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Circulation, Evolution and Transmission of H5N8 virus, 2016–2018

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SUMMARY

Objectives: A second wave of highly pathogenic avian influenza A virus (HPAIV) H5N8 clade 2.3.4.4 has spread globally, causing outbreaks among wild birds and domestic poultry since autumn 2016. The circulation and evolutionary dynamics of the virus remain largely unknown.

Methods: We performed surveillance for H5N8 in Qinghai Lake in China since the emergence of the virus (from 2016 to 2018). By analyzing recovered viruses in Qinghai Lake and all related viruses worldwide (449 strains), we identified the genotypes, estimated their genesis and reassortment, and evaluated their global distribution and transmission.

Results: Through surveillance of wild migratory birds around Qinghai Lake between 2016 and 2018, we revealed that the H5N8 was introduced into Qinghai Lake bird populations (QH-H5N8), with distinct gene constellations in 2016 and 2017. A global analysis of QH-H5N8-related viruses showed that avian influenza viruses with low pathogenicity in wild birds contributed to the high diversity of genotypes; the major reassortment events possibly occurred during the 2016 breeding season and the following winters.

Conclusions: Continued circulation of QH-H5N8-related viruses among wild birds has resulted in the global distribution of high genotypic diversity. Thus, these viruses pose an ongoing threat to wild and domestic bird populations and warrant continuous surveillance.

Introduction

The highly pathogenic H5N1 avian influenza virus (HPAIV) was first identified in China in 1996 [A/goose/Guangdong/1/1996 (Gs/GD)] and infected the human population in Hong Kong in 1997, with 18 recorded cases resulting in six deaths.[1,2][1,2] Since 2003, Gs/GD lineage H5N1 HPAIV infection in humans has been reported in 16 countries, with 860 laboratory-confirmed cases and 454 deaths as of 2018.[3] Although human-to-human transmission has not been documented, H5N1 HPAIV has the potential to cause an influenza pandemic. Outbreaks of H5N1 HPAIV and related viruses in poultry have been reported in Asia, Europe, Africa, and North America and have resulted in considerable economic losses. Based on divergence of the H5 hemagglutinin (HA) gene, the H5N1 virus has evolved into numerous phylogenetic lineages (e.g., Clades 2.2 and 2.3.2),[4] and has reassorted to generate different subtypes since 2008 (e.g., H5N2, H5N5, H5N6, and H5N8; belong to Clade 2.3.4.4).[5]

Importantly, H5 HPAIV has infected migratory birds and may have thus been disseminated to previously unaffected regions. The first global transmission of H5 HPAIV occurred in 2005, when clade 2.2 H5N1 caused an outbreak in Qinghai Lake, China and later spread to Europe and Africa.[6–8] Four more global transmission events of H5 HPAIV have since occurred, including clade 2.3.2 H5N1 in 2009,[9] Group A of clade 2.3.4.4 H5N8 in 2014,[10] clade 2.3.2.1c H5N1 in 2015,[11] and Group B clade 2.3.4.4 H5N8 in 2016.[12] Notably, there have been four outbreaks at Qinghai Lake (in 2005, 2009, 2015, and 2016), which was closely related to global transmission of HPAI influenza viruses since similar viruses were subsequently detected in other regions.[13–17] Qinghai Lake is the largest lake in the Qinghai–Tibetan Plateau and is situated at the intersection of the Central Asian and the East Asian flyways.[18] This critical

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breeding and stopover area for migratory birds supports > 200,000 migrants each year.[19]

The recent Group B H5N8 outbreak in Qinghai Lake was first documented in May 2016[12] and rapidly spread globally, causing fatalities in wild bird and poultry populations in many countries.[20–22] For instance, Group B H5N8 has affected 27 countries in Europe with fatalities in over 50 wild bird species between October 2016 and July 2017.[21] However, the circulation and evolutionary dynamics of the virus remain largely unknown. Here we present the findings from a 3-year surveillance of Qinghai Lake since the emergence of the virus (from 2016 to 2018). By analyzing all related viruses worldwide (449 strains) we identified the genotypes, estimated their genesis and reassortment, and evaluated their global distribution and transmission.

Methods

Sample collection

Feces from apparently healthy birds, cloacal swabs, and organs from carcasses were collected for virus isolation. Fresh and well-separated droppings were sampled and fecal swabs were obtained using sterile swabs. Each sample was placed in a vial containing 2 ml of viral-transport medium, stored at 2–8 °C, and shipped to the laboratory within 12 h for further analysis.[23] For water samples, 40 mL of surface water was collected in a sterilized 50-mL screw-cap plastic vial and stored at 2–8 °C; samples were shipped to laboratory within 2 h and immediately frozen at −80 °C until use. Water samples were collected from a spring in Egg Islet, where is the source of freshwater and bar-headed goose aggregate for breeding.

Virus isolation and sequencing

Before inoculation, swabs with viral-transport medium were thoroughly mixed. The tissues and organs were homogenized in 1 mL cold PBS under sterile conditions. The solid debris in the swab and tissue samples were then pelleted by centrifugation at $5000 \times g$ for 10 min, as previously described.[11] Water samples were treated as previously described.[24] Each water sample was centrifugation at $5000 \times g$ for 10 min and the supernatants were transferred into a sterilized polyethylene plastic tube, under aseptic conditions. Polyethylene glycol 6000, sodium chloride and bovine serum albumin (BSA) were added to final concentrations of 8%, 3% and 0.1%, respectively, mixed gently, set on ice for 8–12 h during which the tube was inverted every 2 h to mix the contents, and centrifuged at 4 °C, $10{,}000 \times g$ for 30 min. The supernatant was discarded, and the precipitate was re-suspended in 1 ml PBS, which contained 2×10^6 U/l penicillin, 2×10^6 U/l amphotericin B, 250 mg/l kitasamycin, 0.5×10^6 U/l nystatin, and 60 mg/l ofloxacin HCl. Then, 0.5 ml of the re-suspended mixture was inoculated within 1 h.

