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Diversity of avian influenza A(H5N6) viruses in wild birds in southern China

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Abstract

The predominance of H5N6 in ducks and continuous human cases have heightened its potential threat to public health in China. Therefore, the detection of emerging variants of H5N6 avian influenza viruses has become a priority for pandemic preparedness. Questions remain as to its origin and circulation within the wild bird reservoir and interactions at the wild–domestic interface. Samples were collected from migratory birds in Poyang Lake, Jiangxi Province, PR China during the routine bird ring survey in 2014–16. Phylogenetic and coalescent analyses were conducted to uncover the evolutionary relationship among viruses circulating in wild birds. Here, we report the potential origin and phylogenetic diversity of H5N6 viruses isolated from wild birds in Poyang Lake. Sequence analyses indicated that Jiangxi H5N6 viruses most likely evolved from Eurasian-derived H5Nx and H6N6 viruses through multiple reassortment events. Crucially, the diversity of the HA gene implies that these Jiangxi H5N6 viruses have diverged into two primary clades – clade 2.3.4.4 and clade 2.3.2.1 c. Phylogenetic analysis revealed two independent pathways of reassortment during 2014–16 that might have facilitated the generation of emerging variants within wild bird populations as well as inter-species infections. Our findings contribute to our understanding of the genetic diversification of H5N6 viruses in the wild bird population. These results highlight the necessity of large-scale surveillance of wild birds in the Poyang Lake area to address the threat of regional epizootic epidemics and attendant pandemics.

INTRODUCTION

Human infections with highly pathogenic avian influenza (HPAI) H5N6 viruses have been reported continuously in PR China since 2014 [1]. In response, constant surveillance of live poultry markets (LPMs) was performed to investigate viral prevalence and evolution in domestic birds. The results of this surveillance indicated that H5N6 has become a dominant subtype in domestic ducks in southern China through the movement of poultry and poultry products [2]. In addition, surveillance in wild waterfowl identified a human-type receptor-binding activity of H5N6 viruses, raising concerns about its potential for transmission to humans [3]. However, large-scale surveillance of avian influenza viruses (AIVs) in wild birds is still rare. Consequently, little is known about the ongoing evolution and diversity of H5N6 within the wild bird reservoir itself, and even less is known about its potential for transmission to domestic birds and its threat to humans [4–7].

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Keywords: H5N6; avian influenza virus; wild birds; reassortment; clade 2.3.2.1c.

Abbreviations: AIVs, avian influenza viruses; HA, hemagglutination; HPAI, highly pathogenic avian influenza; HPD, highest posterior density; LPMs, live poultry markets; MCC, maximum clade credibility; MCMC, Markov chain Monte Carlo; tMRCA, time to the most recent common ancestor.

The sequence data used in this study can be obtained from GISAID EpiFlu database.

Accession numbers of Jiangxi H5N6 viruses isolated in this study. A/duck/Jiangxi/95/2014(H5N6): EPI530051–EPI530058. A/duck/Jiangxi/F31/2014(H5N6): EPI1574368–EPI1574375. A/chicken/Jiangxi/E84/2014(H5N6): EPI1574360–EPI1574367 A/duck/Jiangxi/E7/2014(H5N6): EPI1574352–EPI1574359. A/Mallard/Jiangxi/JXH9/2014(H5N6): EPI1574342–EPI1574349. A/Mallard/Jiangxi/JXH22/2014(H5N6): EPI1574334–EPI1574341. A/quail/Jiangxi/B9/2015(H5N6): EPI1574326–EPI1574333. A/Streptopelia decaocto/Jiangxi/J8/2015(H5N6): EPI1574318–EPI1574325. A/Streptopelia decaocto/Jiangxi/E1/2015(H5N6): EPI1574310–EPI1574317. A/Luscinia cyane/Jiangxi/U2/2016(H5N6): EPI1574302–EPI1574309. A/Streptopelia decaocto/Jiangxi/G6/2016(H5N6): EPI1489545–EPI1489552.

