Virology 530 (2019) 59–64

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Wild birds do not harbor higher diversity of influenza virus internal genes than poultry

Tian Zhang, Huayao Zhao, Jianhua Wang, Guan-Zhu Han*

Jiangsu Key Laboratory for Microbes and Functional Genomics, College of Life Sciences, Nanjing Normal University, Nanjing, Jiangsu 210023, China

ARTICLE INFO

Keywords:
Avian influenza virus
Genetic diversity
Internal genes
Phylogenetic analysis

ABSTRACT

Avian influenza A virus (AIV) has threatened global economy and public health. Wild birds have long been thought to serve as the natural reservoir of influenza virus, and thus it is expected that wild birds harbor higher viral diversity than poultry. Yet, this hypothesis has not been formally tested. Here, we assemble a data set of AIV from 75 regions worldwide over 11 years and compare the genetic diversity of wild bird AIV with that of poultry AIV. We find the genetic diversity of the internal genes of AIV in wild birds is not significantly higher than that in poultry. We propose that the unexpected diversity pattern of AIV internal genes could be explained by the synchronized global sweep of AIV internal genes occurring in the late 1800s and frequent AIV transmission between wild birds and poultry. Our findings might have important implications in understanding the evolution of influenza virus.

1. Introduction

Avian influenza A virus (AIV) poses a great threat to public health and poultry production over the world. Highly pathogenic AIV outbreaks occurred frequently in poultry, and occasionally in wild birds (Chen et al., 2005; Liu et al., 2005; Olsen et al., 2006; Verhagen et al., 2015; Global Consortium for H5N8 and Related Influenza Viruses, 2016). AIV can also cause human infections through direct or indirect contact; of particular note are H5N1 (since 1997) and H7N9 (since 2013) viruses, each of which has been associated with hundreds of fatalities (Zhu et al., 2016; Wang et al., 2017; Lai et al., 2016; Su et al., 2015; Zhou et al., 2017). Moreover, reassortment involving avian and mammalian influenza A viruses led to the three influenza pandemics in the 20th century, that is, 1918 H1N1 Spanish influenza, 1957 H2N2 Asian influenza, 1968 H3N2 Hong Kong influenza (Belshe, 2005; Worobey et al., 2014a, 2014b).

AIV has been isolated from more than 100 wild bird species (Olsen et al., 2006), and infected avian hosts are usually asymptomatic (Olsen et al., 2006; Webster et al., 1992). Wild birds, especially wildfowl and shorebirds, have long been thought to serve as the current reservoir of influenza A virus in nature, because they are the main source of influenza viruses in other species (Olsen et al., 2006; Webster et al., 1992; Horimoto and Kawaoka, 2001). Wild birds harbor influenza viruses of HA (H1-H16) and NA (N1-N9) subtypes, only some of which have been detected in poultry (Joseph et al., 2017). It is expected that wild birds should harbor higher influenza virus diversity than poultry. However, this hypothesis has not been formally tested yet.

In this study, we assembled a data set containing AIV sequences from 75 regions worldwide over 11 years and compared the genetic diversity of wild bird AIV with that of poultry AIV. We found there is no significant difference in genetic diversity between wild bird and poultry AIV internal genes.

2. Results

We performed a systemic analysis of the genetic diversity of influenza virus in wild birds and poultry. First, we estimated the nucleotide diversity (π) for each of the six segments (PA, PB1, PB2, NP, MP, and NS) encoding internal genes. To avoid sampling bias, we randomly sampled 10 and 15 sequences for each region (75 regions across the world; Fig. 1 and S1) and each year (from 2005 to 2015). We assembled a data set consisting of 1334 points with 10 viral sequences and 983 points with 15 viral sequences (521 and 813 points for poultry and wild birds in the 10-sequence data set, respectively; 355 and 628 points for poultry and wild birds in the 15-sequence data set, respectively). These data points are mainly from East and Southeast Asia, North America, and Europe (Fig. 1).

Next, we compared the genetic diversity of six AIV internal gene segments between poultry and wild birds. Phylogenetic analyses suggest two major distinct lineages exist in each AIV internal gene

* Corresponding author.
E-mail address: guanzhu@email.arizona.edu (G.-Z. Han).

https://doi.org/10.1016/j.virol.2019.02.003
Received 25 January 2019; Accepted 5 February 2019
Available online 07 February 2019
0042-6822/ © 2019 Elsevier Inc. All rights reserved.