The treated samples were inoculated into the allantoic cavities of 10-day-old specific pathogen-free embryonated eggs (Beijing Merial Vital Laboratory Animal Technology Co., Beijing, China). After incubation at 37 °C for 48–72 h, the allantoic fluid of the inoculated eggs was collected and tested for the presence of HA-titer using 0.8% chicken red blood cells. The HA-titer positive samples were then confirmed for AIVs with reverse transcription–PCR as previously described.[23] The hosts of AIV-positive samples were identified by mitochondrial analysis as previously described.[25] All gene segments were amplified with Ex Taq DNA polymerase (Takara Bio, Beijing, China) with segment-specific primers.[26] PCR products were purified and sequenced with an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The data were edited and aligned with DNAMAN v.7.0 and BioEdit v.7.0.5.2. Whole-genome sequences of 59 strains of Qinghai Lake H5N8 influenza isolates (QH-H5N8) were determined and deposited in the Global Initiative on Sharing All Influenza Data (GISAID) (accession nos. EPI1143202–EPI1143209, EPI1144044–EPI1144051, EPI1144058–EPI1144071, EPI1144073–EPI1144079, EPI1144081–EPI1144111, EPI1144113–EPI1144190, EPI1144192–EPI1144234, EPI1144243–EPI1144246, EPI1144248–EPI1144258, EPI1144358–EPI1144453, EPI1145047–EPI1145053, EPI1145056–EPI1145069, EPI1145071–EPI1145073, EPI1145075–EPI1145092, EPI1145094–EPI1145129, EPI1145135–EPI1145150, EPI1145781–EPI1145812, EPI1145827–EPI1145834, EPI1145843–EPI1145861, EPI1145863–EPI1145872, EPI1149959–EPI1149961, EPI1149963, EPI1194735, EPI1208842, EPI1208844, and EPI1208985–EPI1208988).

Genetic analysis

All sequence data of influenza A virus available from GISAID and GenBank databases were obtained and combined to one database on March 26, 2018. The analysis datasets were generated by queried each nucleotide sequence of QH-H5N8 virus against the combined database using the blastn program with default parameters. Sequences of the top 1000 hits were collected, which included all the QH-H5N8-related virus and the closest virus. Identical sequences and those without a clear subtype or collection date were removed from each segment dataset. These composed the Dataset 1, and final sequence numbers of each segment were: PB2 540, PB1 589, PA 565, HA 744, NP 547, NA 542, MP 461, and NS 498. Next, 449 strains with complete genome sequences were chosen from the Dataset 1 for further nucleotide mutation and lineage analyses. These were designated the Dataset 2.

The alignment of each segment from Dataset 2 was carried out using Clustal Omega v.1.2.1 with 1000 iterations,[27] and codon alignment was applied. Maximum-likelihood (ML) phylogenetic trees were inferred with RAxML software v.8.2.6 under the GAMMAGTR model with 1000 bootstraps.[28] The ML trees were visualized by FigTree v1.4.3. For each segment, we clustered QH-H5N8-related viruses into different lineages according to the phylogenetic relationships in the phylogenetic tree, the virus characteristics, and the bootstrap value (>80). The genotypes of QH-H5N8 related viruses were nominated according to the combinations of lineages in segment trees. The annotation and visualization of phylogenetic tree for HA segment and related lineages was fulfilled by the R package ggtree.[29]

For the temporal evolution and dynamics analysis of QH-H5N8 related viruses, we first carried out a root-to-tip distance analysis for all viral segments in Dataset 1 by TempEst v1.5[30] to evaluate the feasibility of molecular clock calculation. Then, the BEAST v.2.4.8[31] was used for the calculation of tMRCAs of lineages for each segment, with the GTR+γ nucleotide substitution model and a relaxed clock; the program was performed twice for 500 million iterations with a 10% burn-in. Next, we performed BEAST analysis again on the same dataset, with same parameters to estimate relative genetic diversity by excepting Coalescent Bayesian Skyline. All phylogenetic trees were visualized in FigTree with ascending node order.

Results

Sample collection and isolation of H5N8 viruses at Qinghai lake from 2016 to 2018

Since 2015, we have carried out routine active surveillance of AIV in wild bird populations of Qinghai Lake. In response to the emergence of H5N8 HPAIV in the Qinghai Lake wild bird population in May 2016,[12] surveillance was increased from 2016 through 2017. Samples were collected in April, May, and July 2016; April–

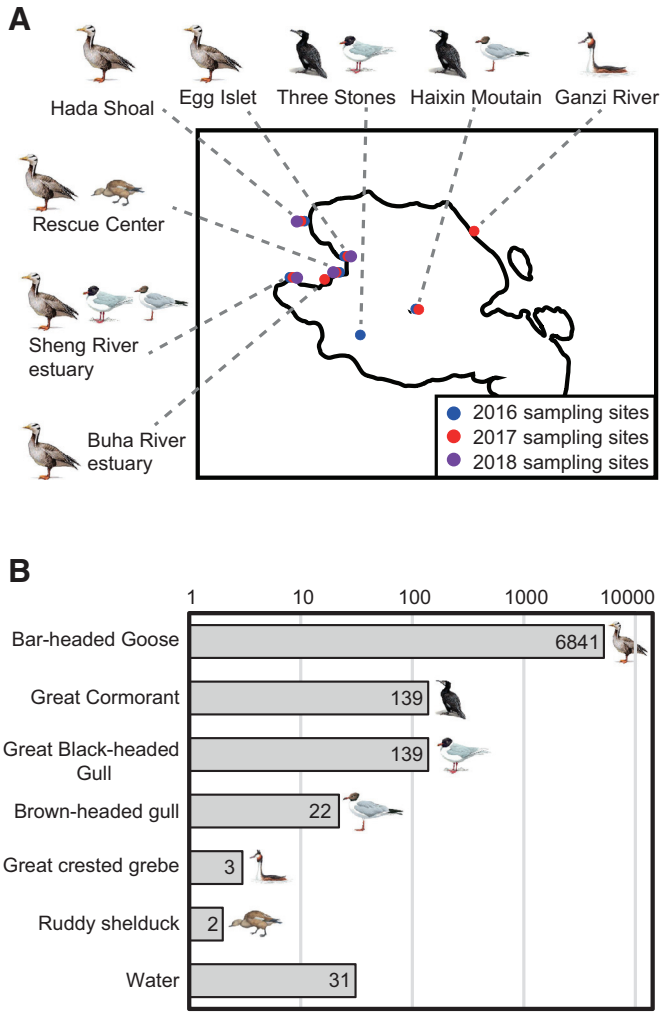

Fig. 1. Overview of sample collection in Qinghai Lake. (A) Wild migratory bird surveillance activities and sampling sites in Qinghai Lake during the breeding season (2016–2018). (B) Samples were collected from various hosts and the sample size of each species is indicated. Detailed sampling information are available as Supplementary Table S1.

