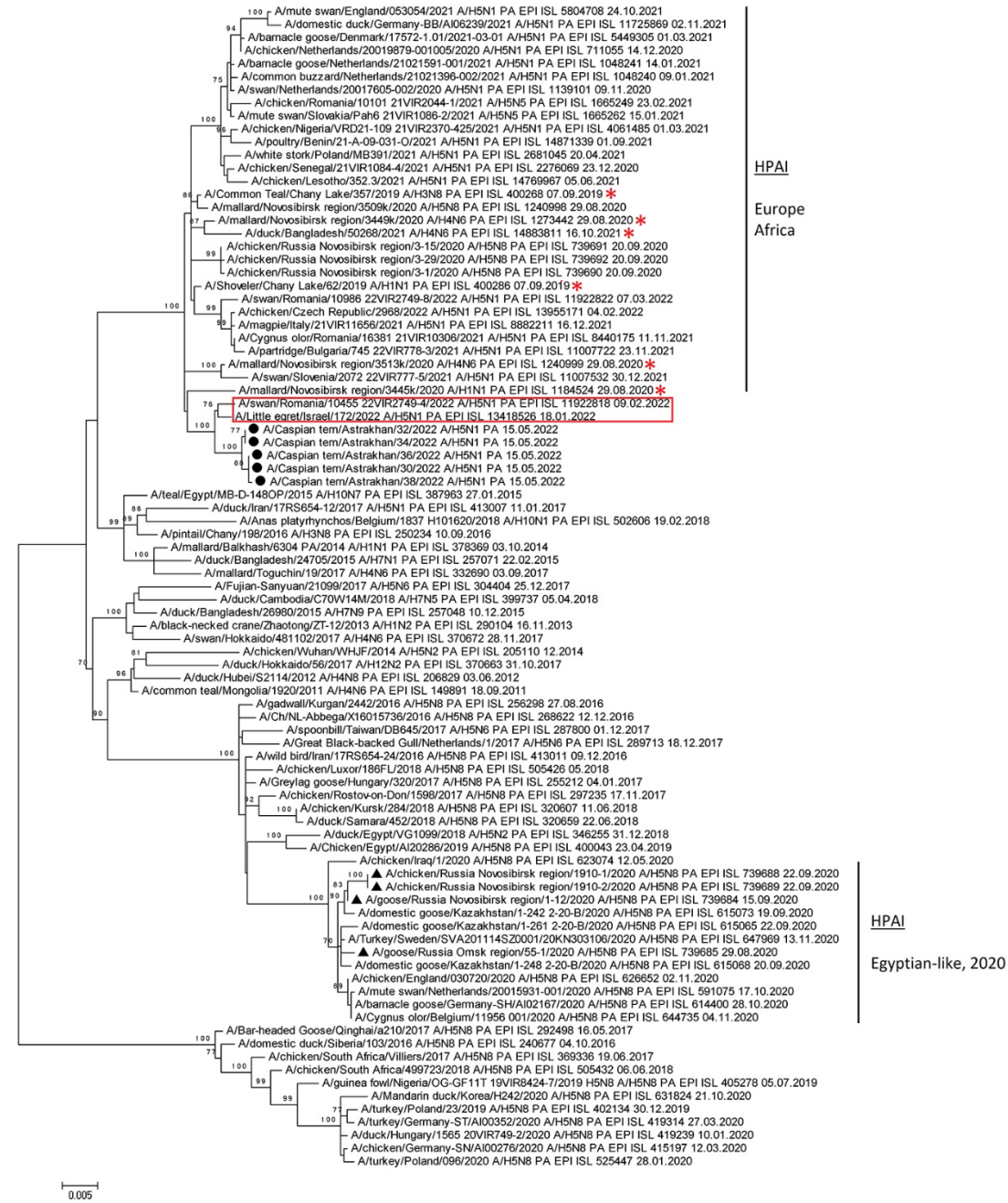
Appendix 1 Figure 4. Phylogenetic analysis of polymerase basic 2 protein gene segments of avian influenza viruses. Trees were constructed by using the maximum-likelihood method. Closed circles indicate the highly pathogenic avian influenza H5N1 virus strains isolated from dead Caspian terns on Maliy Zhemchuzhniy Island, Caspian Sea, Russia in May 2022. Red box indicates virus strains from

Romania closely related to the Caspian Sea strains. Red asterisks indicate low pathogenicity viruses found within highly pathogenic virus groups that were related to the Caspian Sea strains (presumably, highly pathogenic viruses appeared because of reassortment with low pathogenicity viruses). Closed triangles indicate Egyptian-like virus strains from Russia isolated in 2020. Sequences were obtained from the GISAID EpiFlu database (<https://www.gisaid.org>). Scale bar indicates nucleotide substitutions per site. HPAI, high pathogenicity avian influenza; LPAI, low pathogenicity avian influenza.



