The genesis and source of the H7N9 influenza viruses causing human infections in China

Tommy Tsan-Yuk Lam1,2,3*, Jia Wang1,3*, Yongyi Shen1,3,4*, Boping Zhou2, Jian Duan2,3, Chung-Lam Cheung2, Chi Ma1,3, Samantha J. Lyckett2, Connie Yin–Hung Leung2, Xinchun Chen2, Lifeng Li1,2,3, Wenshan Hong1, Yujuan Chai2,3, Linlin Zhou1, Huyi Liang1,2,3, Zhihua Ou1,2,3, Yongmei Liu1,2,3, Amber Farooqui2, David J. Kelvins2, Leo L. M. Poon2,3, David K. Smith1,3, Oliver G. Pybus2,3, Gabriel M. Leung1,3, Yuelong Shu9, Robert G. Webster10, Richard J. Webby10, Joseph S. M. Peiris2,3, Andrew Rambaut1,3, Huachen Zhu1,2,3 & Yi Guan1,2,3

A novel H7N9 influenza A virus first detected in March 2013 has since caused more than 130 human infections in China, resulting in 40 deaths2,4. Preliminary analyses suggest that the virus is a reassortant of H7, N9 and H9N2 avian influenza viruses, and carries some amino acids associated with mammalian receptor binding, raising concerns of a new pandemic1,4,4. However, neither the source populations of the H7N9 outbreak lineage nor the conditions for its genesis are fully known2. Using a combination of active surveillance, screening of virus archives, and evolutionary analyses, here we show that H7 viruses probably transferred from domestic duck to chicken populations in China on at least two independent occasions. We show that the H7 viruses subsequently reassorted with enzootic H9N2 viruses to generate the H7N9 outbreak lineage, and a related previously unrecognized H7N7 lineage. The H7N9 outbreak lineage has spread over a large geographic region and is prevalent in chickens at live poultry markets, which are thought to be the immediate source of human infections. Whether the H7N9 outbreak lineage has, or will, become enzootic in China and neighbouring regions requires further investigation. The discovery here of a related H7N7 influenza virus in chickens that has the ability to infect mammals experimentally, suggests that H7 viruses may pose threats beyond the current outbreak. The continuing prevalence of H7 viruses in poultry could lead to the generation of highly pathogenic variants and further sporadic human infections, with a continued risk of the virus acquiring human-to-human transmissibility.

After the initial reports of H7N9 influenza infection in humans, field surveillance was conducted during 4–18 April 2013 in Wenzhou (Zhejiang province, 500 km south of Shanghai) and Rizhao (Shandong province, 600 km north of Shanghai), which both border the main outbreak region, and in Shenzhen (Guangdong province, 1,200 km south of Shanghai), an area that has not reported human cases (Supplementary Fig. 1). A total of 1,341 pairs of oropharyngeal and cloacal samples were collected from chickens, ducks, geese, pigeons, partridges and quail. A further 1,006 faecal and water samples from live poultry markets (LPMs), farms and wetlands were also collected (Supplementary Table 1). A total of 388 haemagglutinin–positive agents were isolated (10.5% of samples), of which 60 and 85 represented H7 and H9 influenza A viruses. The remaining positive isolates represented other subtypes of influenza A virus or avian paramyxovirus (Supplementary Table 1).

H7 influenza A viruses were only detected in Wenzhou and Rizhao, and only in LPMs. All H7 isolates from Rizhao were H7N9 viruses, whereas those from Wenzhou were all H7N7 viruses, except for two duck isolates that were H7N2 and H7N3 viruses. All H9 isolates were H9N2 viruses (80 from LPMs, 5 from farms). At LPMs in Wenzhou, the H7 virus was at its highest prevalence in chickens (10.1%; 46 out of 457), followed by ducks (2.4%; 3 out of 125) and pigeons (1.6%; 3 out of 188). In Rizhao, LPM H7N9 viruses were only found in chickens (0.7%; 8 out of 1,113). Of the chicken isolates, 100% of H7N9, 65.3% of H7N7 and 94.8% of H9N2 viruses were from oropharyngeal swabs (Supplementary Table 1), suggesting that these H7N9 and H7N7 viruses might replicate in the upper respiratory tract of terrestrial poultry, similar to the enzootic H9N2 viruses6.

