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# Sequential Reassortments Underlie Diverse Influenza H7N9 Genotypes in China

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## SUMMARY

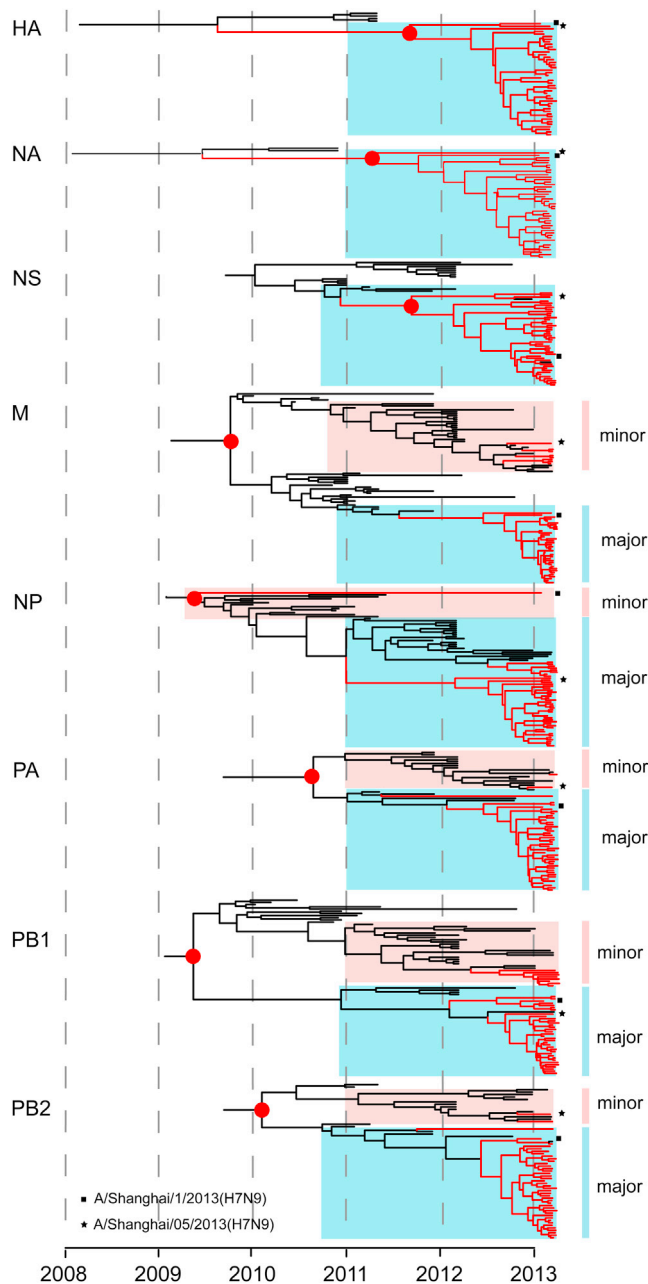
Initial genetic characterizations have suggested that the influenza A (H7N9) viruses responsible for the current outbreak in China are novel reassortants. However, little is known about the pathways of their evolution and, in particular, the generation of diverse viral genotypes. Here we report an in-depth evolutionary analysis of whole-genome sequence data of 45 H7N9 and 42 H9N2 viruses isolated from humans, poultry, and wild birds during recent influenza surveillance efforts in China. Our analysis shows that the H7N9 viruses were generated by at least two steps of sequential reassortments involving distinct H9N2 donor viruses in different hosts. The first reassortment likely occurred in wild birds and the second in domestic birds in east China in early 2012. Our study identifies the pathways for the generation of diverse H7N9 genotypes in China and highlights the importance of monitoring multiple sources for effective surveillance of potential influenza outbreaks.

## INTRODUCTION

Unlike the previous H7N9 viruses, which had never been reported to infect humans (Parry, 2013), the novel H7N9 viruses in the current outbreak in China (Gao et al., 2013; Li et al., 2013), referred to as A/China/2013(H7N9), have caused more than 130 human infections and 40 deaths since the report of human infections in east China in late March 2013 (World Health Organization, 2013; Gao et al., 2013). The potential of A/China/2013(H7N9) viruses to evolve into strains more readily transmissible among humans (Zhu et al., 2013) and the emergence of drug-resistant strains (Hu et al., 2013) are a significant global

concern (Schenk et al., 2013; Uyeki and Cox, 2013). A thorough understanding of the evolutionary history of the A/China/2013(H7N9) viruses that emerged in east China is of critical importance for formulating proper measures for surveillance and control of these viruses.