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Twelve supplementary tables and eighteen supplementary figures are available with the online version of this article

Poyang Lake, the largest freshwater body in PR China, is a key wintering site [8] situated in the East Asia–Australia migratory flyway [9]. Its diverse natural ecological landscape supports congregation and hence virus gene exchange among different bird species. Moreover, free-range poultry in surrounding croplands have frequent contact with wild migratory birds [10]. Therefore, Poyang Lake is an ideal site to investigate viral dynamics within the wild bird reservoir and interactions at the wild–domestic interface. Previous analyses have shown that through long-term migration migratory ducks at Poyang Lake could have transmitted H5N1 viruses to geographically remote areas [11]. Additionally, direct interactions between migratory and domestic ducks at Poyang Lake may have given rise to the close genetic relationship between viruses isolated from wild and domestic bird species [12]. Questions remain as to whether H5N6 viruses have also undergone extensive genetic reassortment events, generating multiple new variants and facilitating transmission to domestic birds. It is essential that extensive surveillance in both wild and domestic birds should be implemented to narrow this knowledge gap.

In this paper, we present results of H5N6 viruses isolated from wild and domestic birds in Poyang Lake, Jiangxi Province, PR China in 2014–16. We assessed the prevalence of AIVs and H5N6 viruses in different bird species. We performed phylogenetic and coalescent analyses to characterize their evolutionary relationship with other AIV subtypes. To best of the authors' knowledge, no H5N6 clade 2.3.2.1 c has been detected in wild birds in China. Our results highlight that large-scale viral surveillance among wild birds in Poyang Lake should be a priority for pandemic preparedness.

METHODS

Ethical statement

All animal work was approved by the Beijing Association for Science and Technology [approval SYXK (Beijing) 2007–0023]. The laboratory animal research was performed in the microbiology laboratory of China Agricultural University, adhering to Beijing Laboratory Animal Welfare and Ethics guidelines issued by the Beijing Administration Committee of Laboratory Animals and China Agricultural University Institutional Animal Care and Use Committee guidelines (ID: SKLAB-B-2010–003). All experiments in this study were performed in biosafety level 3 containment approved by the Ministry of Agriculture of PR China.

Surveillance of avian influenza viruses

We captured migratory birds [13] in Poyang Lake, Jiangxi Province during the routine bird ring survey in the wintering seasons of 2014–16. Tracheal and cloacal swab samples were subsequently collected from these migratory birds. To investigate interactions between wild and domestic birds, we used active surveillance to collect samples from apparently healthy domestic ducks and chickens in the surrounding rice fields as well. Upon collection, samples were preserved in sample solution [phosphate-buffered saline (PBS) (7.2) containing 0.1% 2×10^6 U l^{-1} penicillin G, 2×10^6 U l^{-1} amphotericin B, 250 mg l^{-1} kitasamycin, 0.5×10^6 U l^{-1} nystatin, 60 mg l^{-1} ofloxacin HCl and 400 mg l^{-1} streptomycin sulfate) in the refrigerator (4 °C) and subsequently shipped to the laboratory and stored frozen at −80 °C for a maximum of 10 days. Virus isolation using these specimens was conducted in 9–11-day-old specific pathogen-free embryonated chicken eggs. A haemagglutination-Inhibition test was carried out following the classical procedure [14]. Among these samples, 14 isolates from wild birds and 157 isolates from domestic birds showed haemagglutination activity. Viral RNAs were extracted from allantoic fluid of these positive samples using the RNeasy Mini kit (Qiagen, Hilden, Germany). The superscript III reverse transcription PCR (RT-PCR) kit (Invitrogen, USA) was used for reverse transcription. The subtype of each of the positive samples was determined using subtype-specific PCRs [15, 16]. All gene segments of the H5N6 strains were amplified by using a Phusion high-Fidelity PCR system (New England Biolabs, Ipswich, MA, USA) adhering to the manufacturer's instructions [17]. Sequencing of each segment was subsequently performed as individual amplicons using an Applied Biosystems Automated 3730xl DNA Analyzer. Sequences were submitted to the GISAID EpiFlu database under the accession numbers provided in Table S1 (available in the online version of this article).