June 2017; and May and June 2018, which is the breeding period at Qinghai Lake. Surveillance was carried out at four main sites—i.e., Egg Islet, Hada Shoal, Sheng River estuary, and Buha River estuary (Fig. 1(A) and Supplementary Table S1). A total of 7177 samples were collected, of which most were fresh droppings (7137/7177, 99.44%) and the remainder were organ ($n = 7$), swab ($n = 2$), and water ($n = 31$) samples. Most samples (6841/7177, 95.32%) were from bar-headed goose (Anser indicus); the others were from other species (Fig. 1(B) and Supplementary Table S1).

In 2016, we isolated 50 H5N8 viruses, with a total isolation rate of 2.98% (50/1679 samples; Supplementary Table S1). In April 2016 (before the outbreak), one H5N8 virus strain was recovered from bar-headed goose (1/20 samples). In May 2016 (during the outbreak), we isolated 44 H5N8 virus strains from fecal samples of bar-headed goose (44/1376 samples) and two from water samples (2/11 samples). In July 2016 (after the outbreak), three H5N8 viruses were recovered from great cormorant (3/272 samples). No outbreaks were recorded in 2017, and therefore, the rate of isolation decreased significantly from the previous year, although the sample size increased to 3876. Only nine H5N8 viruses were isolated for a total isolation rate of 0.23% (9/3876 samples; Supplementary Table S1). However, no H5N8 viruses were isolated from 1622 samples during the breeding season in 2018 (Supplementary Table S1). H5N8 viruses were recovered from water samples for 2 consecutive years (2016 and 2017).

Phylogenetics and genotypes of QH-H5N8-related viruses

To understand the genetic relationship of QH-H5N8 viruses with other viruses, we carried out a phylogenetic analysis of H5N8 relevant sequences from public databases. In the HA phylogenetic tree, 58 of all 59 QH-H5N8 strains belonging to the group B H5N8 lineage along with second-wave H5N8 viruses from around the world (Fig. 2 and Supplementary Fig. S1). Only one strain A/Bar-headed goose/Qinghai/p23/2016 (H5N8) clustered with domestic poultry H5N6 viruses that circulated in China during 2015–2016. Most of the 2016 QH-H5N8 isolates (49 of 50 strains) formed a distinct lineage, indicating that the 2016 outbreak was caused by a single virus strain. Notably, one 2016 strain A/Bar-headed goose/Qinghai/XXI122/2016 (H5N8) falling into another monophyletic branch and closely related to Korean strains found in wild birds and chicken. All the 2017 QH-H5N8 isolates clustered together and formed a monophyletic branch that differed from 2016 QH-H5N8 strains. As for the NA tree, QH-H5N8 strains formed a similar topology as in the HA tree (Supplementary Fig. S2F). These results suggested that at least two variants, from an unknown common ancestral H5 virus, were introduced into Qinghai Lake in early-summer 2016. In 2017, a different ancestral strain was introduced into birds.

We performed phylogenetic analyses of internal genes and classified them into different lineages according to nucleotide similarity ($< 98\%$), tree topology and bootstrap values ($> 80\%$)[32] (Supplementary Fig. S2). According to the sublineages of internal genes (originated from wild bird gene pool LPAI-1, LPAI-2 et al. or domestic poultry LPAI-p), all QH-H5N8-related viruses were assigned as 18 distinct genotypes (Supplementary Table S2). The majority of 2016 QH-H5N8 strains were designated as Genotype I. tMRCA of Genotype I was estimated to January 2016 [95% highest posterior density (HPD), January 2015–March 2016] (Fig. 3(C)). Strain A/Bar-headed goose/Qinghai/p23/2016 (H5N8), which had all genes except NA and was closely related to H5N6 viruses that were assigned as Genotype II. The 2017 QH-H5N8 strains were designated as Genotype IV. tMRCA of Genotype IV was estimated to January 2016 [95% highest posterior density (HPD), January 2015–March 2016] (Fig. 3(C)) Phylogenetic analysis of internal genes also indicates multiple and separate introductions of the H5N8 viruses into Qinghai Lake, during 2016–2017. The H5N8 virus isolated in April, either in 2016 or 2017, was closely related with subsequently circulated strains in individual year, indicating the H5N8 virus was introduced into Qinghai Lake as early as April. The tMRCA of Genotype I and IV indicate both two genotype viruses emerged before they were introduction into Qinghai Lake. It should be addressed that the viruses recovered from water samples clustered with the H5N8 viruses circulated in the same year, further provide evidence that the water may play role for disseminating virus among wild birds.

Frequent reassortments underlie the diverse genotypes of QH-H5N8-related viruses

Based on phylogenetic analysis and genotyping, we constructed the schematic map of H5N8 reassortments. The earliest QH-H5N8 isolate was the result of reassortment of poultry H5N8 viruses circulated among domestic waterfowl in eastern China in 2013 and 2014, with wild bird low pathogenicity (LP)AIVs causing the outbreak in Qinghai Lake.[12] The gene constellation of these viruses was Genotype I (Fig. 3(A)). A series of reassortments then occurred within wild bird populations through replacement of distinct LPAIV genes since autumn 2016. The first period was from August to