Based on phylogenetic analysis of seven isolates from humans and domestic birds (Chen et al., 2013; Gao et al., 2013; Shi et al., 2013), it has been proposed that the internal genes of the A/China/2013(H7N9) viruses are derived from avian H9N2 viruses that circulated recently in China (Gao et al., 2013; Shi et al., 2013; Kageyama et al., 2013; Liu et al., 2013), while the genes encoding viral hemagglutinin (HA) and neuraminidase (NA) are from avian H7N7/H7N9 viruses of Eurasian origin (Kageyama et al., 2013; Liu et al., 2013). Recently, Lam et al. further proposed that the HA of the A/China/2013(H7N9) viruses was from the H7 viruses, which probably transferred from domestic duck to chicken populations in China, while the NA of the A/China/2013(H7N9) viruses was more likely related to H11N9 and H2N9 viruses, which had been found in migratory birds in Hong Kong in 2010–2011 (Lam et al., 2013). Beyond this, however, little is known about the details of how the viruses evolved, including the donor viruses, timing, and pathways of reassortment. Moreover, among the seven sequenced A/China/2013(H7N9) viruses, the nucleocapsid (NP) from one virus (A/Shanghai/1/2013) (Kageyama et al., 2013) and polymerase basic protein 1 (PB1) from another virus (A/pigeon/Shanghai/S1069/2013) (Shi et al., 2013) appear to belong to two different lineages from the other six gene segments. This genetic divergence is even more evident when 38 new H7N9 isolates were sequenced (see Results below). How such genetic divergence was generated remains unknown. We have now further sequenced 42 H9N2 viruses isolated from domestic and wild birds in east China from 2012 to 2013. An in-depth phylogenetic analysis of all available H7N9 and H9N2 viruses has enabled us to define the possible donor viruses, the timing, and pathways of reassortment through which the A/China/2013(H7N9) viruses were evolved.



**Figure 1. An Integrated Picture of Genomic Divergence of A/China/2013(H7N9) Viruses**

The phylogenetic clades that included the 45 A/China/2013(H7N9) viruses are taken from the dated phylogeny of each of the eight gene segments constructed by molecular phylogenetic analysis and are further aligned onto the same timescale. The red and black branches represent the 45 A/China/2013(H7N9) viruses and other related viruses, respectively. The inferred most recent common ancestors (MRCA) of the 45 A/China/2013(H7N9) viruses are indicated by red dots. Phylogenetic clades of eight gene segments are highlighted with colored boxes. A/Shanghai/1/2013(H7N9) and A/Shanghai/05/2013(H7N9) viruses are indicated with black squares and stars, respectively. See also Figure S1 and Table S1.