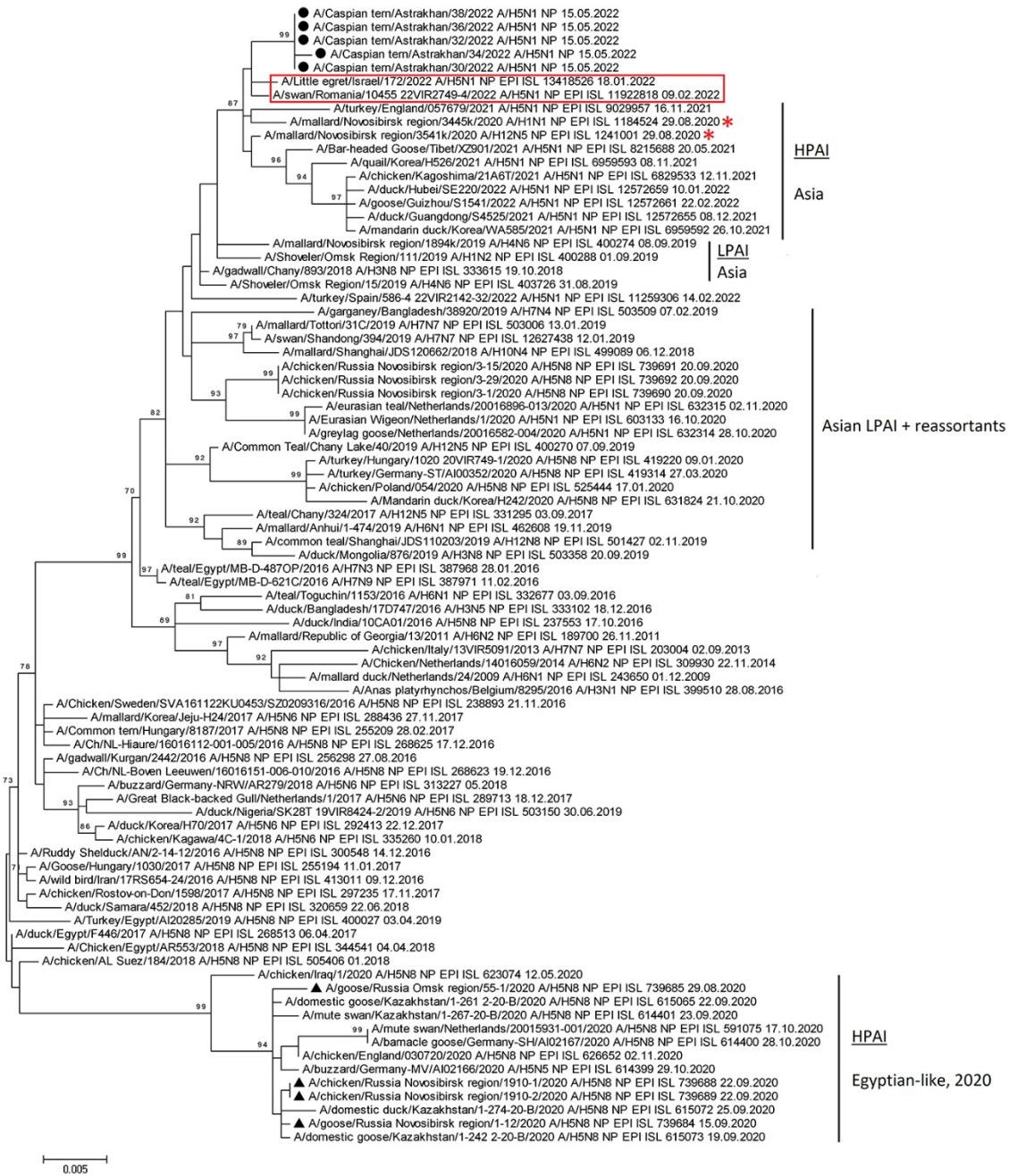
Appendix 1 Figure 5. Phylogenetic analysis of polymerase basic 1 protein gene segments of avian influenza viruses. Trees were constructed by using the maximum-likelihood method. Closed circles indicate the highly pathogenic avian influenza H5N1 virus strains isolated from dead Caspian terns on Malyy Zhemchuzhnyy Island, Caspian Sea, Russia in May 2022. Red box indicates virus strains from Israel