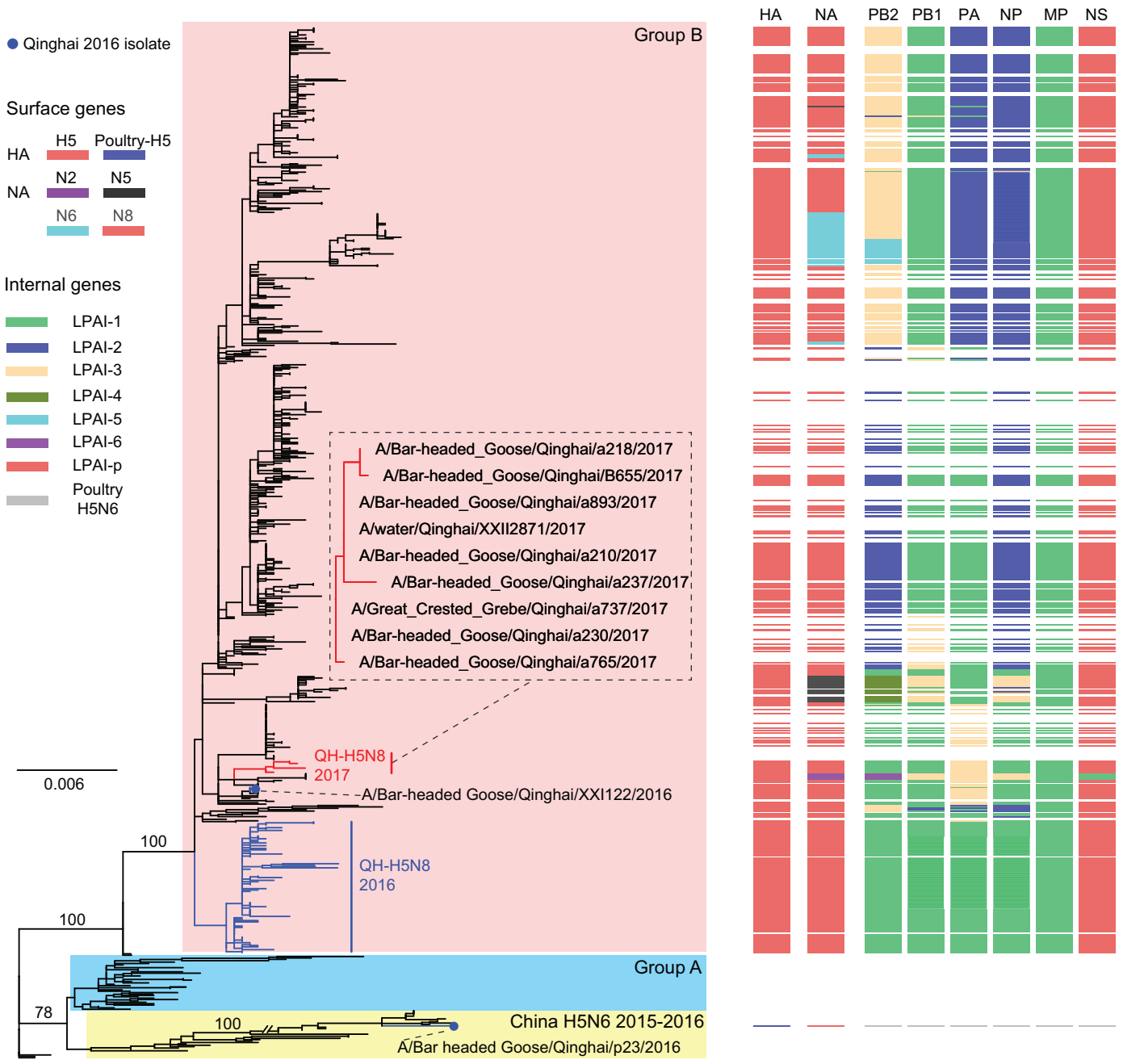

Fig. 2. Phylogenetic analysis of QH-H5N8-related viruses. The part of the tree is representing maximum likelihood phylogeny of HA gene from QH-H5N8-related viruses. In the tree, QH-H5N8 viruses are marked by different colors (2016, blue branches and solid circles; 2017, red branches). For QH-H5N8-related viruses with complete genomes, the clade origins of each gene segment are indicated by different colored bars. Scale bars represent substitutions per site. Detailed phylogenetic trees are available as Supplementary Figs. S1 and S2.

October 2016, when five genotypes of H5 virus including Genotypes III ($n = 143$), IV ($n = 36$), V (H5N5, $n = 14$), VI ($n = 1$), and VII ($n = 81$)—all of which were derived from Genotype I—were identified (Fig. 3(A) and (B) and Supplementary Table S2). In late 2016 and early 2017, eight more novel genotypes emerged following further reassortment with LPAIV gene pools in wild birds; these were Genotypes VIII (H5N5, $n = 1$), IX ($n = 17$), X ($n = 1$), XI ($n = 1$), XII (H5N5, $n = 2$), XIII (H5N6, $n = 25$), XIV ($n = 1$), and XV ($n = 1$) (Fig. 3(A) and (B) and Supplementary Table S2). Four genotypes (VIII, XI, XIII, and XIV) were derived from Genotype III. Genotypes XII, XV, IX, and X were derived from genotypes V, VI, VII, and I, respectively (Fig. 3(A)). In autumn 2017 and early 2018, a third group of reassortants emerged that were classified as Genotype XVI ($n = 1$), XVII (H5N6, $n = 17$), and XVIII (H5N2, $n = 5$) and were derived from Genotypes IV and XIII (Fig. 3(A)). In general, of all the analyzed QH-H5N8-related viruses, Genotypes I, III, and VII accounted for 68.2% of recorded strains (306/449; Fig. 3(B) and Supplementary Table S2).

To identify the timeline of reassortment of the QH-H5N8-related viruses, we estimated tMRCA using BEAST.[31] tMRCA of Genotype I was estimated to January 2016 [95% highest posterior density (HPD), January 2015–March 2016] when reassortment event likely occurred before this time (Figs. 3(C) and Supplementary Figs. S3 and S4). tMRCAs of Genotypes III–VII were all traced to the period from June to August, 2016 (95% HPD, December 2015–October 2016), while those of Genotypes VIII–XV were