These samples were sequenced to investigate the evolutionary history of avian influenza viruses implicated in the current outbreak of H7N9 infections of humans and poultry. Full genome sequences were obtained for 34 H7N7, 4 H7N9 and 19 H9N2 isolates. The H7 and N7/N9 genes of 16 mixed H7/H9 infections were sequenced (Supplementary Table 1), as were 3 H7N9 and 3 H7N7 samples that had multiple H9N2-like internal gene segments. The H7 haemagglutinin gene sequences of the H7N9 viruses isolated from chickens in Rizhao formed a tight monophyletic group (Fig. 1a, lineage ‘b’) with previously reported human and avian viruses from the current H7N9 outbreak. This was most closely related to a group comprising mainly H7N7 viruses obtained from Wenzhou chickens, ducks and pigeons (Fig. 1a, lineage ‘c’). All viruses isolated from chickens in these two groups had internal gene complexes that were closely related to those present in co-circulating H9N2 viruses.

To examine the genesis of these H7N9 and H7N7 viruses, we sequenced 197 archived isolates of H7, N9, N7 and H9N2 viruses, obtained during previous influenza surveillance between 2000 and 2013 in southern China (Supplementary Fig. 1). These sequences were analysed together with those obtained in our post-outbreak surveillance, plus all closely related sequences from public databases (see Methods). H7 influenza viruses from East Asian migratory waterfowl were introduced into domestic ducks in China on several occasions during the past decade (Fig. 1a and Supplementary Fig. 2). In 2009–2010, H7 viruses with live NA subtypes were found in duck farms and LPMs in Jiangxi, suggesting an epidemiological bridge from migratory birds to sentinel farm ducks and then to market birds7 (Supplementary Fig. 2). These introductions to domestic birds persisted for less than two years, except for the introduction of H7N3 viruses (initially isolated in Fujian and Zhejiang in 2010–2011) that led to the 2013 H7N9 outbreak lineage viruses and the H7N7 viruses from Wenzhou (Fig. 1a, lineage ‘a’).

Previous analyses have suggested that the N9 gene of the H7N9 outbreak lineage was derived from wild birds in Europe and Korea14.
Figure 1 | Phylogenies of haemagglutinin, neuraminidase and PB2 genes.

a-d, Phylogenies of H7 haemagglutinin (n = 46) (a), N9 neuraminidase (n = 34) (b), N7 neuraminidase (n = 25) (c) and PB2 (n = 93) (d) genes. Sequences reported in this study have their taxon names shown in bold. Genotypes of the influenza viruses are shown on the right (a–c) as eight coloured blocks representing each gene segment (from left to right: PB2, PB1, polymerase acidic, haemagglutinin, nucleoprotein, neuraminidase, matrix and non-structural; absent if the sequence is unavailable) with the colour indicating the subtype (for haemagglutinin, neuraminidase) or lineage (internal genes) indicated by the solid vertical line in d) of that segment. Bootstrap support values (%) from 1,000 pseudoreplicates are shown for selected lineages. Support values for lineages ‘a’–‘d’ were all 100%. The scale bar to the left of each tree represents 0.01 substitutions per site. Asterisks in b denote N9 sub-lineages linking the viruses of domestic ducks and wild birds. Host species are: Ck (chicken), Dk (duck), Gs (goose), Md (mallard), Nsh (Northern shoveler), Pg (pigeon), Pt (pintail), SCk (silkie chicken), SbD (spot-billed duck), Te (teal). Viruses from different hosts are indicated by: humans, circles; chickens, blue squares; ducks, purple triangles; geese, green squares.
However, our data show that, for this gene, more closely related H11N9 and H2N9 viruses are found in migratory wild birds in Hong Kong in 2010–2011 (Fig. 1b, asterisk). Phylogenetic linkage between the viruses of these birds and those of domestic ducks in China can be observed at least twice (Fig. 1b, asterisk) before the emergence of the N9 gene in the 2013 outbreak (Fig. 1b and Supplementary Fig. 3).

These data also allow us to reconstruct the genesis of the novel H7N7 lineage closely related to the H7N9 outbreak strain. The N7 of this lineage (Fig. 1c, lineage ‘d’; Supplementary Figs 5–10) arose from H7N7 viruses present in the domestic waterfowl of China since at least 2010 (Fig. 1c and Supplementary Fig. 4). After entering domestic ducks, these early H7N7 viruses and H7N3 viruses (which had internal genes from a sub-lineage, ZJ-5, of the wild bird viral gene pool) co-circulated in these birds in eastern China during 2010–2011 (Fig. 1) and gave rise to H7N7 viruses with ZJ-5 internal genes (WZ-Dk-H7N7; Fig. 1a). H7N7 viruses were also introduced to chickens and by reassortment obtained internal genes from co-circulating H9N2 viruses, generating the H7N7 viruses found in Wenzhou (WZ-Ck-H7N7; Figs 1 and 2).