Phylogenetic and coalescent analyses

Publicly available sequences of influenza A viruses were downloaded from the GenBank and GISAID databases. To reduce the size of this dataset, we firstly removed identical sequences by keeping the sequence with the earliest date. With the remaining sequences, we constructed a maximum-likelihood tree and used the clustering algorithm (cut-off value: 98%) to select representative sequences. Additionally, we used a BLAST search to identify closely related sequences (with >98% identity). Sequences identified by the clustering algorithm and BLAST search were integrated as a dataset of representative sequences for subsequent analyses. Details of reference sequences analysed in this study are provided in Tables S2–9. Molecular phylogenetic analyses were conducted using MEGA 6.0 software [18] and the maximum-likelihood method based on the Kimura two-parameter model [19] with 1000 bootstrap replicates. Clade classification of the haemagglutination (HA) gene was made adhering to the clade designation recommended by the World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (OIE) [20]. Jiangxi H5N6 viruses were subsequently assigned to different groups according to the phylogeny topology for bootstrap values of >75%. To further investigate the relationship between the internal genes of Jiangxi H5N6 viruses and those of local non-H5 viruses, two H3N6 viruses [21] and one H3N8 virus [22, 23] isolated from wild birds in Poyang Lake were incorporated.

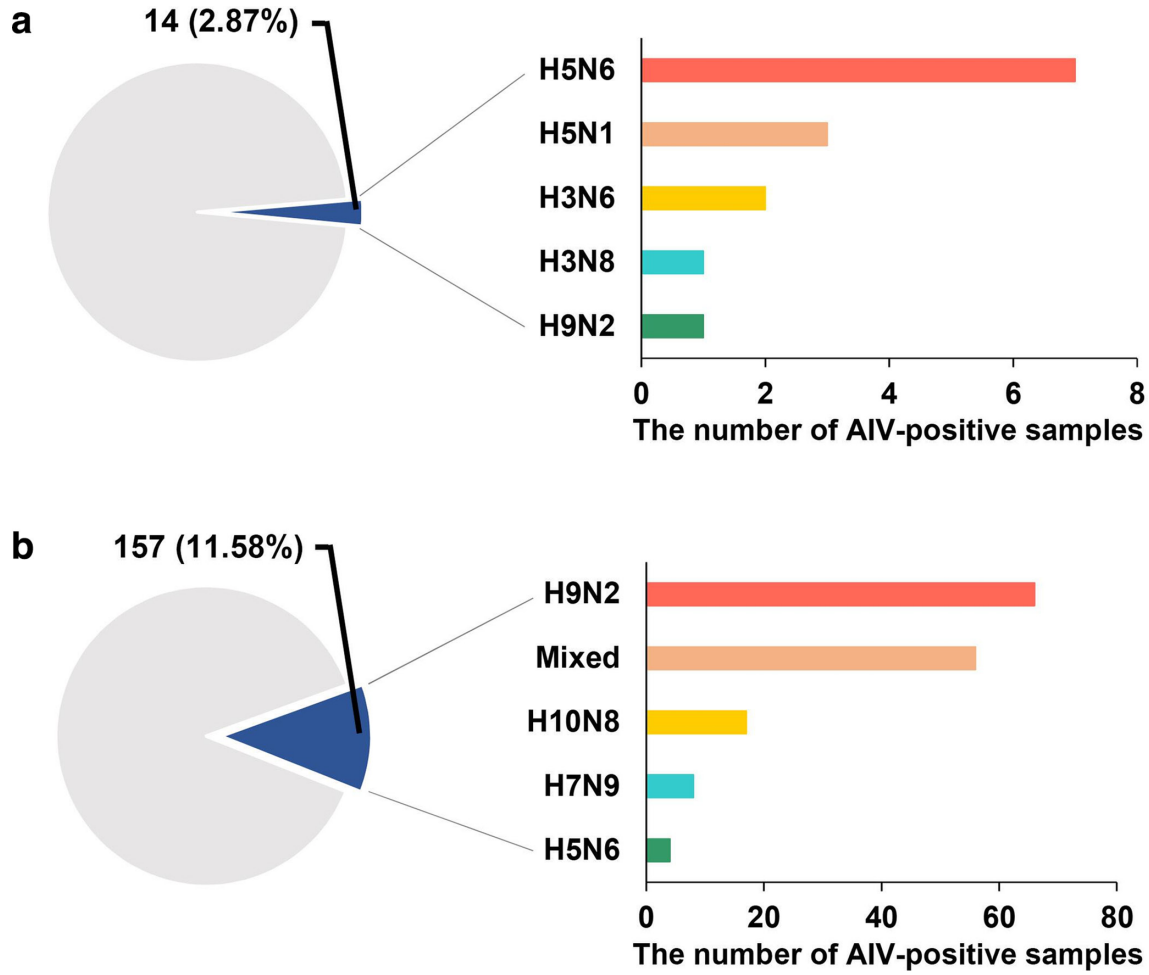

Fig. 1. Surveillance of avian influenza viruses. Amongst the 488 samples collected from wild birds (a) and the 1365 samples from domestic poultry (b), the number and proportion of samples with haemagglutination activity are presented. Sample size for each subtype is distinguished by colour.