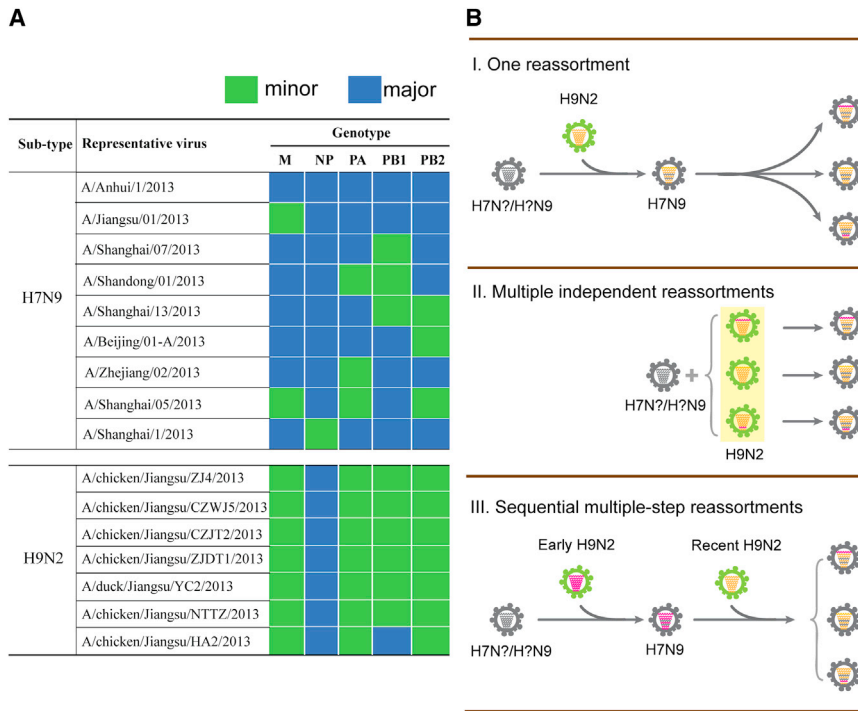
## RESULTS

### Genetic Divergence of Internal Genes of A/China/2013(H7N9) Viruses

We used molecular clock analysis (Drummond and Rambaut, 2007) to compute the phylogenies and the time of most recent common ancestor (tMRCA) (Smith et al., 2009), or timing of divergence, for each gene segment of the 45 A/China/2013(H7N9) viruses and other related influenza viruses. The phylogenetic clades that include A/China/2013(H7N9) viruses were identified based on sequence similarity, timing of divergence, and average genetic distance (see Experimental Procedures) (Figure S1). To better compare the timing and pattern of divergence, we further integrated the evolutionary analysis of each gene segment of the 45 A/China/2013(H7N9) viruses onto the same timescale. As shown in Figure 1, gene segments HA, NA, and NS each belonged to a single clade with moderate divergence. The tMRCA of HA, NA, and NS were estimated to be June, September, and July 2011, respectively (Table S1), which were not significantly different based on Bayes factor (BF) (Kass and Raftery, 1995) tests. However, each of the other five gene segments (M, NP, PA, PB1, and PB2) was much more divergent and can be grouped into two clades. The tMRCA of the two clades was dated to be between mid-2009 and mid-2010 (Figure 1 and Table S1), which is significantly earlier than tMRCA of HA, NA, and NS. The two clades for each of the five internal genes included a significantly different number of A/China/2013(H7N9) viruses and thus are referred to as minor (m) and major (M) clades. Based on this, the 45 H7N9 viruses were classified into nine genotypes, namely viruses containing no minor gene segment (M), minor clade of NP (m-NP), minor clade of M (m-M), minor clade of PA (m-PA), minor clade of PB1 (m-PB1), minor clade of PB2 (m-PB2), minor clades of PA and PB1 (m-PA|PB1), minor clades of PB1 and PB2 (m-PB1|PB2), and minor clades of M, PA, and PB2 (m-M|PA|PB2) (Figure 2A and Table S2). Of the 45 H7N9 viruses, 24 belonged to M, 18 contained the minor clade of only one internal gene, and 3 contained minor clades of two or three internal genes. Thus, compared to HA, NA, and NS, the other five internal gene segments show much greater divergence. The diverse genotypes of the A/China/2013(H7N9) viruses suggest complex genetic events involved in their evolution.

### Pathways for A/China/2013(H7N9) Evolution

We investigated possible genetic events that contribute to the generation of A/China/2013(H7N9) viruses with diverse genotypes. Given the significant difference in the timing of divergence between the five internal genes and HA/NA/NS, it is unlikely that all A/China/2013(H7N9) viruses were evolved from a single ancestor virus that was generated by an one-time reassortment event between a H7N7/H7N9 virus and a H9N2 virus (Figure 2B, pathway I). Rather, the A/China/2013(H7N9) viruses were likely generated through either multiple independent reassortments or sequential multistep reassortments with different H9N2 viruses (Figure 2B, pathways II and III). In pathway II, the internal genes of A/China/2013(H7N9) were inherited as a whole from H9N2 viruses, which requires the donor H9N2 viruses carrying all of the nine different genotypes. To test this prediction, we determined the whole-genome sequences of 42 H9N2 viruses



**Figure 2. Possible Evolutionary Pathways toward Generation of Diverse Genotypes of A/China/2013(H7N9) Viruses**

(A) Summary of genotypes for the 45 A/China/2013(H7N9) viruses and their potential donor-like H9N2 viruses. The minor and major clades of each of the five internal genes (M, NP, PA, PB1, and PB2) are represented by green and blue rectangles. The genotype of an H7N9 or H9N2 virus is an ensemble of the clades of the five internal genes.