and Romania closely related to the Caspian Sea strains. Closed triangles indicate Egyptian-like virus strains from Russia isolated in 2020. Sequences were obtained from the GISAID EpiFlu database (<https://www.gisaid.org>). Scale bar indicates nucleotide substitutions per site. HPAI, high pathogenicity avian influenza; LPAI, low pathogenicity avian influenza.



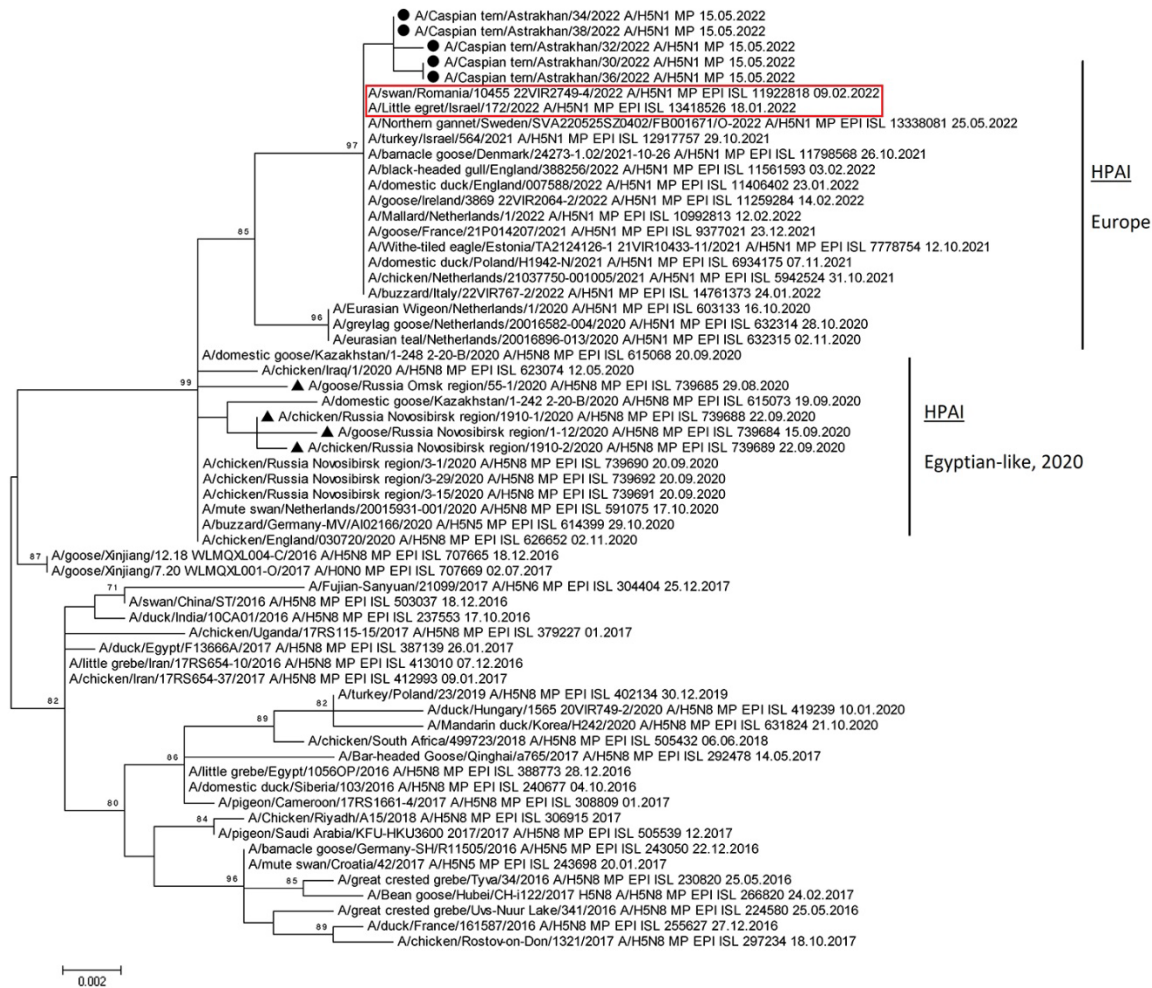
Appendix 1 Figure 6. Phylogenetic analysis of polymerase acidic protein gene segments of avian influenza viruses. Trees were constructed by using the maximum-likelihood method. Red asterisks