The H9N2 viruses that contributed internal genes to the H7N9 and H7N7 lineages were formed by reassortment of the major H9N2 lineage in China (SH-F/98) with a Eurasian wild bird virus, from which the former acquired a polymerase basic 2 (PB2) segment, creating a separate sub-lineage (ZJ-HJ/07; Fig. 2 and Supplementary Figs 5–10). The internal genes of the ZJ-HJ/07 sub-lineage form two distinct subgroups (Supplementary Figs 5–10, marked ‘α’ and ‘β’), based on the PB2 segment phylogeny. Reassortment between α and β led to the internal gene cassettes of the H7N9 and H7N7 viruses having an NS gene from α and other internal genes from β (Fig. 2).

Even though the H7N9 and H7N7 chicken isolates obtained their internal gene cassettes from similar H9N2 viruses, all of their six internal genes formed distinct sublineages by subtype (Fig. 1d and Supplementary Figs 5–10). This indicates that the H7N9 and H7N7 viruses resulted from two independent interspecies transmissions to chickens, most likely from domestic ducks, and subsequent reassortment events. Some H7N9 and H7N7 viruses had one or two internal genes that fell outside these sublineages and instead clustered with other co-circulating H9N2 chicken viruses (Supplementary Figs 5–10). Thus, further reassortments with other H9N2 viruses continued to occur in chickens, increasing the genetic diversity of the H7N9 and H7N7 viruses.

Both the H7N9 and H7N7 lineages have three major sources of their eight gene segments (Fig. 2). Although the H7N7 virus suggests a path of two consecutive reassortment events (Fig. 2, yellow arrows), the evidence is not as clear for H7N9 viruses (Fig. 2, purple arrows). Two models are supported for the generation of the H7N9 outbreak lineage by molecular clock dating of divergence times: either a single reassortment event between mid-2011 and mid-2012, or a reassortment between H7 and N9 viruses in 2011 to generate an H7N9 ancestor, probably in ducks, followed by an interspecies transmission and a second reassortment with chicken H9N2 viruses from mid-2011 to late 2012 (Supplementary Fig. 11; substitution rates are shown in Supplementary Table 2). However, alternative reassortant pathways and hosts cannot be definitively excluded due to the limited numbers of H7 and N9 viruses identified during outbreak surveillance, and it is possible for either the H7 or N9 viruses to have first reassorted with H9N2 viruses.

The H7N9 and H7N7 viruses have similar, but independent evolutionary origins. Their haemagglutinin genes originated from H7 viruses that have been introduced to and established among the domestic ducks in China since 2010. At that time, these duck H7 viruses had internal genes from the ZJ-5 sub-lineage, as did H3N3, H1N2 and H11N3 duck viruses found in southern and eastern China during 2009–2013 (Fig. 1). An N9 virus that was a related precursor of the H7N9 viruses (Dk/IX/21714/11; H11N9), had polymerase genes from the ZJ-5 sub-lineage (Fig. 1b, asterisk; Fig. 1d), linking duck N9 viruses to the duck H7N3 precursors of the H7N9 lineage.

The haemagglutinin of the H7N9 outbreak lineage (including viruses isolated from Rizhao, but excluding Shanghai/1/2013 and Ck/ZJ/DTID/ZJU011/2013) had the amino acid substitution Gln235Leu/Ile (position 226 in H3 numbering) indicating that this substitution, which favours α2,6 sialic acid binding, seems to have originated in chickens. The equivalent substitution in chicken H9N2 viruses has been observed since the late 1990s and was linked to virus replication in the upper respiratory tract of birds6. The haemagglutinins of the H7N7 lineage from Wenzhou, and their closely related duck viruses (Fig. 1a), do not have this substitution. Of the other amino acid substitutions linked to α2,6 sialic acid binding seen in the H7N9 viruses (Gly195Val, Ala146Ser; 186, 138 in H3 numbering)6, only Gly195Val is seen in one H7N7 virus (Supplementary Fig. 12). However, two recent studies have shown that, despite these mutations, the H7 haemagglutinin has limited binding to human receptors8,9. Both the neuraminidase proteins of the H7N7 and H7N9 chicken viruses have overlapping deletions (20 amino acids in N7, positions 53–72; five amino acids in N9, positions 69–73) in the stalk region, which are often observed in influenza viruses established in terrestrial poultry10. Duck H7 viruses (N7, N3 and N2) from Wenzhou did not have a stalk deletion. The Glu627Lys and Asp701Asn substitutions in PB2 normally seen in mammalian viruses11 are only found in human H7N9 isolates (but not in all) and not in the chicken H7N9 and H7N7 viruses obtained here. Although it cannot be excluded, there is no evidence from these data to suggest that...
these viruses are mammalian adapted or that a mammalian intermediate host was involved in the human H7N9 infections.