To estimate the time to the most recent common ancestor (tMRCA) of each group, Bayesian Markov chain Monte Carlo (MCMC) analysis was performed via BEAST v1.7.5 [24]. The SRD06 substitution model and the Bayesian Skyride population prior were chosen [25, 26]. The uncorrelated relaxed lognormal clock was used to allow for the rate variation among branches [27]. The Bayesian MCMC was run for 2×10^7 steps, with sampling every 5000 steps. The convergence of relevant parameters was assessed using the effective sample size >200 in Tracer v1.6 [27, 28]. A maximum clade credibility (MCC) tree was generated after removing the first 10–15% burn-in states. The uncertainty of the estimated tMRCA was quantified using the 95% highest posterior density (HPD). Estimates of nucleotide substitution rates for each gene are presented in Table S10.

RESULTS

Surveillance results

We obtained a total of 488 samples from 15 wild bird species and 1356 samples from domestic ducks and chickens (Tables S11 and S12). The isolation rates for AIVs were 2.87% (14/488) for wild birds and 11.58% (157/1356) for domestic birds (Fig. 1). Among these AIV-positive samples, seven from wild birds were identified as H5N6, which were designated as A/Streptopelia decaocto/Jiangxi/E1/2015(H5N6) (abbreviated as Sd/JX/E1), A/Streptopelia decaocto/Jiangxi/G6/2016(H5N6) (Sd/JX/G6), A/Streptopelia decaocto/Jiangxi/J8/2015(H5N6) (Sd/JX/J8), A/Mallard/Jiangxi/JXH22/2014(H5N6) (Md/JX/H22), A/Mallard/Jiangxi/JXH9/2014(H5N6) (Md/JX/H9), A/Luscinia cyane/Jiangxi/U2/2016(H5N6) (Lc/JX/U2) and A/quail/Jiangxi/B9/2015(H5N6) (Qa/JX/B9). The four H5N6 samples from domestic birds were named A/duck/Jiangxi/95/2014(H5N6)