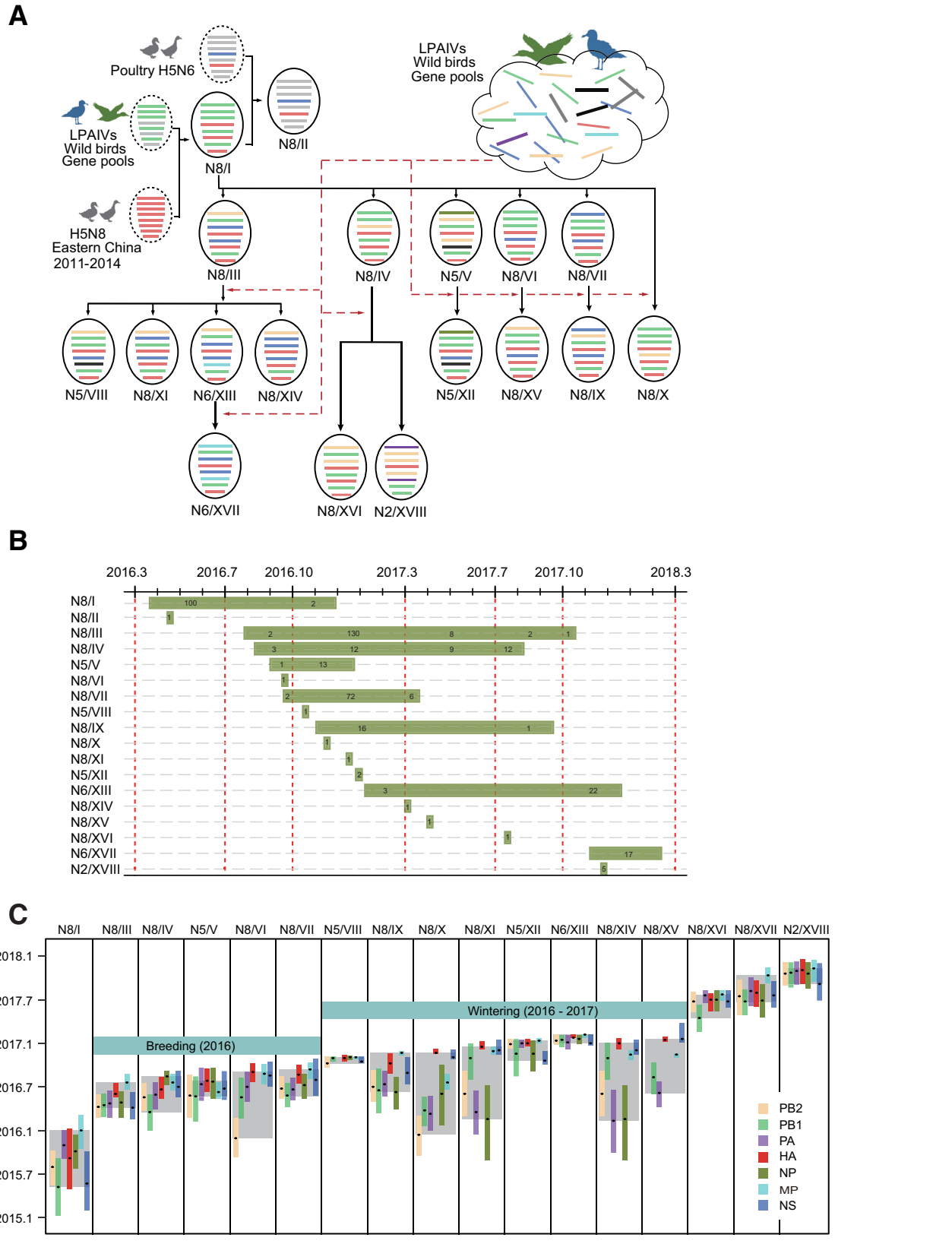

Fig. 3. Genesis and circulation of QH-H5N8-related viruses. (A) Hypothetical reassortment pathway of QH-H5N8-related viruses. Virus particles are shown as colored ovals containing horizontal bars that represent the eight gene segments (from top to bottom: PB2, PB1, PA, HA, NP, NA, M, and NS). Gene segments are colored according to their origin. Dashed virions indicate unidentified viruses. (B) Timeline of emergence of different genotypes of QH-H5N8-related viruses. Gray bars represent duration of circulation of QH-H5N8-related viruses. Based on the birds' migration cycle, the duration of circulation of QH-H5N8-related viruses (2016–2018) were divided into six short period with red dash lines: March to July 2016, August to October 2016, November 2016 to February 2017, March to July 2017, August to October 2017 and November 2017 to March 2018. The numbers of each genotype series in different period were indicated. (C) Molecular dating of QH-H5N8-related viruses. Events contributing to the establishment of QH-H5N8-related viruses estimated using multiple influenza segments. tMRCA (indicated by a black point) is size-scaled by the posterior probability (0–1.0), and the 95% highest posterior density is color-coded by segment. Gray shading represents tMRCA of multiple segments. Detailed phylogenetic trees are available as Supplementary Figs. S2–4.