The chicken H7N7 viruses carry only some of the molecular markers seen in the human H7N9 isolates (Supplementary Fig. 12), but they may still have the potential to infect humans or mammals. To assess infectivity in mammals, two groups (n = 6 each) of ferrets were inoculated with Chicken/Wenzhou/610/2013 (H7N7) at 10^5.5 or 10^6.5 plaque-forming units (p.f.u.). Both groups shed virus from 2 days post-inoculation (d.p.i.) (high dose) and 3 d.p.i. (low dose), and virus could be detected in rectal swabs at 4 d.p.i. in both groups (Supplementary Fig. 13). Infectious virus and positive nucleoprotein-stained cells could be detected in the nasal turbinate, and also in the trachea, lungs and hilar lymph nodes, with marginally higher levels from the high dose group at 3 and 5 d.p.i. (Supplementary Figs 13 and 14). This shows that the H7N7 lineage viruses can cause significant infection in mammals under experimental conditions, although virus shedding is lower than those of the 2009 pandemic H1N1 and 2013 human H7N9 viruses.

These findings provide a comprehensive picture of the creation and establishment of the H7N9 viruses that have infected humans. Domestic ducks seem to act as key intermediate hosts by acquiring and maintaining diverse influenza viruses from migratory birds, by facilitating the generation of different combinations of H7 and N9 or N7 subtype viruses, and by transmitting these viruses to chickens. After transmission, reassortment with enzootic H9N2 viruses formed the current H7N9 or H7N7 viruses seen in chickens. This probably led to outbreaks in chickens, resulting in the rapid spread of the novel reassortant H7N9 lineage through LPMs, which then became the source of human infections. The cessation of human infections after the closure of LPMs, after a precedent set during the Hong Kong H5N1 ‘bird flu’ incident in 1997 (ref. 14), strongly supports this proposition.

The detection of H7N7 chicken viruses in Wenzhou that, like H7N9 viruses, have the potential to infect mammals suggests the current pandemic threat extends beyond the H7N9 virus. Even though human infections with the H7N9 virus seem to be under control, it is too early to know whether this virus has been eradicated from chickens over a larger geographic region. It is possible that H7N9 or H7N7 viruses are still present and may become enzootic in poultry. To control H7N9 and related viruses ultimately, it is necessary to reconsider the management of LPMs in urban areas. Long-term influenza surveillance remains essential for early warning of novel reassortant viruses and interspecies transmission events.

**METHODS SUMMARY**

Oropharyngeal, cloacal or faecal samples were taken from poultry at LPMs, farms and from wild birds at wetlands in regions flanking the recent H7N9 influenza outbreak. These samples were assessed for the presence of influenza viruses and, from wild birds at wetlands in regions flanking the recent H7N9 influenza outbreaks. These samples were assessed for the presence of influenza viruses and, from wild birds at wetlands in regions flanking the recent H7N9 influenza outbreak.

**Full Methods**

and any associated references are available in the online version of the paper.

Received 22 May; accepted 1 August 2013.
Published online 21 August 2013.