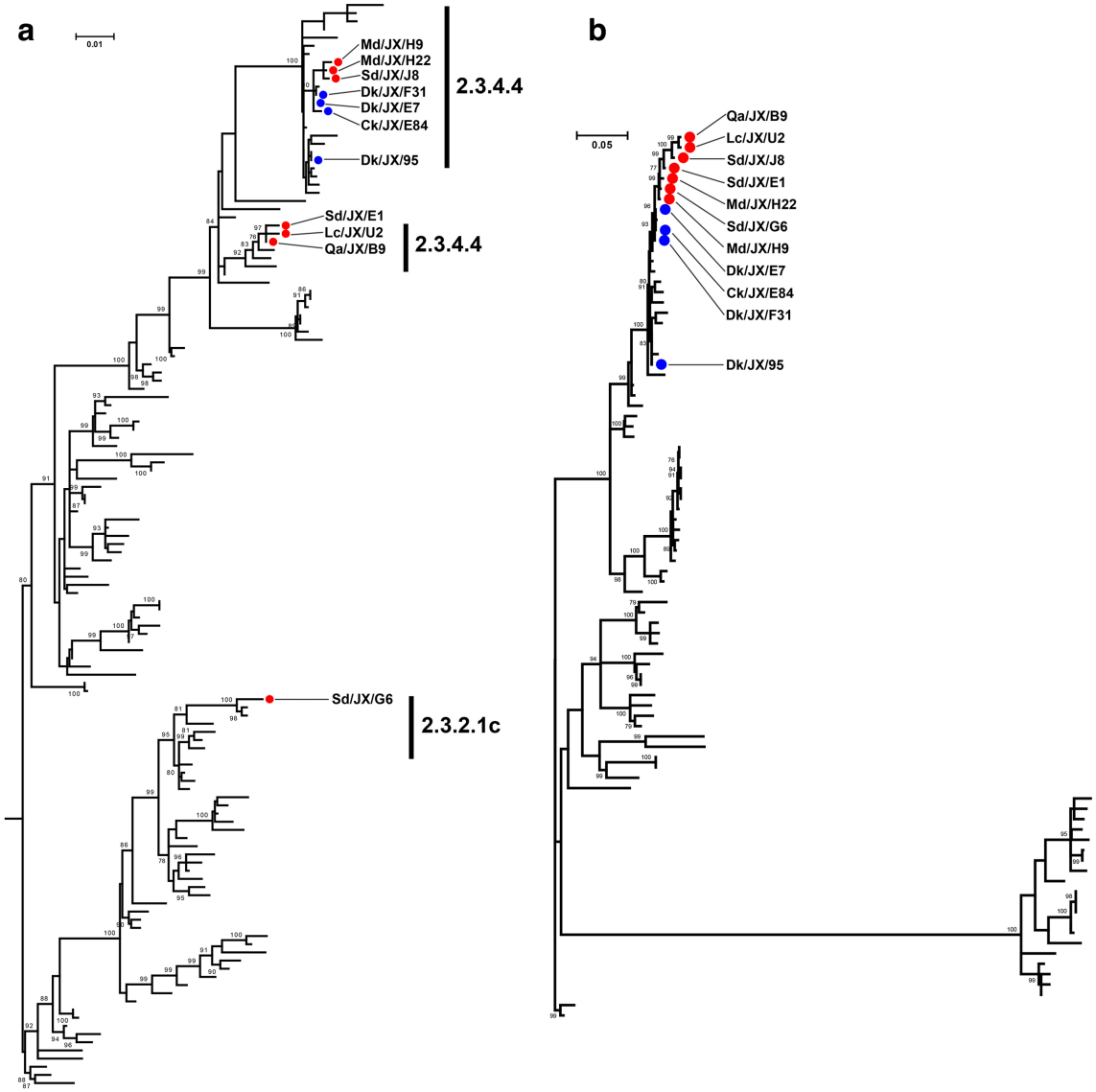

Fig. 2. Phylogenetic analysis of HA and NA genes of Jiangxi H5N6 viruses. The phylogeny of HA and NA genes was inferred using the maximum-likelihood method with 1000 bootstrap replicates. Jiangxi H5N6 viruses in wild birds are highlighted with red dots and those in domestic birds are highlighted with blue dots. (a) Phylogeny of HA genes of clade 2.3 viruses. In addition to the Jiangxi H5N6 viruses, publicly available H5 sequences obtained from GenBank, GISAID and reference sequences for clade classification are also included. The vertical bars and clade designation show that the Jiangxi H5N6 viruses belong to three groups. Bootstrap values >75% are shown on the branch. (b) Phylogeny of the NA gene. In addition to Jiangxi H5N6 viruses, publicly available N6 sequences from GenBank and GISAID are also included.

(Dk/JX/95), A/duck/Jiangxi/E7/2014(H5N6) (Dk/JX/E7), A/chicken/Jiangxi/E84/2014(H5N6) (Ck/JX/E84) and A/duck/Jiangxi/F31/2014(H5N6) (Dk/JX/F31).

Evolutionary analysis of HA and NA genes of Jiangxi H5N6 viruses from wild birds

The HA phylogeny revealed that Jiangxi H5N6 viruses diverged into three phylogenetically distinct groups (Figs 2a and S1). Of note, a novel reassortant, Sd/JX/G6 virus, was clustered together with Asian H5N1 viruses and belonged exclusively to clade 2.3.2.1 c. By contrast, all the other viruses belonged to clade 2.3.4.4 and formed two groups. More specifically, three viruses from wild birds (i.e. Md/JX/H9, Md/JX/H22 and Sd/JX/J8) formed a single group with all poultry viruses isolated in Jiangxi and recent H5N6 viruses from southern China and Vietnam. This group fall into subgroup C of clade 2.3.4.4 (Fig. S18). The other group consisted of three other viruses from wild birds, which were evolutionarily close to H5N6 and H5N8 viruses from domestic ducks circulating in eastern China in 2013–14. This group belongs to subgroup B of the clade 2.3.4.4 viruses. (Fig. S18). Additionally, the H5 phylogeny

Table 1. The estimated time to the most recent common ancestor (tMRCA). For each gene, group type, i.e. wild/domestic bird group and wild bird group, the median estimates and 95% HPD of the tMRCA for each phylogenetic group are shown