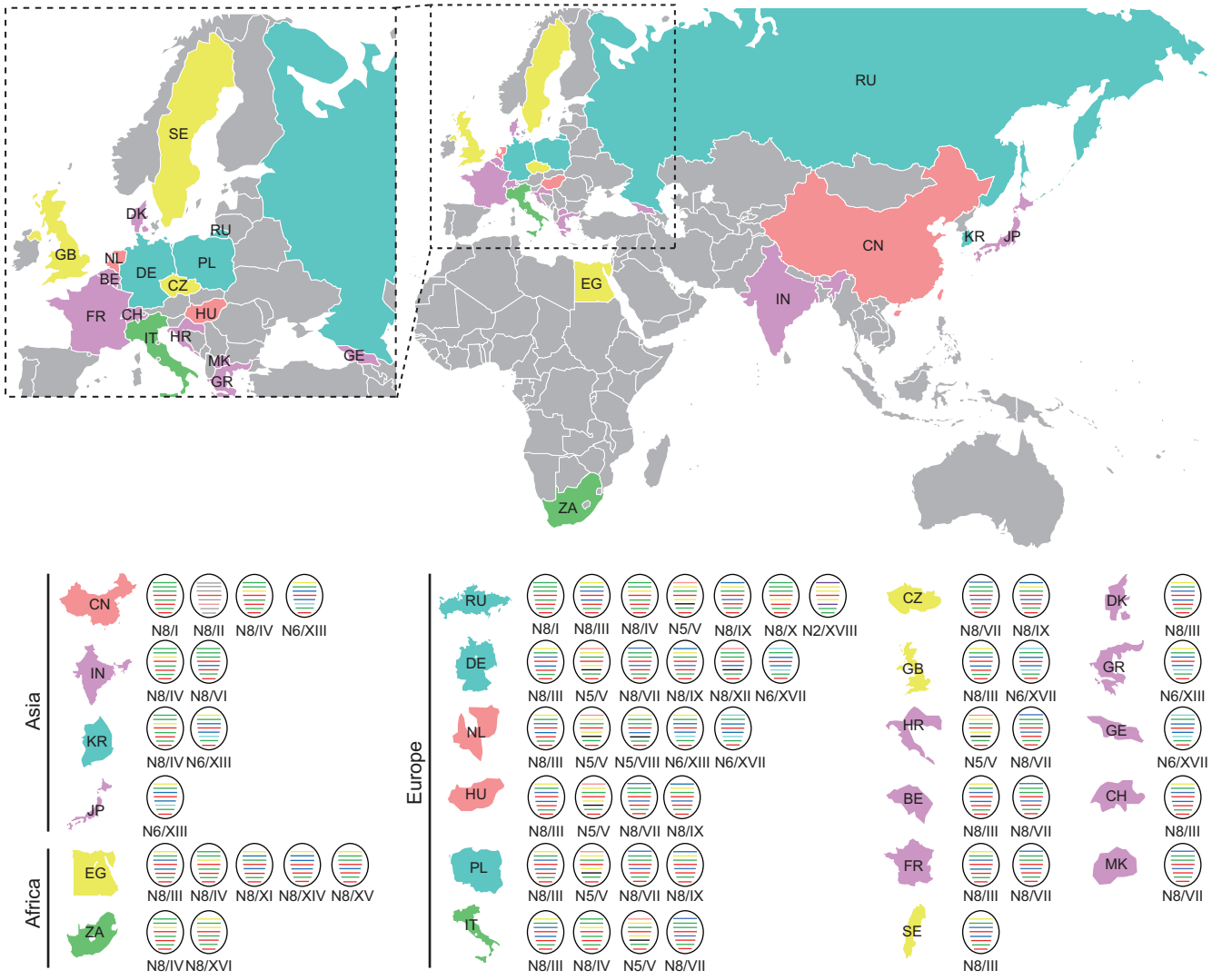

Fig. 4. Geographical distribution of QH-H5N8-related viruses. The countries in the map are filled in different colors depending on the number of QH-H5N8-related virus reported: red, > 50; blue, > 20; green, > 10; yellow, > 5; purple, > 1. Country names are abbreviated as follows: BE, Belgium; CH, Switzerland; CN, China; CZ, Czech Republic; DE, Germany; DK, Denmark; GB, England; GE, Georgia; FR, France; GR, Greece; HR, Croatia; HU, Hungary; IN, India; IT, Italy; EG, Egypt; KR, Korea; JP, Japan; MK, Republic of Macedonia; NL, The Netherlands; PL, Poland; RU, Russia; SE, Sweden; ZA, South Africa. See also Supplementary Fig. S5.

dated to the period from October 2016 to January 2017 (95% HPD, September 2015–March 2017) (Fig. 3(C) and Supplementary Fig. S3 and S4). Although the reassortment event may earlier than the estimated tMRCA, the molecular dating results further suggest sequential reassortments that produced multiple genotypes of second-wave H5N8 and descendent viruses.

Geographic distribution and spread of QH-H5N8-related viruses

We mapped the geographic distribution of all identified QH-H5N8-related viruses and found that they had dispersed to Asia, Europe, and Africa, affecting 23 countries (four in Asia, seventeen in Europe, and two in Africa) (Fig. 4, and Supplementary Fig. S5 and Table S2). Isolates in European countries had the highest diversity, with 11 distinct genotypes identified. Moreover, at least four genotypes were detected in six countries including Russia ($n = 7$), Germany ($n = 6$), Netherlands ($n = 5$), Poland ($n = 4$), Hungary ($n = 4$), and Italy ($n = 4$). In Africa, five genotypes (III, IV, XI, XIV, and XV) were present in Egypt and two (IV and XVI) circulated in South Africa. Five virus genotypes circulated in Asia with four in China (I, II, IV, and XIII), two in India (IV and VI), two in Korea (IV and XIII), and one in Japan (XIII) (Fig. 4, and Supplementary Fig. S5 and Table S2). It should be noted that only Genotype IV viruses have been identified in all three continents, and this genotype has been re-introduced into Qinghai Lake in 2017.

We investigated the global transmissions paths of QH-H5N8-related viruses and found that multiple migration flyways may have been involved in the spread of these viruses. During Spring 2016, Genotype I H5N8 viruses that may have originated in the wintering areas around the Bay of Bengal[33] possibly spread north along the Central Asian Flyway (Fig. 5(A)), leading to their introduction to Qinghai Lake and an outbreak in 2016. Continuous bird migration resulted in the spread of the virus to Uvs–Nuur Lake (Russia/Mongolia) and Siberia, where birds breed. Within the