(B) Proposed reassortment pathways for the generation of the diverse genotypes of A/China/2013(H7N9) viruses. H7N9/H?N9 represent the donor viruses that contributed the HA and NA genes for A/China/2013 viruses. See also Figure S3.

isolated from domestic chicken and ducks and a wild bird in east China from 2012 to 2013 (see [Experimental Procedures](#)) (Table S3). Although the newly sequenced H9N2 viruses were more closely related to A/China/2013(H7N9) viruses than other previously sequenced H9N2 viruses (see the viruses marked in blue in [Figures 3](#) and [S2](#)), we did not observe the similar diverse genotypes in the internal genes in the newly isolated H9N2 viruses as in A/China/2013(H7N9) viruses ([Figures 2A](#) and [S3](#)). Thus, the different genotypes of the internal genes of A/China/2013(H7N9) viruses are unlikely to be inherited as a whole from the H9N2 viruses, leaving the sequential multistep reassortments with distinct H9N2 viruses as the likely pathway for the generation of diverse A/China/2013(H7N9) genotypes.

#### Identification of Donor-like Viruses Involved in the Most Recent Reassortment Event

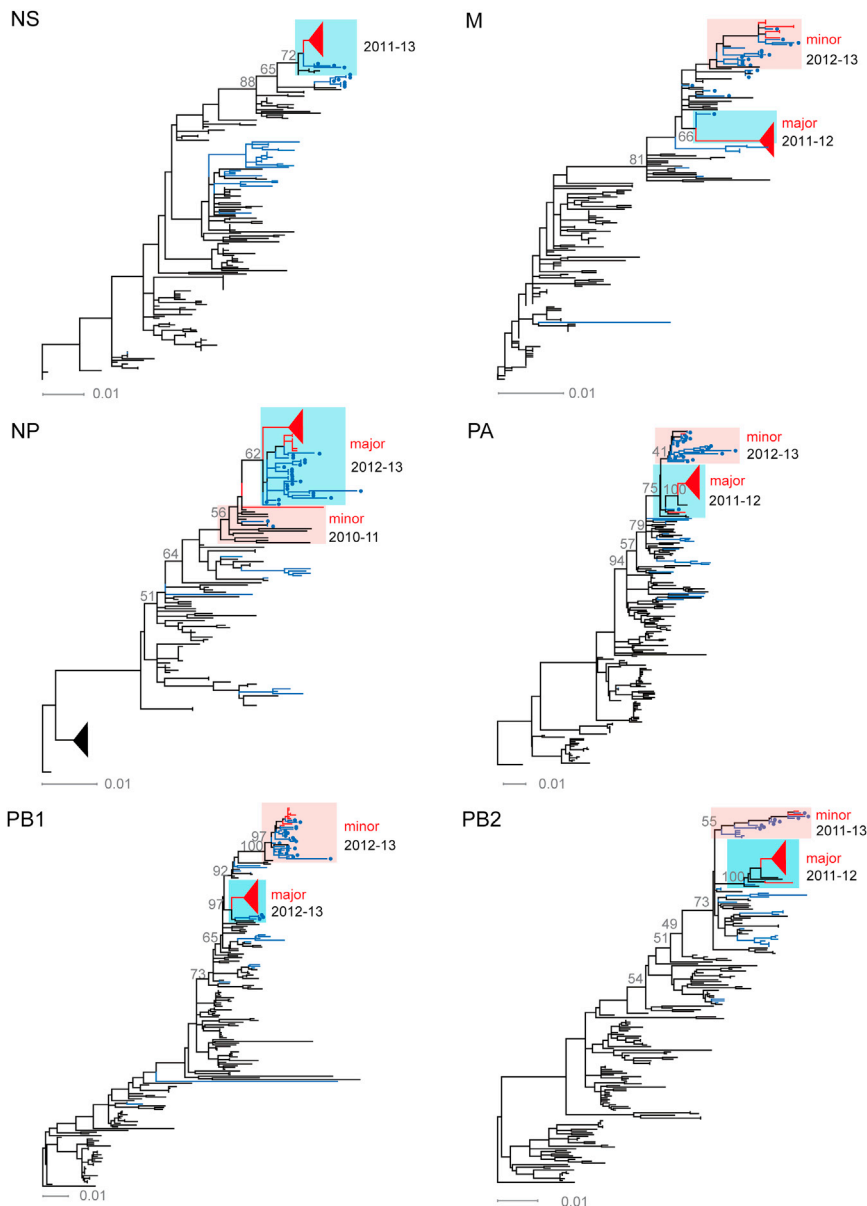
Since it is hard to identify the exact viruses that offer gene segments for a reassorted virus, the viruses of high genetic similarity to the reassorted virus can be regarded as donor-like viruses. To identify donor-like viruses and further determine the reassortment pathways in the generation of the A/China/2013(H7N9) viruses, we first examined how the two clades of each of the five internal genes were derived from H9N2 viruses. We noticed that for each of the five internal genes, one clade contained H9N2 viruses isolated in 2013, while the other clade contained H9N2 viruses from 2012 and earlier ([Figure 3](#)). Notably, the H9N2 viruses from 2013 were closely related to the A/China/2013(H7N9) viruses in the same clade, whereas the H9N2 viruses from 2012 or earlier had a greater divergence from the A/China/2013(H7N9) viruses in the same clade. Based on the assumption that the H9N2 viruses in the same clade can be potential donor-like viruses, all possible donor-like H9N2 viruses for either clade of the five gene segments of the