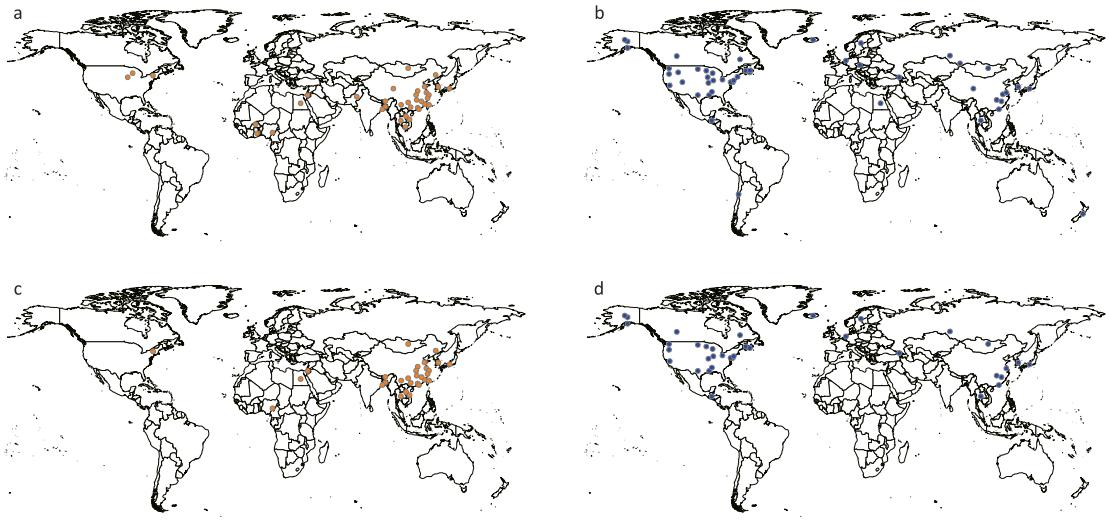

Fig. 1. Global avian influenza virus sampling. Filled circles indicate the sampling regions of avian influenza virus in poultry (a and c) and wild birds (b and d) for 10 sequence data sets (a and b) and 15 sequence data sets (c and d).

fragment according to viral geographic source, that is, western and eastern hemispheric lineages (Worobey et al., 2014a, 2014b). In eastern hemisphere, we found that the AIV internal gene segments of wild birds do not exhibit significantly higher genetic diversity than these of poultry in both the 10-sequence ($p = 0.90$ for MP, $p = 0.09$ for NP, $p = 0.50$ for NS, $p = 0.55$ for PA, $p = 0.72$ for PB1, $p = 0.31$ for PB2) and 15-sequence data sets ($p = 0.22$ for MP, $p = 0.63$ for NS, $p = 0.57$ for PA, $p = 0.23$ for PB1, $p = 0.18$ for PB2), except the NP segment where wild bird AIV has weakly higher genetic diversity in the 15-sequence data set ($p = 0.04$) (Fig. 2). In western hemisphere, the sampling of AIV is generally not extensive in poultry (Fig. 1). Based on the data available, our analyses show that the AIV internal gene segments of wild birds share similar level of genetic diversity with those of poultry the 10-sequence ($p = 0.87$ for MP, $p = 0.20$ for PA, $p = 0.88$ for PB1, $p = 0.05$ for PB2) and 15-sequence data sets ($p = 0.57$ for NP, $p = 0.10$ for PA, $p = 0.65$ for PB1, $p = 0.14$ for PB2), except the NP segment whereby wild bird AIV has weakly higher genetic diversity in the 10-sequence data set ($p = 0.03$) (Fig. 2). Moreover, for each internal gene segment, we compared diversity of combined poultry and wild bird AIV with that of poultry AIV and wild bird AIV, respectively, and found no significant difference (Fig. S2). Taken together, our results provide no evidence for higher genetic diversity of internal gene segments in wild bird AIV than in poultry AIV.