indicate low pathogenicity viruses found within highly pathogenic virus groups that were related to the Caspian Sea strains (presumably, highly pathogenic viruses appeared because of reassortment with low pathogenicity viruses). Red box indicates virus strains from Romania closely related to the Caspian Sea strains. Closed circles indicate the highly pathogenic avian influenza H5N1 virus strains isolated from dead Caspian terns on Maliy Zhemchuzhniy Island, Caspian Sea, Russia in May 2022. Closed triangles indicate Egyptian-like virus strains from Russia isolated in 2020. Sequences were obtained from the GISAID EpiFlu database (<https://www.gisaid.org>). Scale bar indicates nucleotide substitutions per site. HPAI, high pathogenicity avian influenza; LPAI, low pathogenicity avian influenza.

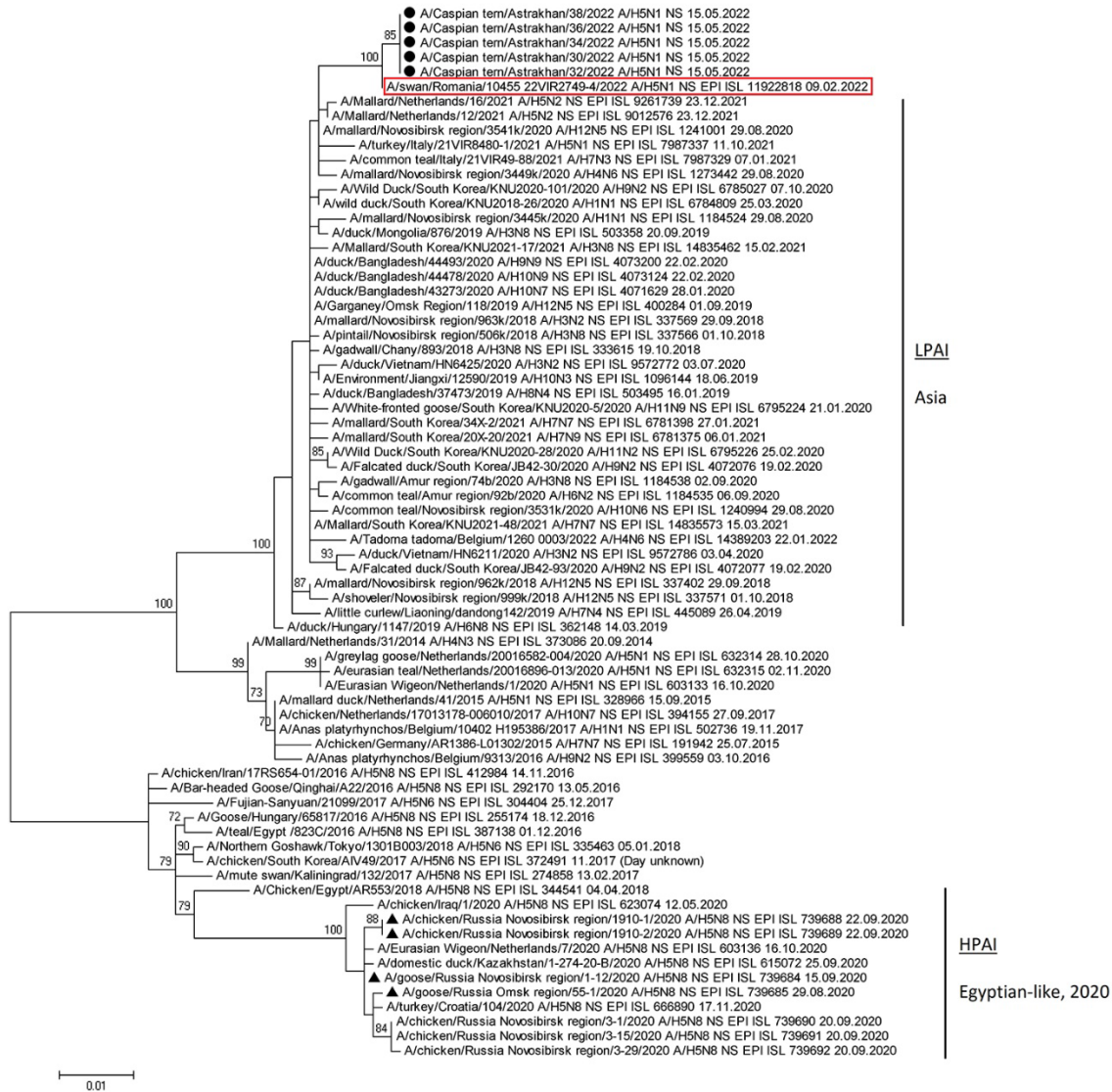


Appendix 1 Figure 7. Phylogenetic analysis of nucleoprotein gene segments of avian influenza viruses. Trees were constructed by using the maximum-likelihood method. Closed circles indicate the highly pathogenic avian influenza H5N1 virus strains isolated from dead Caspian terns on Maliy Zhemchuzhnyi Island, Caspian Sea, Russia in May 2022. Red box indicates virus strains from Israel and Romania closely related to the Caspian Sea strains. Red asterisks indicate low pathogenicity viruses found within highly pathogenic virus groups that were related to the Caspian Sea strains (presumably, highly pathogenic viruses appeared because of reassortment with low pathogenicity viruses). Closed triangles

indicate Egyptian-like virus strains from Russia isolated in 2020. Sequences were obtained from the GISAID EpiFlu database (<https://www.gisaid.org>). Scale bar indicates nucleotide substitutions per site. HPAI, high pathogenicity avian influenza; LPAI, low pathogenicity avian influenza.