1. Gao, R. et al. Human infection with a novel avian-origin influenza A (H7N9) virus. *N. Engl. J. Med.* 368, 1888–1897 (2013).
2. World Health Organization. Number of confirmed human cases of avian influenza A(H7N9) reported to World Health Organization; http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/ReportWebH7N9Number.pdf (2013).
3. Liu, D. et al. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. *Lancet* 381, 1926–1932 (2013).
4. Kageyama, T. et al. Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013. *Euro Surveill.* 18, 20453 (2013).
5. Hviidtndahl, M., Normile, D. & Cohen, J. Influenza. Despite large research effort, H7N9 continues to baffle. *Science* 340, 414–419 (2013).
6. Guo, Y. et al. Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. *Virology* 267, 279–288 (2000).
7. Duan, L. et al. Influenza virus surveillance in migratory ducks and sentinel ducks at Poyang Lake, China. *Influenza Other Resp. Viruses* 5 (suppl. 1), 65–68 (2011).
8. Thararakaman, K. et al. Glycan receptor binding of the influenza A virus H7N9 hemagglutinin. *Cell* 153, 1486–1493 (2013).
9. Xiong, X. et al. Receptor binding by an H7N9 influenza virus from humans. *Nature* 499, 496–499 (2013).
10. Cheung, C. L. et al. Establishment of influenza A virus (H6N1) in minor poultry species in southern China. *J. Virol.* 81, 10402–10412 (2007).
11. Yamada, S. et al. Biological and structural characterization of a host-adapting amino acid in influenza virus. *PLoS Pathog.* 6, e1001034 (2010).
12. Zhu, H. et al. Infectively, transmission, and pathology of human-isolated H7N9 influenza virus in ferrets and pigs. *Science* 341, 183–186 (2013).
13. Xu, J., Lu, S., Wang, H. & Chen, C. Reducing exposure to avian influenza H7N9. *Lancet* 381, 1815–1816 (2013).
14. Shortridge, K. F. et al. Interspecies transmission of influenza viruses: H5N1 virus and a Hong Kong SAR perspective. *Vet. Microbiol.* 74, 141–147 (2000).
15. Anisimova, M., Gil, M., Duftyward, J. F., Dessimoz, C. & Gascuel, O. Survey of branch support methods demonstrates accuracy, power, and robustness of fast likelihood-based approximation schemes. *Syst. Biol.* 60, 685–699 (2011).
16. Guindon, S. et al. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321 (2010).
17. Stamatakis, A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22, 2688–2690 (2006).
18. Drummond, A. J., Suchard, M. A., Xie, D. & Rambaut, A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol. Biol. Evol.* 29, 1969–1973 (2012).
19. Murrell, B. et al. Detecting individual sites subject to episodic diversifying selection. *PLoS Genet.* 8, e1002764 (2012).
20. Pond, S. L., Frost, D. S. & Muse, S. V. HyPhy: hypothesis testing using phylogenies. *Bioinformatics* 21, 676–679 (2005).
21. Pupko, T., Pe’er, I., Shamir, R. & Graur, D. A fast algorithm for joint reconstruction of ancestral amino acid sequences. *Mol. Biol. Evol.* 17, 890–896 (2000).

**Supplementary Information** is available in the online version of the paper.

**Acknowledgements**

We thank our colleagues from the Joint Influenza Research Centre (SUNMC/HKU) and the State Key Laboratory of Emerging Infectious Diseases for their technical assistance. This study was supported by the National Institutes of Health (National Institute of Allergy and Infectious Diseases contract HSN266200700005C), Li Ka Shing Foundation, the Area of Excellence Scheme of the University Grants Committee of the Hong Kong SAR (grant AoE/M-12/06), Shenzhen Peacock Plan High-End Talents Program (KQT20120203), the University Development Fund (HKU) and the Innovation and Technology Commission of the Hong Kong Government. T.T.-Y.L. was supported in part by a Newton International Fellowship of the Royal Society. MetaBio’s involvement was supported by the US Agency for International Development (USAID) Emerging Pandemic Threats Program, PRECEDE project, under the terms of Cooperative Agreement Number GH4-A-00-09-00010-00. The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under Grant Agreement No. 278433-PREDIMICS, ERC Grant agreement no. 260864 and the Wellcome Trust (grant 092807) to A.R. and S.J.L.

**Author Contributions**

Y.G., H.Z. and T.T.-Y.L. conceived the study; J.W., Y. Shen, B.Z., L.D., C.Y.-H., L., W., Z.D. and X.C. conducted surveillance; H.Z., J.W., C.-L., C.M., L.L., Y.C., L.Z., H.L., Y.L., A.F. and D.J.K., performed virus isolation, sequencing and animal experiments; T.T.-Y.L., A.R., O.G.P., H.Z., D.K.S., S.J.L., L.L.M.P., J.S.M.P., G.M.L., Y. Shi, R.G.W., R.J.W. and Y.G. contributed to the analysis; D.K.S. and T.T.-Y.L. wrote the manuscript; Y.G., H.Z., O.G.P. and A.R. edited the manuscript.