We also compared the number of HA and NA subtypes in both poultry and wild bird AIV data sets. We found wild bird AIV generally has larger number of HA and NA subtypes than poultry AIV in both eastern hemisphere ($p < 0.01$) and western hemisphere ($p < 0.05$), consistent with previous finding that more HA and NA subtypes of AIV were isolated in wild birds (Olsen et al., 2006) (Fig. 3). However, for nucleotide diversity of HA and NA segments, we found no general pattern: the diversity of poultry AIV H5 and N1 is higher than that of wild birds in eastern hemisphere ($p < 0.01$), wild bird AIV N2 exhibits higher genetic diversity than poultry AIV N2 in western hemisphere ($p < 0.01$), and there is no significant difference in genetic diversity between wild bird and poultry N2 in eastern hemisphere ($p = 0.90$) as well as between wild bird and poultry H5 in western hemisphere ($p = 0.94$) (Fig. 4). We estimated the synonymous and nonsynonymous diversity of HA and NA and found the results are identical to the overall genetic diversity (Fig. S3).

3. Discussion

Intuitively, the larger number of HA and NA subtypes of AIV in wild birds seems to be inconsistent with the similar genetic diversity of internal gene segments between wild bird AIV and poultry AIV. We think the evolutionary history of influenza virus could explain this paradoxical pattern of AIV diversity. Phylogenetic analyses suggest the AIV internal gene segments (except the NS segment that was incompletely swept) underwent a synchronized global sweep beginning in the late 1800s, explaining the restricted genetic diversity of internal gene segments (Worobey et al., 2014a, 2014b). All the diversity of internal gene segments (except the NS gene segment) originated from an avian virus, possibly from domestic birds, after cross-transmission from horse (Worobey et al., 2014a, 2014b). If the modern diversity of internal gene segments did originate from poultry and frequent AIV transmission occurred between poultry and wild birds (Fig. 5b), it is unsurprising to observe that wild bird AIV internal genes do not have higher diversity than poultry AIV internal genes. On the other hand, the global sweep of internal gene segments does not affect the pre-existing diversity of HA and NA (Worobey et al., 2014a, 2014b). It follows that wild bird AIV might have larger number of HA and NA subtypes.

Given that wild birds have been widely thought to be the natural reservoir influenza virus (Olsen et al., 2006; Webster et al., 1992; Horimoto and Kawaoka, 2001), the transmission from poultry to wild birds are thought to sporadic or limited (Joseph et al., 2017) (Fig. 5a). To further test the possibility of AIV transmission from poultry to wild birds (Fig. 5), we estimated the transmission rate of AIV between the wild birds and poultry for internal genes. We found that AIV could be transmitted from wild birds to poultry as well as from poultry to wild birds. Although the transmission rate from wild birds to poultry is generally higher than that from poultry to wild birds, the transmission from poultry to wild birds is not negligible (Table 1).

Taken together, our analyses suggest that internal gene segments of wild bird AIV do not have higher genetic diversity than those of poultry AIV. It appears to be not a good metaphor to assume wild birds serve as the reservoir of AIV internal gene segments (Fig. 5a). The transmission of AIV between wild birds and domestic birds are more complex than a single dominant route from wild birds to poultry as thought (Fig. 5a). Our studies came with several caveats: i) Although AIV seems to be extensively surveilled, especially in North America, Europe, and Asia, the data of AIV isolated in a specific region over years are still limited. ii) The sampling location of AIV was often labeled by the level of