ing that they have likely contributed these internal gene segments to the A/China/2013(H7N9) viruses.

For the minor clade of NP and the major clades of M, PA, PB1, and PB2, we were unable to identify any virus among 119 fully sequenced H9N2 viruses isolated from domestic birds and a wild bird during 2008–2013 that contained all the five gene segments for the five clades ([Figure S3](#)). However, A/Shanghai/1/2013(H7N9), referred to as SH/1, had the gene segments for the five clades that were genetically close to the H9N2 viruses from 2012 or earlier ([Figure 3](#)). Moreover, based on phylogenetic analysis, the HA and NA of SH/1 virus appeared to have an earlier timing of divergence among the A/China/2013(H7N9) viruses ([Figures 1](#) and [S1](#)). Therefore, the SH/1-like virus is likely another donor-like virus in the most recent reassortment event, which contributed not only HA and NA but also the gene segments that define the minor clade of NP and the major clades of M, PA, PB1, and PB2.

Similar to SH/1, A/Shanghai/05/2013(H7N9) virus, referred to as SH/05, also had HA and NA from an earlier timing of divergence. However, almost all the internal genes (except PB1) were closely related to those of A/chicken/Jiangsu/ZJ4/2013(H9N2)-like viruses. Thus, SH/05 is likely a product of the most recent reassortment event. Furthermore, we noticed that all six internal genes of A/Jiangsu/HA2/2013(H9N2) virus were almost identical to those of SH/05, with sequence similarities from 98.33% to 99.88%, but not to other 2013 H9N2 isolates ([Figures 2A](#) and [S2](#)). Thus, the A/Jiangsu/HA2/2013(H9N2) virus itself appears to be a counterpart reassortant between SH/1-like H7N9 virus and A/chicken/Jiangsu/ZJ4/2013(H9N2)-like virus, providing further evidence for the most recent reassortment event involving these two viruses. The combination of the internal genes from A/chicken/Jiangsu/ZJ4/2013-like H9N2 viruses and SH/1-like H7N9 viruses in the most recent reassortment





**Figure 3. Phylogenetic Relationships of the Six Internal Genes among 45 A/China/2013(H7N9) Viruses and Closely Related H9N2 Viruses**

Phylogenetic clades of each gene segment are highlighted with colored boxes. The isolation time of H9N2 viruses in each clade is given. Red triangles represent many H7N9 viruses that are tightly grouped together. Blue dots and lines indicate our newly sequenced H9N2 viruses isolated from east China during 2009–2013. The red and black branches represent the 45 A/China/2013(H7N9) viruses and other related viruses, respectively. Supporting bootstrap values are given in numbers. All branch lengths were drawn to a scale of nucleotide substitutions per site. See also Figure S2.

recent surveillance of avian influenza viruses from wild birds in east China, A/Mallard/Zhejiang/136/2013(H9N2), which was genetically closer to SH/1 virus than H9N2 viruses from domestic birds (Figure S3). These results, although preliminary, suggest that wild birds are likely the source of donor H9N2 viruses for the internal genes of the SH/1-like viruses.