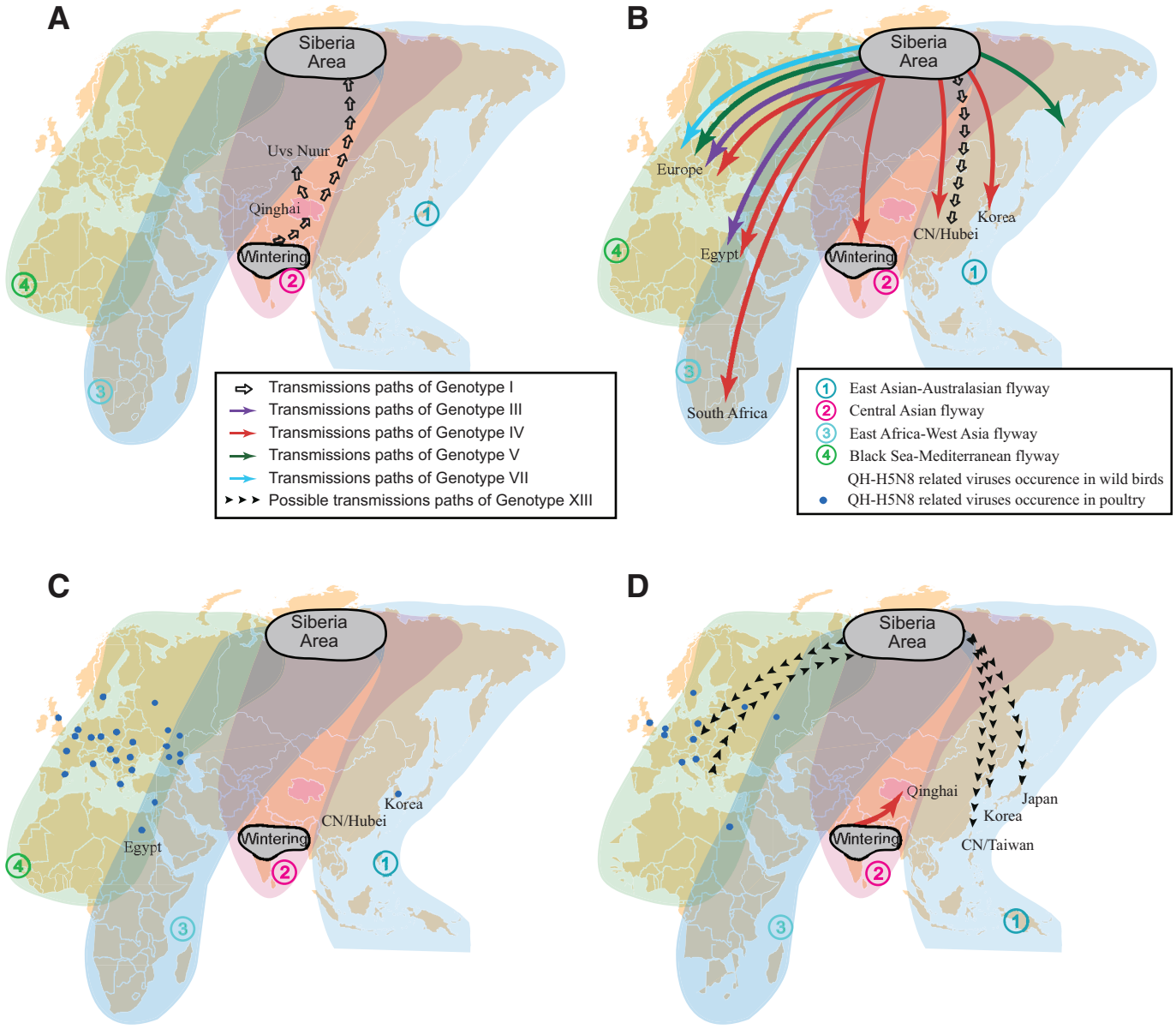

Fig. 5. Map of global transmission of QH-H5N8-related viruses. Migratory flyways of wild bird populations are adapted from related literature[10,40], which numbered and shaded in different colors. Based on phylogeny of eight segments, virus isolation information and wild bird flyway, the dissemination of QH-H5N8-related viruses was divided into four periods: (A) 2016 Spring; (B) 2016 Autumn; (C) 2016–2017 Wintering season; and (D) after 2017 Spring. Dissemination of different genotypes of QH-H5N8-related viruses are indicated in different arrows. The red and blue dots illustrate the QH-H5N8-related occurrence in wild and domestic birds.

breeding area of Siberia where multiple migratory flyways converge, Genotype I H5N8 virus reassorted with LPAIVs, producing novel genotypes (Fig. 5(B)). During fall migration, Genotypes I, III, IV, V, and VII emerged and were disseminated along multiple flyways into Europe, Africa, and South and East Asia, and circulated locally in Europe, Black Sea–Mediterranean area, South Africa, and the Bay of Bengal, even spreading to domestic poultry populations (Fig. 5(C)). Six novel genotypes (VIII–XV) emerged during this period in late 2016 and early 2017. In Spring 2017 when wild birds entered another migration cycle, H5N8 viruses were re-introduced into Qinghai Lake possibly through the Central Asian Flyway, and Genotype I replaced by Genotype IV (Fig. 5(D)).

Evolutionary dynamics of QH-H5N8-related viruses

To estimate the population dynamics of QH-H5N8 and related viruses, we generated skyline plots (Fig. 6(A)), which revealed that the genetic diversity increased sharply during March and April 2016 before an outbreak in Qinghai Lake in 2016. This increase was followed by a plateau, implying that the virus experienced a steady period. A second increase was observed in late 2016 and early 2017, when outbreaks occurred in wild and domestic bird populations in Europe. After the spring of 2017, the genetic diversity declined steadily until 2018. The substitution rates of the eight segments of QH-H5N8 and related viruses were estimated (Fig. 6(B)); overall, nucleotide substitution rates ranged from 5.55×10^{-3} (NP) to 8.37×10^{-3} (MP), which were within the expected range for RNA viruses.[34, 35] HA, NA, MP, and NS showed higher substitution rates than the remaining genes, which consisted of the polymerase and NP genes (Fig. 6(B)).

Discussion

Our findings demonstrate that the H5N8 virus was introduced into Qinghai Lake as early as April 2016 (Fig. 2, Supplementary Tables S1 and S2 and Figs. S1 and S2). Thereafter, the virus

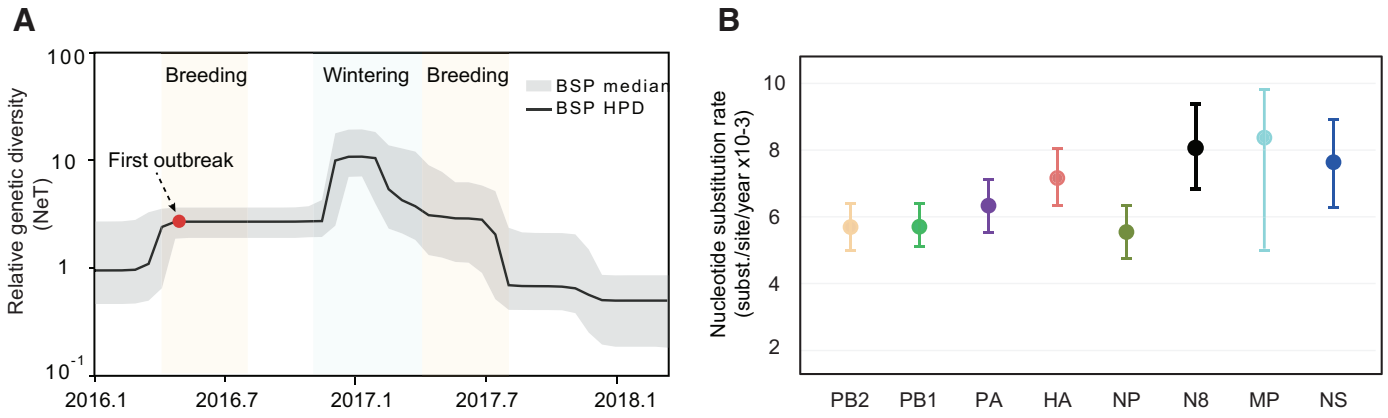

Fig. 6. Evolutionary dynamics of QH-H5N8-related viruses. (A) Bayesian skyline plot (BSP) of the HA gene showing changes in relative genetic diversity of QH-H5N8-related viruses. The thick solid black line is the median estimate, and the grey area is the 95% HPD of the genetic diversity estimates. The horizontal shaded blocks represent breeding and wintering seasons of migratory birds. The red dot denotes the first outbreak event of those viruses. (B) Evolutionary rates (substitutions per site per year $\times 10^{-3}$) and 95% HPD values of the eight gene segments of QH-H5N8-related viruses.