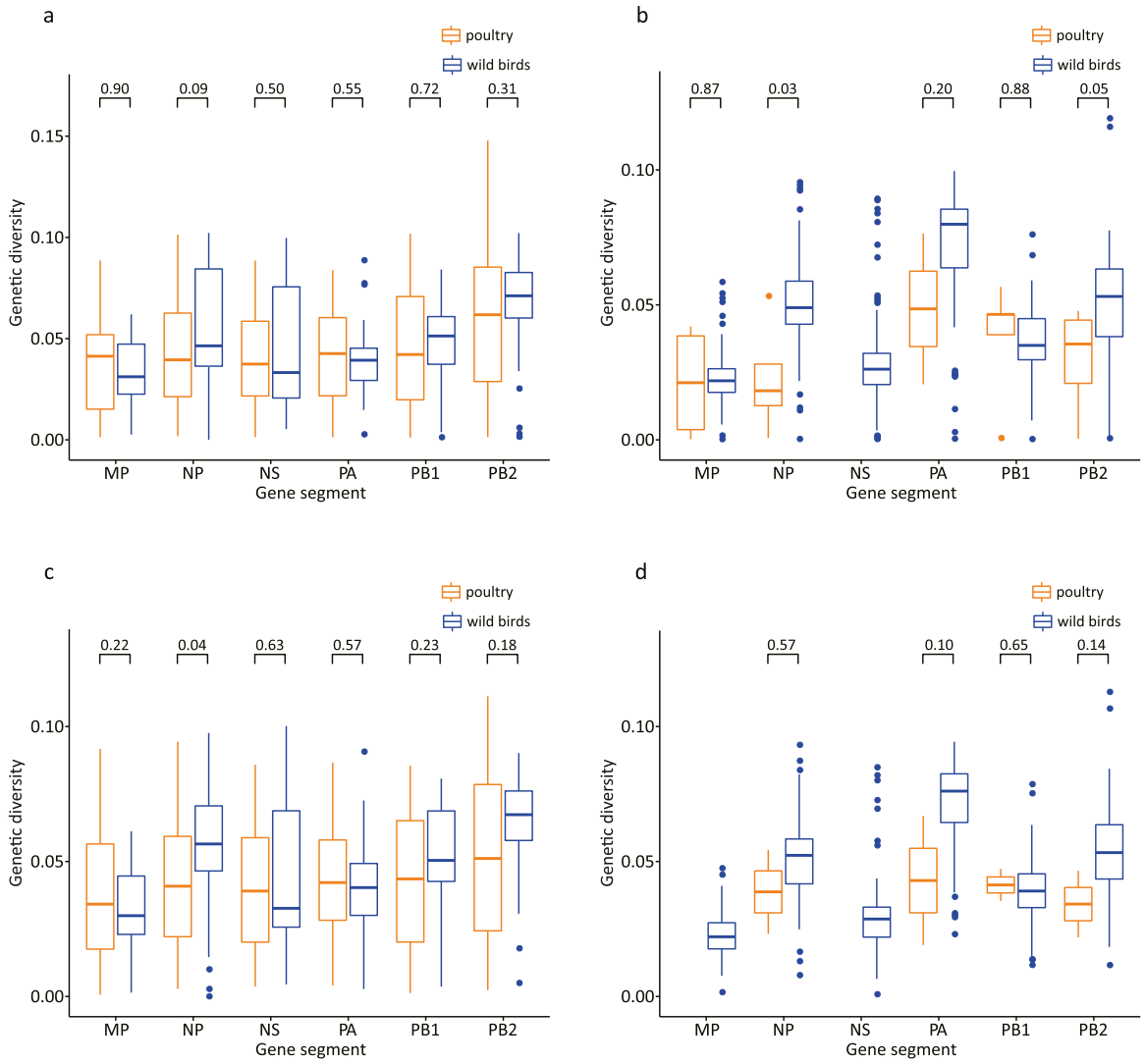

Fig. 2. Comparison of genetic diversity of internal gene segments between wild bird and poultry AIV. a. Comparison between wild bird and poultry AIV in eastern hemisphere using 10 sequences randomly sampled for each region and each year. **b.** Comparison between wild bird and poultry AIV in western hemisphere using 10 sequences randomly sampled for each region and each year. **c.** Comparison between wild bird and poultry AIV in eastern hemisphere using 15 sequences randomly sampled for each region and each year. **d.** Comparison between wild bird and poultry AIV in western hemisphere using 15 sequences randomly sampled for each region and each year. The poultry and wild bird AIV data are highlighted in orange and blue colors, respectively. The statistical significance was tested using student's *t*-test or Wilcoxon-Mann-Whitney test.

administrative region, such as states for USA and provinces for China. However, the area of regions is not positively correlated to the genetic diversity for most of the data sets (Table S1). Nevertheless, further extensive surveillance of AIV in wild birds and poultry will help fully understand the diversity and transmission of AIV and potentially design control measures of avian influenza.