As for the origin of HA and NA of SH/1-like virus, although previous reports have identified HA of A/duck/Zhejiang/12/2011(H7N3) and NA of A/wild bird/Korea/A14/2011(H7N9) as the genetically closest relatives (Gao et al., 2013; Kagayama et al., 2013; Liu et al., 2013), they still showed significant genetic divergence. The tMRCAs for HAs of SH/1-like virus and A/duck/Zhejiang/12/2011(H7N3) virus and the tMRCAs for NAs of SH/1-like virus and A/wild bird/Korea/A14/2011(H7N9) were both estimated to be May 2009 (Figure S1 and Table S1). Based on their significant divergence, it is not possible to determine

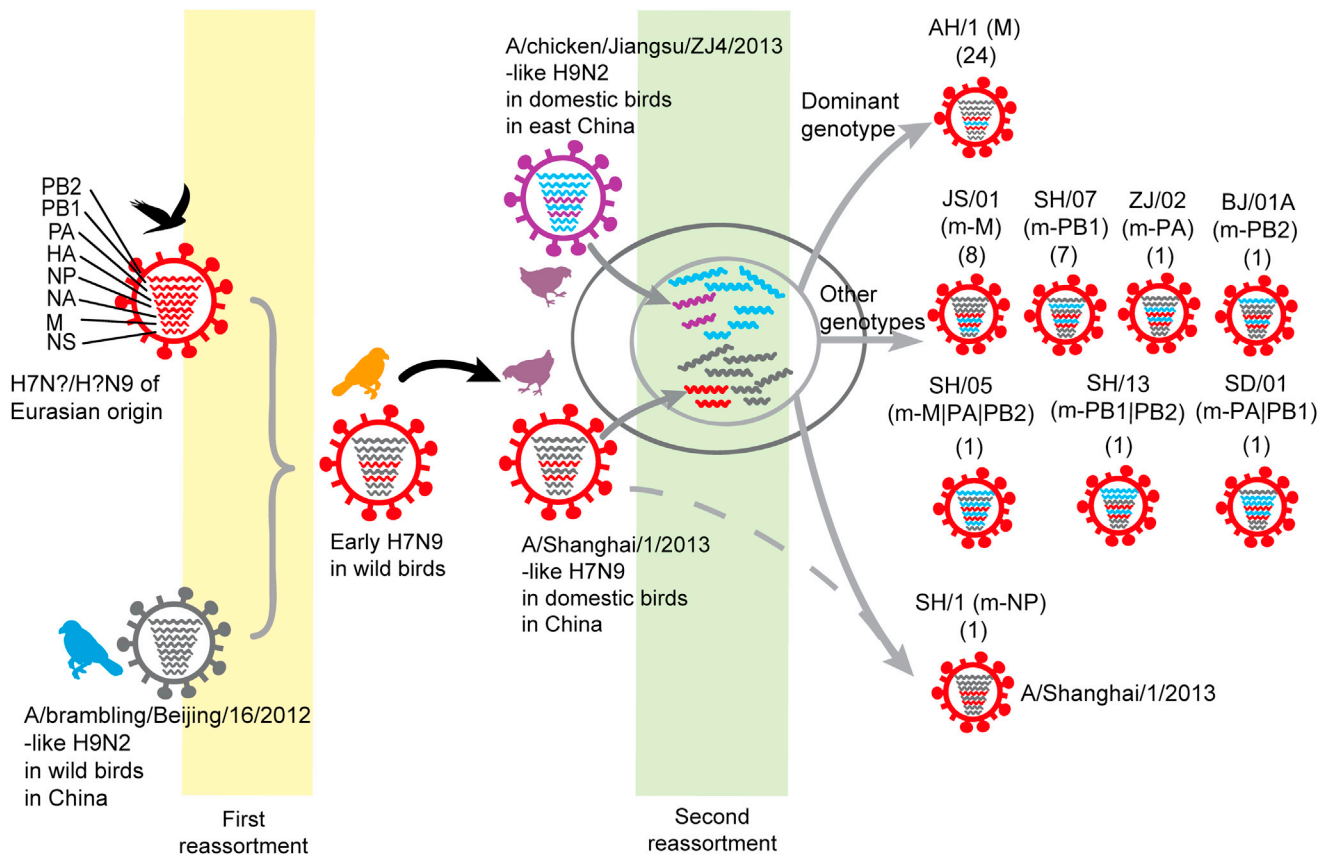
event also underlies the diverse genotypes of the A/China/2013(H7N9) viruses.