**Author Information**

All sequences generated by this study have been deposited in GenBank under accession numbers KF258943–KF260956 and KF297287–KF297322 (Supplementary Table 3). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to H.Z. (zhuhch@hku.hk) or Y.G. (gyuan@hku.hk).

©2013 Macmillan Publishers Limited. All rights reserved.
METHODS

Influenza virus surveillance in Zhejiang, Shandong and Guangdong provinces. LPMs, poultry farms and a wetland were surveyed for influenza viruses. If accessible, paired oropharyngeal and cloacal samples were taken from birds, otherwise, samples from isolated individual fecal droppings were collected. Some drinking water samples were also collected. Two cities that flanked the initial outbreak around Shanghai and a more distant city were surveyed (Supplementary Fig. 1), as these locations might have viruses related to the outbreak and/or viruses similar to those that led to the genesis of the outbreak virus. Sampling was conducted immediately before the closure of markets and/or culling of the birds.

Sampling was conducted at LPMs, farms and a wetland in Wenzhou, Zhejiang, a city approximately 500 km south of Shanghai, from 7 to 10 April 2013. Poultry from LPMs and farms were sampled at Zhishan, Shandong, a city approximately 600 km north of Shanghai, on 17 and 18 April 2013. Sampling was also carried out at LPMs in Shenzhen, a major urban centre adjoining Hong Kong and approximately 1,200 km south of Shanghai, on 4, 6 and 7 April 2013. Details of the numbers and types of poultry sampled are given in Supplementary Table 1. After approximately 1,200 km south of Shanghai, on 4, 6 and 7 April 2013. Details of the number of a tree branch were compared, and the differences represent the substitutions that occurred along that branch. Substitutions in the positively selected codons identified by the mixed effects model of evolution21 in Datamonkey22 are highlighted in Supplementary Fig. 12.

Animal infection experiments. Four- to five-month-old female Angora ferrets (Mustela putorius furo) were obtained through a laboratory ferret breeding program at the Wuxi Sangosho and confirmed to be influenza virus free by virus isolation in Madin–Darby canine kidney cells (ATCC) from nasal washes and rectal swabs and sero-negative by HAI assay against contemporary swine and human influenza viruses (swine: H1 and H3; human: seasonal H1N1, H3N2 and influenza B), and avian H5N1 and H9N2 that are enzootic in China, and the chicken H7N7 virus to be tested in this study. A minimum number of animals necessary to obtain reproducible results were used according to the ethical guidelines. Ferrets were randomly allocated across the treatment groups, to which investigators were not blinded.

A group of six ferrets were intranasally inoculated with a dose of 10^6.5 p.f.u. (or 10^8 to 10^50% tissue culture infectious dose (TCID_{50}) of the Chicken/Wenzhou/610/2013 (H7N9) virus (high dose group), and a second group were inoculated with 10^6 p.f.u. (or 10^8 TCID_{50}) of viruses (low dose group). Nasal and rectal washes were taken daily from each individual and titrated using standard TCID_{50} assays. At 3 and 5 d.p.i., three ferrets from each group were euthanized and tissues from each major organ (pulmonary lobes, nasal turbinates, upper and lower trachea and laryngeal lymph nodes) were collected for virus titration (TCID_{50} assays), RNA extraction and qRT–PCR, and studies of tissue pathology (hematoxylin and eosin and viral nucleoprotein staining), following protocols described previously23. All animal experiment protocols were reviewed and approved by the Institutional Ethical Review Board (IERB) of Shantou University Medical College (ref no. SUMC2013-111), and all animal care protocols were in accordance with the approved guidelines of the University of the Use of Live Animals in Teaching & Research. All experiments with H7N7 and H7N9 viruses were conducted in biosafety level 3 (BSL3) facilities, using enhanced BSL3 practices for the animal work and following practices in the approved institutional guidelines.
27. Lam, T. T. et al. Systematic phylogenetic analysis of influenza A virus reveals many novel mosaic genome segments. *Infect. Genet. Evol.* **18**, 367–378 (2013).

28. Delport, W., Poon, A. F., Frost, S. D. & Kosakovsky Pond, S. L. Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. *Bioinformatics* **26**, 2455–2457 (2010).