4. Materials and methods

4.1. Data collection

All the complete nucleotide sequences of AIV internal gene (PA, PB1, PB2, NP, MP, and NS) and external gene (H5, N1, and N2) segments collected from 2005 to 2015 were retrieved from Influenza Virus Resource (https://www.ncbi.nlm.nih.gov/genomes/FLU/) (Hatcher et al., 2017). Regions with more than 10 sequences each year were selected for further analyses. The regions represent states for USA, provinces for China, federal subjects for Russia, provinces for Canada, and small countries as a whole. Most of the regions are smaller than 300,000 km^2 (Fig. S1). The sampling sites were mapped by using R packages ggplot2, ggmap, sp, maptools, and maps. These sequences were classified into wild birds and poultry based on the information from original literature and/or strain name. Because the NS segment was incompletely swept (Worobey et al., 2014b) and the NS lineage "B" strains might confound our analysis, we excluded the NS segment sequences of lineage "B" identified through phylogenetic analyses. The sequences were aligned using MAFFT (Katoh and Standley, 2013). The phylogenetic analyses were performed using an approximately maximum likelihood method implemented in FastTree 2.0 (Price et al., 2010).

4.2. Genetic diversity estimation

For each internal gene segment, we randomly sampled 10 and 15 sequences for each region (75 regions) and each year (from 2005 to 2015). We also randomly sampled 5 sequences of poultry AIV and 5 sequences of wild bird AIV sampled in the same region and in the same year. The sequences were aligned using MAFFT (Katoh et al., 2013). The nucleotide diversity (π) of each sequence data set was estimated using DnaSP v5 (Librado and Rozas, 2009). The number of HA and NA