### Origin of SH/1(H7N9)-like Virus

We further sought to determine how SH/1(H7N9)-like virus was derived from the previous reassortment event. We were not able to reliably identify any virus among 119 fully sequenced H9N2 viruses isolated from domestic birds during 2008–2013 that shared the same clades of all five internal gene segments as SH/1 virus (Figure S3). However, through phylogenetic analysis of individual internal genes (Figures S1 and S2) and all six internal genes together, we identified A/brambling/Beijing/16/2012(H9N2), the only H9N2 virus isolated from a wild bird with whole genome information available, as the most closely related to SH/1 with sequence similarities from 97.33% (NP) to 99.16% (PB2). Furthermore, we identified another H9N2 virus from a

with confidence that A/duck/Zhejiang/12/2011(H7N3) and A/wild bird/Korea/A14/2011(H7N9) are direct donor-like viruses for SH/1-like virus.

To gain insight into the origin of the HA and NA, we examined the avian influenza surveillance data from both domestic and wild birds in east China (see Experimental Procedures). Except the A/China/2013(H7N9) viruses detected in early 2013, few viruses of H7 subtype or N9 subtype were detected in domestic birds in the recent years (Figure S4 and Table S4), while other subtypes were found at much higher frequencies. These results suggest that the HA and NA of SH/1-like viruses were probably not from domestic birds in China but from wild birds for which there was a lack of sufficient surveillance data. These observations suggest that the reassortment event to generate the SH/1-like viruses likely occurred between two viruses from wild birds. Thus, the generation of A/China/2013 viruses involves at



**Figure 4. Sequential Two-Step Reassortments for the Generation of Diverse A/China/2013(H7N9) Genotypes**

The donor-like viruses and hosts for the first and second reassortment events are indicated. Genotypes of all 45 A/China/2013(H7N9) viruses are shown as an outcome of the second reassortment in 2012.

least two reassortment events with distinct H9N2 viruses at different times and in different hosts.

#### Timing of A/China/2013(H7N9) Virus Generation

We further investigated when the two reassortment events occurred during the generation of A/China/2013(H7N9) viruses. Given that the first assembly of the virus most likely occurred in wild birds, the lack of sufficient viruses isolated from wild birds precludes the accurate dating of the first reassortment event. However, the tMRCAs of the five internal genes for the donor-like H9N2 viruses and the reassorted H7N9 viruses were all dated to 2012 (Table S1). Thus, the most recent reassortment event likely occurred in early 2012.

#### DISCUSSION

Through extensive influenza surveillance in humans, poultry, and wild birds in east China and in-depth evolutionary analysis of sequence data, we have reconstructed evolutionary pathways toward generation of the novel A/China/2013(H7N9) viruses (Figure 4). The at least two steps of sequential reassortment with distinct H9N2 viruses provide a mechanism for the generation of diverse A/China/2013(H7N9) genotypes. The first reassortment event likely happened between A/brambling/Beijing/16/2012(H9N2)-like viruses and H7N9?/H7N9 viruses in wild birds,

generating the SH/1-like viruses. The resulting viruses likely transmitted to domestic birds in China and further underwent a second reassortment with more recent H9N2 viruses that were circulating in poultry in east China. In the second (recent) reassortment, which was estimated to have occurred in early 2012, the reassortment of gene segments from SH/1-like viruses and A/chicken/Jiangsu/ZJ4/2013(H9N2)-like viruses leads to the emergence of diverse genotypes of the novel H7N9 viruses in China. It is interesting to note that SH/1 and SH/05 viruses show notable divergence from the majority of other H7N9 viruses in HA and NA (Figure 1), indicating that in addition to reassortment, gene mutations contribute to the adaptation of the viruses in poultry. Given that the generation of influenza viruses often involves both frequent reassortments and rapid gene mutations (Marshall et al., 2013; Nelson et al., 2008; Neumann et al., 2009; Rambaut et al., 2008), we suggest that in addition to the two major reassortment events, the gene mutations as well as other minor reassortment events could contribute to the genetic diversity of the novel A/China/2013(H7N9) viruses.

Due to the difficulty in obtaining sufficient viral samples from wild birds, it was not possible to determine precisely the source of the HA and NA of A/China/2013(H7N9) viruses. Despite extensive efforts, we have not been able to identify donor-like viruses for HA and NA. Nevertheless, by integrating the surveillance data from infections in humans, poultry, and wild birds, we suggest

that the HA and NA were most likely derived from viruses carried by wild birds. This not only re-enforces the important role of wild birds in the emergence of novel influenza viruses but also highlights the necessity of integrating multiple data sources for effective influenza surveillance.