continued to circulate in wild bird populations, causing fatalities in May 2016[12]; this was accompanied by a high rate of infection among wild bird populations during the 2016 breeding season. In 2017, the viruses were re-introduced into Qinghai Lake, with a gene constellation differing from that of the 2016 strains (Figs. 2 and 3(A)). The virus had a low circulation among wild bird populations and did not cause an outbreak in wild birds in Qinghai Lake in 2017.

The establishment of QH-H5N8—likely Genotype I—was presumed to have occurred at the end of the 2015–2016 wintering season when birds in South Asia began to migrate north towards Qinghai Lake. Notably, viral genes of Genotype I included those from domestic poultry H5N8 viruses (HA, NA, and NS) that were circulating in East China[12] as well as several gene segments that were likely derived from viruses present in Bangladesh.[33] The virus containing gene segments originated from different hosts and large distance strongly suggested wild birds and their migration may contribute the genesis of H5N8. Until now, the place and the species that involved gene flow between two areas remains unknown. Although previous satellite tracking data from multiple Anatidae species indicate Central Asian and East Asian-Australasian Flyways were spatially distinct,[36,37] it is possible that unknown species of wild birds migrate directly between two flyways, or that waterfowls at unknown wetlands link these two flyways through shared stopovers.

Although no H5N8 outbreak was reported in South Asia prior to that in Qinghai Lake, QH-H5N8 was possibly established in domestic poultry or wild bird populations, before their flight to Qinghai Lake from the South Asian wintering sites. Satellite tracking data in Central Asian Flyway have revealed a direct spatio-temporal link between areas identified at highest HPAI H5N1 risk in India and Bangladesh, and the wild bird outbreaks in Qinghai Lake, Mongolia, and Russia.[38,39] Abundant species of wild birds that wintering in India and Bangladesh and breeding in Qinghai Lake may involve the genesis and spread of QH-H5N8, such as ruddy shelduck (*Tadorna ferruginea*) and great black-headed gull (*Larus ichthyaetus*). Data show that birds marked with satellite transmitters in Qinghai Lake were found wintering in the eastern Indian subcontinent to the Bay of Bengal in areas where domestic and wild bird flocks intermix.[40,41] On the wintering grounds, birds were found feeding in sites where they could intermix with domestic bird flocks. Two distinct H5 HPAIV lineages—Clade 2.2 in 2005[42,43] and Clade 2.3.2 in 2009[9,44,45]—reportedly caused outbreaks first in Qinghai Lake and later in Europe, similar to QH-H5N8. Although the genesis of these two clades remains unclear because of the lack of surveillance data, it is likely that these viruses were established in a manner similar to QH-H5N8.

After Group B H5N8 viruses were established among wild birds in Qinghai Lake, they reassorted with diversified genes from Eurasian LPAIVs during dissemination. This resulted in the emergence of at least 18 genotypes worldwide, including the novel H5N2, H5N5, and H5N6 viruses[44–48] (Fig. 3(A)). Our findings indicated that sequential reassortments underlie genotypic diversity. A similar pattern was reported after Group A H5N8 viruses spread into North America in 2014 during autumn bird migration and reassorted with local LPAIVs.[49–52] In this study, 10/18 genotypes were transient and were detected for less than 1 month (Supplementary Table S2), suggesting that the majority of novel reassortants are not well adapted to wild birds. Four genotypes circulated for more than 10 months (Supplementary Table S2): Genotype III spread to Europe and Africa, Genotype IV was transmitted back to Qinghai Lake, Genotype IX remained prevalent in Europe, and Genotype XIII (H5N6) was disseminated to the East Asia–Australasia flyway (Figs. 4 and 5). This implies that some reassortants can adapt to diverse species of wild birds and can thus be transported to different areas, although additional surveillance data are needed to confirm this supposition. Most novel genotypes were estimated to emerge during the 2016 breeding season and 2016–2017 wintering season (Fig. 3) when large numbers and species of wild birds aggregated, allowing viral cross-species transmission and adaptation. It is worth noting that 15/18 genotypes—including three H5N5, two H5N6, and one H5N2 reassortant—were detected in domestic ducks and chickens in Europe, Africa, and Asia (Fig. 4 and Supplementary Table S2). Since 2005, the Clade 2.2 QH-H5N1-related viruses have become endemic in Egypt[7] and have caused 359 human infections with 120 deaths[51,53] after being introduced to domestic bird population possibly through bird migration.[54,55] Whether QH-H5N8-related viruses can become enzootic and pose a continuous threat to human health is of particular concern, although no infections in humans have been reported to date. It is therefore essential to continue surveillance of QH-H5N8-related viruses in poultry in the coming years.

We did not detect any H5-positive samples in Qinghai Lake in 2018, and only sporadic fatal cases in wild birds have been recorded in Europe up to spring 2018.[20] Given that HPAIV Clades 2.2, 2.3.2, and 2.3.2.1c QH-H5N1 have not been reported in migratory birds after circulating for several years following the first outbreak (Clade 2.2, 2005–2008; Clade 2.3.2, 2009–2011; and Clade 2.3.2.1c, 2014–2015; according to viral sequences from public databases), QH-H5N8-related viruses will likely not be detected

from wild bird populations within a couple of years. However, H5 HPAIV has persistently circulated among poultry and has diversified for over two decades. The frequent introduction of poultry-origin H5 HPAIV genes into wild birds is one of the main causes of H5 outbreaks in migratory bird populations. Therefore, the de novo introduction of viruses cannot be ruled out in the future if H5 HPAIV in poultry are not well controlled.

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Conflict of interest

None declared.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.07.005.

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