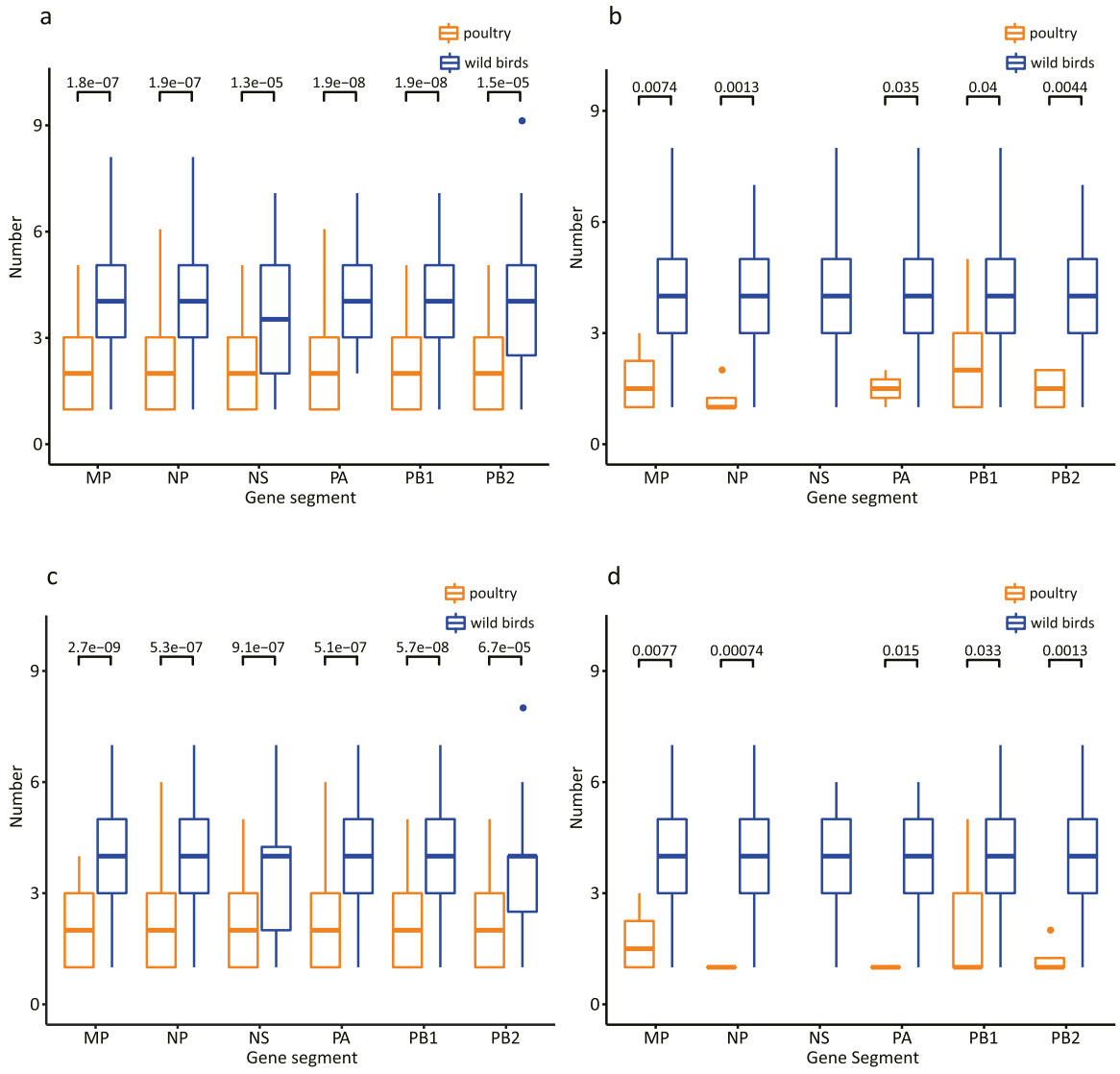

Fig. 3. Comparison of number of HA and NA subtypes among six internal gene segments between wild bird and poultry AIV. a. Comparison of number of HA subtypes between wild bird and poultry AIV in eastern hemisphere using 10 sequences randomly sampled for each region and each year. **b.** Comparison of number of HA subtypes between wild bird and poultry AIV in western hemisphere using 10 sequences randomly sampled for each region and each year. **c.** Comparison of number of NA subtypes between wild bird and poultry AIV in eastern hemisphere using 10 sequences randomly sampled for each region and each year. **d.** Comparison of number of NA subtypes between wild bird and poultry AIV in western hemisphere using 10 sequences randomly sampled for each region and each year. The poultry and wild bird AIV data are highlighted in orange and blue colors, respectively. The statistical significance was tested using student's *t*-test or Wilcoxon-Mann-Whitney test.

subtypes were counted for each internal gene data set with 10 or 15 sequences.

4.3. Statistical tests

To test whether there is significant difference between the AIV diversity of wild birds and that of poultry, we used student's *t*-test for data set following a normal distribution and Wilcoxon-Mann-Whitney test for other data sets. Whether a data set follows a normal distribution was determined by using Shapiro-Wilk test. All the statistical tests were performed by using R.

4.4. Transition rate estimation

Because limited number of AIV sequences were sampled in poultry of western hemisphere, we only estimated AIV transition rates between wild birds and poultry in eastern hemisphere. To avoid the effect sampling bias on transition rate estimation, we randomly sampled equal number (~ 300–400 based on the number of wild bird AIV sequences available) of AIV internal gene sequences from wild birds and poultry. For each internal gene segment, phylogenetic analyses were conducted using a maximum likelihood method implemented in IQ-TREE (Nguyen et al., 2015). We used BayesTraits V3 (http://www.evolution.rdg.ac.uk/BayesTraitsV3.0.1/BayesTraitsV3.0.1.html) to estimate AIV transition rates between wild birds and poultry. We used MultiState algorithm to reconstruct how different traits that fit a finite number of discrete states evolve on phylogenetic trees. Markov chain Monte Carlo (MCMC) methods were applied to sample from the posterior distributions of transition rates, depicting the comparative analysis of a binary discrete character. We considered the character with two states, wild bird and poultry. Based on a phylogenetic tree and on different states at the tips of the phylogeny, we estimated the transmission rates between wild birds and poultry. The MCMC chain consists of 101,000 iterations, of which we sampled every 1000 iterations. We