## EXPERIMENTAL PROCEDURES

To identify the specific evolutionary pathways toward the origins of the novel H7N9 viruses in recent outbreaks in China, we have carried out a large-scale surveillance of H7N9 viruses and their donor H9N2 viruses in humans, poultry, and wild birds in east China. The genomic sequences of 35 A/China/2013(H7N9) viruses isolated from humans, 3 A/China/2013(H7N9) viruses isolated from poultry, 41 H9N2 viruses isolated from domestic birds during 2009–2013, and 1 H9N2 virus isolated from wild birds in April 2013 were determined (see [Supplemental Experimental Procedures](#)). Seven previously reported A/China/2013(H7N9) viruses were downloaded from the Global Initiative on Sharing Avian Influenza Data (GISAID) database (<http://platform.gisaid.org/epi3/frontend#200476>) while other sequences used for evolutionary analysis were downloaded from Influenza Virus Resources (Bao et al., 2008). Phylogenetic trees were constructed for each gene segment (PB2, PB1, PA, HA, NP, NA, M, and NS) and the whole genome with MEGA version 5 (Tamura et al., 2011) independently (see [Supplemental Experimental Procedures](#)). For molecular clock analysis, the Bayesian Markov chain Monte Carlo approach in BEAST v1.7.5 (Drummond and Rambaut, 2007) was used to estimate the rate of viral evolution and dates of divergence for each genomic segment of A/China/2013(H7N9) viruses (see [Supplemental Experimental Procedures](#)). The phylogenetic clades of the six internal gene segments of A/China/2013(H7N9) viruses were defined based on a genotype classification method that considers topologies of phylogenetic trees, genetic distances, and sequence similarities among viruses (Dong et al., 2011). In our analysis, we identified A/China/2013(H7N9) clade as a phylogenetic branch within which the timing of divergence of all the viruses was within two and a half years and the average genetic distance between viruses was less than 0.025 (Figures S1 and S2). For each gene segment, the H9N2 viruses that belonged to the same phylogenetic clade of A/China/2013(H7N9) viruses were identified as potential donor-like H9N2 viruses for the given gene segment of these H7N9 viruses.

## ACCESSION NUMBERS

The GISAID accession number of the sequences reported in this paper are: EPI466535, EPI466531, EPI466528, EPI466521, EPI466515, EPI466509, EPI466502, EPI466494, EPI466480, EPI466472, EPI466456, EPI466448, EPI466440, EPI466432, EPI466424, EPI466416, EPI466408, EPI466400, EPI466392, EPI466384, EPI466376, EPI466368, EPI466360, EPI466352, EPI466344, EPI466336, EPI466328, EPI466320, EPI466312, EPI466304, EPI466296, EPI466288, EPI466280, EPI466272, EPI466264, EPI466256, EPI466248, EPI466240, EPI466464, EPI466212, EPI466486, EPI466529, EPI466519, EPI466513, EPI466507, EPI466500, EPI466492, EPI466478, EPI466470, EPI466454, EPI466446, EPI466438, EPI466430, EPI466422, EPI466414, EPI466406, EPI466398, EPI466390, EPI466382, EPI466374, EPI466366, EPI466358, EPI466350, EPI466342, EPI466334, EPI466326, EPI466318, EPI466310, EPI466302, EPI466294, EPI466286, EPI466278, EPI466270, EPI466262, EPI466254, EPI466246, EPI466239, EPI466462, EPI466213, EPI466489, EPI466534, EPI466533, EPI466524, EPI466523, EPI466503, EPI466496, EPI466482, EPI466474, EPI466458, EPI466450, EPI466442, EPI466434, EPI466426, EPI466418, EPI466410, EPI466402, EPI466394, EPI466386, EPI466378, EPI466370, EPI466362, EPI466354, EPI466346, EPI466338, EPI466330, EPI466322, EPI466314, EPI466306, EPI466298, EPI466290, EPI466282, EPI466274, EPI466266, EPI466258, EPI466250, EPI466242, EPI466466, EPI466216, EPI466483, EPI466530, EPI466526, EPI466517, EPI466516, EPI466506, EPI466497, EPI466495, EPI466475, EPI466467, EPI466451, EPI466443, EPI466443, EPI466435, EPI466427, EPI466419, EPI466411, EPI466403, EPI466395, EPI466387, EPI466379, EPI466371, EPI466363, EPI466355, EPI466347, EPI466339, EPI466331, EPI466323, EPI466315, EPI466307, EPI466299, EPI466291, EPI466283,

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## SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2013.09.001>.

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