Appendix 1 Figure 8. Phylogenetic analysis of matrix protein gene segments of avian influenza viruses. Trees were constructed by using the maximum-likelihood method. Closed circles indicate the highly pathogenic avian influenza H5N1 virus strains isolated from dead Caspian terns on Maliy Zhemchuzhnyi Island, Caspian Sea, Russia in May 2022. Red box indicates virus strains from Israel and Romania closely related to the Caspian Sea strains. Closed triangles indicate Egyptian-like virus strains from Russia isolated in 2020. Sequences were obtained from the GISAID EpiFlu database (<https://www.gisaid.org>). Scale bar indicates nucleotide substitutions per site. HPAI, high pathogenicity avian influenza; LPAI, low pathogenicity avian influenza.



Appendix 1 Figure 9. Phylogenetic analysis of nonstructural protein gene segments of avian influenza viruses. Trees were constructed by using the maximum-likelihood method. Closed circles indicate the highly pathogenic avian influenza H5N1 virus strains isolated from dead Caspian terns on Maliy Zhemchuzhniy Island, Caspian Sea, Russia in May 2022. Red box indicates virus strains from Israel and Romania closely related to the Caspian Sea strains. Closed triangles indicate Egyptian-like virus strains from Russia isolated in 2020. Sequences were obtained from the GISAID EpiFlu database (<https://www.gisaid.org>). Scale bar indicates nucleotide substitutions per site. HPAI, high pathogenicity avian influenza; LPAI, low pathogenicity avian influenza.



Appendix 1 Figure 10. Reconstructive analysis of flight routes according to data from the Russian Bird Ringing Center. Numbers represent different bird species: 1, *Anser anser*; 2, *Cygnus olor*; 3, *Anas platyrhynchos*; 4, *Anas crecca*; 5, *Anas strepera*; 6, *Anas penelope*; 7, *Anas acuta*; 8, *Anas querquedula*; 9, *Anas clypeata*; 10, *Netta rufina*; 11, *Aythya ferina*; 12, *Bucephala clangula*; 13, *Pandion haliaetus*; 14, *Arenaria interpres*; 15, *Himantopus himantopus*; 16, *Calidris ferruginea*; 17, *Calidris alba*; 18, *Larus ichthyaetus*; 19, *Larus minutus*; 20, *Larus fuscus*; 21, *Larus cachinnans*; 22, *Larus canus*; 23, *Larus genei*. Image and descriptions are from (4), licensed under CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0>).