Gene	Group type	tMRCA (95% HPD)
HA	Wild/domestic bird	20 March 2012 (24 September 2011–4 August 2012)
	Wild bird	6 July 2011 (22 September 2010–2 April 2012)
	Wild bird	27 April 2011 (11 August 2010–12 November 2011)
NA	Wild/domestic bird	12 June 2011 (8 June 2011–17 July 2011)
PB2	Wild/domestic bird	15 January 2009 (3 April 2008–18 October 2009)
	Wild bird	2 September 1992 (11 January 1983–14 April 1998)
	Wild bird	NA
PB1	Wild/domestic bird	15 July 2009 (6 October 2008–13 May 2010)
	Wild bird	5 November 2010 (11 February 2010–7 June 2011)
	Wild bird	16 July 2013 (9 November 2011–14 July 2014)
PA	Wild/domestic bird	23 April 2011 (1 November 2010–23 June 2011)
	Wild bird	11 May 2012 (22 April 2011–2 July 2013)
NP	Wild/domestic bird	12 September 2010 (11 July 2010–7 November 2010)
	Wild bird	24 September 2012 (9 February 2012–13 May 2013)
M	Wild/domestic bird	15 March 2008 (15 October 2007–28 February 2009)
	Wild bird	29 October 2014 (22 April 2014–1 January 2015)
NS	Wild/domestic bird	13 March 2009 (3 March 2008–15 April 2010)
	Wild bird	9 December 2010 (20 March 2009–2 June 2012)

suggested two different origins of the HA gene. The virus in the clade 2.3.2.1 c group might be of Eurasian H5N1 origin and the estimated tMRCA was 27 April 2011 (95% HPD: 11 August 2010–12 November 2011), while H5N6 clade 2.3.4.4 viruses might have originated from H5N8 viruses in China, and the corresponding tMRCA for the two groups was 20 March 2012 (24 September 2011–4 August 2012) and 6 July 2011 (22 September 2010–2 April 2012), respectively (Table 1, Fig. S9). The NA phylogeny showed that all Jiangxi H5N6 viruses formed a single group, which was likely derived from LPAI H6N6 in Guangdong Province, PR China in 2010–11 (Figs 2b and S2). The median estimate of tMRCA of the NA gene was 12 June 2011 (6 June–17 July 2011) (Table 1, Fig. S10).

Evolutionary analysis of internal genes of Jiangxi H5N6 viruses from wild birds

Phylogenetic analyses suggested two types of groups in each internal gene (Figs 3, S3–S8). The wild/domestic birds group includes isolates from both domestic and wild birds. These isolates were genetically close to the recent Eurasian H5N6 viruses, which originated from Eurasian-derived H5N1 viruses. By contrast, the wild birds group exclusively contains wild birds isolates, which clustered with viruses from multiple subtypes, including H5N1, H5N6, H5N8, H7N3, H7N9 and H9N2. Nevertheless, the origin of viruses in the wild birds group was unclear. Furthermore, the median estimates of tMRCA suggested two independent evolutionary processes of Jiangxi H5N6 viruses in each group. It is estimated that the median divergence time of viruses in the wild/domestic birds group was between March 2009 and November 2011. By contrast, the divergence time of viruses in the wild birds group was approximately 1–3 years later in most internal genes (except for the PB2 gene) (Table 1, Fig. S11–16).

Molecular characteristics of Jiangxi H5N6 viruses from wild birds

All Jiangxi H5N6 viruses had multiple basic amino acids at the HA cleavage site and contained two motifs (RERRRKR/GL and REKRRKR/GL), suggesting their high pathogenicity in poultry. Additionally, the HA gene had Q226 and G228 (H3 numbering) at the receptor binding sites, which are associated with an adaptation to avian-like receptors [29]. The NA stalk of these viruses possessed an 11 amino acid deletion (positions 58–68), which may enhance viral adaptation to domestic poultry and increase virulence to mammals [30, 31]. However, there were no mutations for Q591K, E627K and D701N in the PB2 gene, indicating inefficient replication in mammals [32]. It is remarkable that all viruses isolated from domestic poultry harboured a truncated PB1-F2 protein with a length of 57 amino acids. By contrast, three viruses isolated from wild birds possessed a truncated form of the protein (i.e. Sd/JX/G6, 57 amino acid fragment with C-terminal end truncated; Qa/JX/B9 and Lc/JX/U2, 52 amino acid fragment with N-terminal truncated), and the rest had complete PB1-F2 proteins. These truncated proteins may contribute to the enhanced virulence in mammals [33]. Furthermore, no drug resistance-associated mutations were detected in NA (H274Y) and M2 (S31N) proteins, suggesting that these isolates are sensitive to NA and M inhibitors [34].