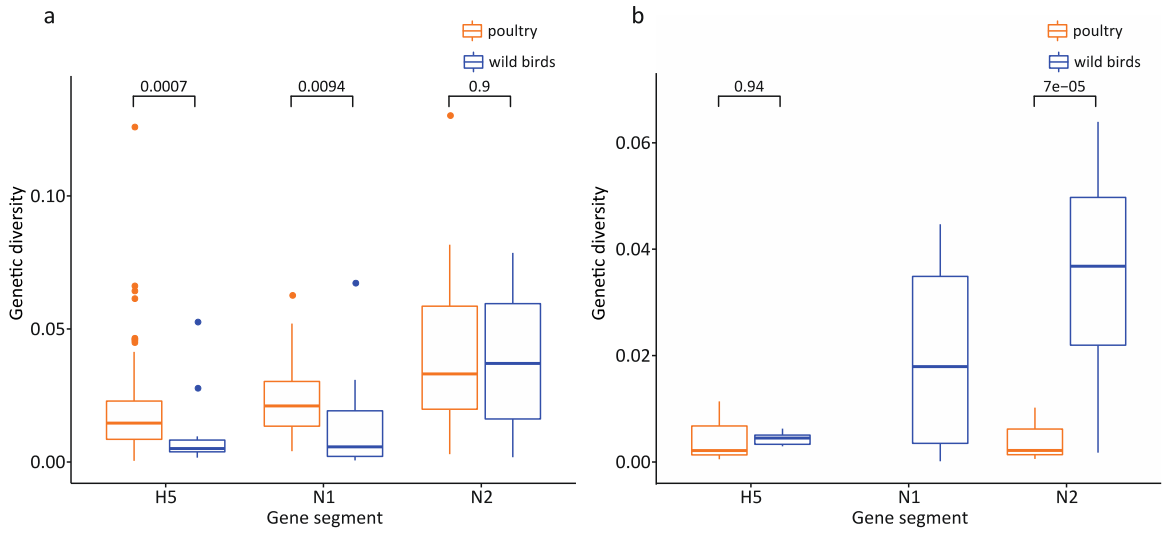

Fig. 4. Comparison of genetic diversity of HA and NA segments between wild bird and poultry AIV. a. Comparison between wild bird and poultry AIV in eastern hemisphere using 10 sequences randomly sampled for each region and each year. **b.** Comparison between wild bird and poultry AIV in western hemisphere using 10 sequences randomly sampled for each region and each year. The poultry and wild bird AIV data are highlighted in orange and blue colors, respectively. The statistical significance was tested using student's *t*-test or Wilcoxon-Mann-Whitney test.

calculated the median of output to illustrate the rate of transmission varied in each sample.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31701091), the Natural Science Foundation of Jiangsu Province (BK20161016), the Program for Jiangsu Excellent Scientific and Technological Innovation Team (17CXTD00014), and the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

Table 1
Avian influenza virus transition rate between poultry and wild birds.

Gene segment	Median transition rate from poultry to wild birds (95% HPD)	Median transition rate from wild birds to poultry (95% HPD)
MP	11.92 (6.56–17.37)	22.59 (16.10–30.64)
NP	6.31 (2.44–11.12)	17.82 (12.99–23.59)
NS1	7.09 (2.72–12.56)	29.63 (20.60–39.61)
PA	5.37 (2.37–8.76)	14.67 (9.84–20.13)
PB1	5.69 (2.93–9.50)	15.63 (10.58–20.37)
PB2	6.86 (3.12–11.26)	16.52 (11.04–22.31)

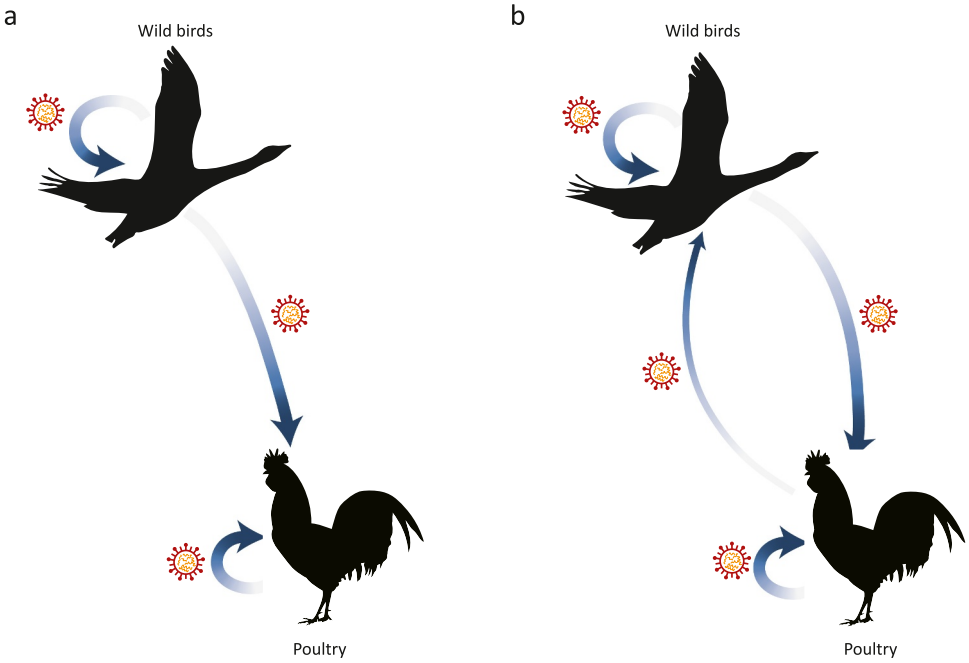

Fig. 5. Transmission of AIV between wild birds and poultry. AIV is circulating among wild birds and poultry. **a.** AIV is mainly transmitted from wild birds to poultry. **b.** AIV is transmitted frequently between wild birds and poultry.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.02.003.

References

Belshe, R.B., 2005. The origins of pandemic influenza–lessons from the 1918 virus. N. Engl. J. Med. 353, 2209–2211.

Chen, H., Smith, G.J., Zhang, S.Y., Qin, K., Wang, J., Li, K.S., Webster, R.G., Peiris, J.S., Guan, Y., 2005. Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature 436, 191–192.

Global Consortium for H5N8 and Related Influenza Viruses, 2016. Role for migratory wild birds in the global spread of avian influenza H5N8. Science 354, 213–217.

Hatcher, E.L., Zhdanov, S.A., Bao, Y., Blinkova, O., Nawrocki, E.P., Ostapchuck, Y., Schaffer, A.A., Brister, J.R., 2017. Virus variation resource – improved response to emergent viral outbreaks. Nucleic Acids Res. 45, D482–D490.

Horimoto, T., Kawaoka, Y., 2001. Pandemic threat posed by avian influenza A viruses. Clin. Microbiol. Rev. 14, 129–149.

Joseph, U., Su, Y.C., Vijaykrishna, D., Smith, G.J., 2017. The ecology and adaptive evolution of influenza A interspecies transmission. Influenza Other Respir. Virus. 11, 74–84.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780.

Lai, S., et al., 2016. Global epidemiology of avian influenza A H5N1 virus infection in humans, 1997–2015: a systematic review of individual case data. Lancet Infect. Dis. 16, e108–e118.

Librado, P., Rozas, J., 2009. DnaSPv5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.

Liu, J., et al., 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309, 1206.

Nguyen, L.T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 2, 268–274.

Olsen, B., Munster, V.J., Wallensten, A., Waldenstrom, J., Osterhaus, A.D., Fouchier, R.A., 2006. Global patterns of influenza a virus in wild birds. Science 312, 384–388.

Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2–approximately maximum-likelihood trees for large alignments. PLoS One 5, e9490.

Su, S., Bi, Y., Wong, G., Gray, G.C., Gao, G.F., Li, S., 2015. Epidemiology, evolution, and recent outbreaks of avian influenza virus in China. J. Virol. 89, 8671–8676.

Verhagen, J.H., Herfst, S., Fouchier, R.A., 2015. Infectious disease. How a virus travels the world. Science 347, 616–617.

Wang, X., et al., 2017. Epidemiology of avian influenza A H7N9 virus in human beings across five epidemics in mainland China, 2013-17: an epidemiological study of laboratory-confirmed case series. Lancet Infect. Dis. 17, 822–832.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y., 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56, 152–179.

Worobey, M., Han, G.Z., Rambaut, A., 2014a. A synchronized global sweep of the internal genes of modern avian influenza virus. Nature 508, 254–257.

Worobey, M., Han, G.Z., Rambaut, A., 2014b. Genesis and pathogenesis of the 1918 pandemic H1N1 influenza Avirus. Proc. Natl. Acad. Sci. USA 111, 8107–8112.

Zhou, L., et al., 2017. Sudden increase in human infection with avian influenza A(H7N9) virus in China, September-December 2016. West. Pac. Surveill. Response J. 8, 6–14.

Zhu, H., Lam, T.T., Smith, D.K., Guan, Y., 2016. Emergence and development of H7N9 influenza viruses in China. Curr. Opin. Virol. 16, 106–113.