DISCUSSION

Given the diverse natural ecosystem in Poyang Lake, it is not surprising that we identified a high diversity of H5N6

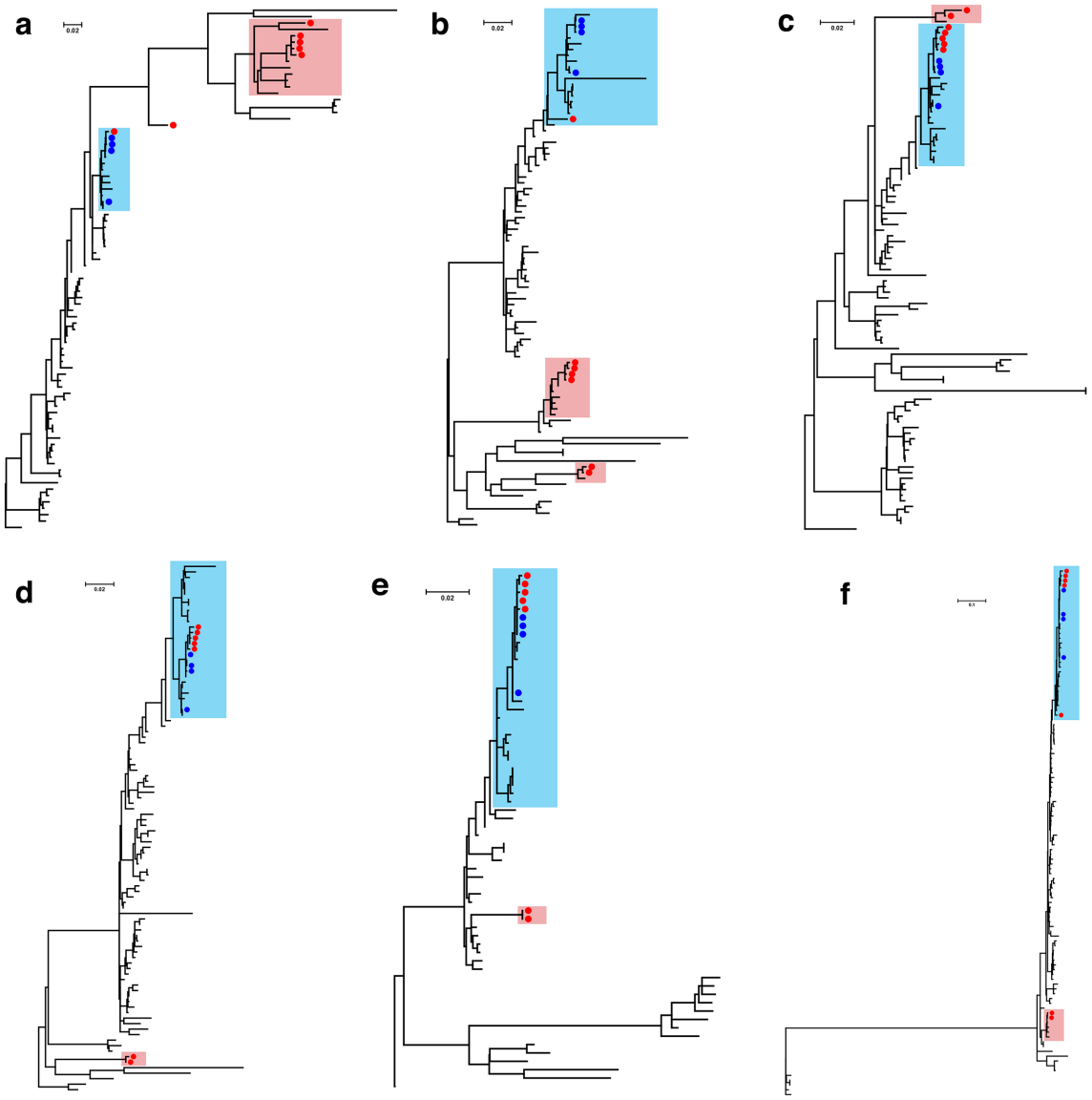

Fig. 3. Phylogenetic analysis of internal genes of Jiangxi H5N6 viruses. Phylogeny of (a) PB2, (b) PB1, (c) PA, (d) NP, (e) M and (f) NS were inferred using the maximum-likelihood method. Jiangxi H5N6 viruses in wild birds are highlighted with red dots and those in domestic birds are highlighted with blue dots. Accordingly, the wild birds group are shown in red rectangles and the wild/domestic birds group are shown in blue rectangles.

viruses coupled with two independent pathways of reassortment. First, the relationships among AIV subtypes are highly diversified within the wild bird reservoir. Without exception, H5N6 viruses in the wild bird group are genetically related to multiple AIV subtypes; those in the wild/domestic bird group clustered exclusively with H5N6 viruses. The congregation of various bird species over winter in Poyang Lake might have led to this additional viral diversity, enabling inter-subtype viral reassortment within the wild bird population. Interestingly, H5N6 clade 2.3.2 viruses have only been detected in live bird markets in Vietnam and Hunan Province, PR China. As such, all clade 2.3.2.1 c viruses from wild birds were of the H5N1 subtype and no variants had been isolated up to now. Therefore, the isolation of Sd/JX/G6 virus raises a concern about the likelihood that clade 2.3.2.1 c viruses in wild birds will reassort with different neuraminidase subtypes.

Consistent with the high nucleotide identity of viruses isolated from wild and domestic birds [35], the genetic relatedness of viruses isolated from wild and domestic birds implies that the likelihood of virus exchange between wild and domestic birds through frequent contacts is high at Poyang Lake – a crucial over-winter sites for migratory birds. In light of this, bird congregation at both breeding [36, 37] and over-winter sites may have provided

an opportunity for the close contact between wild and domestic birds, facilitating inter-species viral transmission and reassortment.

During our surveillance, it was observed that wild waterfowl, e.g. egrets, have frequent interactions with domestic ducks by sharing common water bodies and surrounding rice paddies (Fig. S17a), which might have facilitated transmission of H5N6 viruses from the natural gene pool to ducks. Note that domestic ducks are generally regarded as the interface transferring AIVs from wild birds to chickens [[6, 37–39]. We therefore speculate that sharing outdoor areas might have enabled the subsequent spillover of H5N6 from domestic ducks to chickens. Alternatively, wild terrestrial birds, e.g. Eurasian collared doves, are frequently observed around houses (Fig. S17b) where free-ranging chickens are raised (Fig. S17c). Therefore, we infer a high probability of H5N6 transmission from terrestrial birds to domestic poultry at these common habitats. This inference is consistent with pilot studies that suggested an increased risk of viral transmission between these two bird species [40, 41]. On the basis of the tMRCA, the divergence of viruses circulating among wild and domestic birds may have occurred 1–3 years earlier compared with those within wild bird populations. Therefore, we hypothesize that inter-species transmission might have enhanced the divergence of viruses at the wild–domestic interface. Accordingly, we suggest that the modification of traditional farming practice is a cost-effective approach to successfully limit inter-species viral dissemination and infection. We believe that it would consequently avert the threat of regional epizootic epidemics and potential pandemics.

Care should be taken when interpreting our findings. The isolation rate of AIVs and phylogenetic analyses should not be interpreted as representative features for the entire wild bird population, but should be verified spanning different spatiotemporal settings. In addition, amongst the 157 AIV-positive samples isolated from domestic birds, only 4 H5N6 viruses were sequenced. Therefore, our analysis may not represent the gene pool of AIVs in domestic birds in Poyang Lake. Furthermore, H5 clade 2.3.4.4. viruses have diverged into four genetic subgroups together with their worldwide diffusions [41, 42]. The H5N6 clade 2.3.4.4 viruses isolated in our study fall into two subgroups, indicating that genetic variation among these H5N6 viruses from Poyang Lake does not fully reflect the overall variations of clade 2.3.4.4 viruses (Fig. S18). Future studies would benefit from using extensive surveillance and sequenced data to elucidate a comprehensive pattern of viral dynamics in birds.

In conclusion, phylogenetic diversity implies that H5N6 viruses have undergone continuous evolution both within the wild bird reservoir and at the wild–domestic interface. The natural environment in the Poyang Lake area may be the primary factor driving the reassortment of H5N6 viruses. The unknown origin and large uncertainty in the divergent time of viruses within the wild bird population mean that extensive surveillance of avian influenza viruses is required in the future.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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