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Genetic characterization of a rare H12N3 avian influenza virus isolated from a green-winged teal in Japan

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Abstract This study reports on the genetic characterization of an avian influenza virus, subtype H12N3, isolated from an Eurasian green-winged teal (*Anas crecca*) in Japan in 2009. The entire genome sequence of the isolate was analyzed, and phylogenetic analyses were conducted to characterize the evolutionary history of the isolate. Phylogenetic analysis of the hemagglutinin and neuraminidase genes indicated that the virus belonged to the Eurasian-like avian lineage. Molecular dating indicated that this H12 virus is likely a multiple reassortant influenza A virus. This is the first reported characterization of influenza A virus subtype H12N3 isolated in Japan and these data contribute to the accumulation of knowledge on the genetic diversity and generation of novel influenza A viruses.

Keywords Avian influenza · Wild birds · H12N3 · Genetic · Japan

Avian influenza viruses (AIVs) are classified on the basis of the antigenic properties of two surface proteins, the hemagglutinin (HA) and neuraminidase (NA), by which the viruses are grouped into 16 subtypes of HA (H1–H16) and 9 NA (N1–N9) [1]. In addition, AIVs are also classified by their pathogenicity in chickens, which differentiates highly pathogenic avian influenza (HPAI) virus and low pathogenic avian influenza (LPAI) virus. To date, only H5 and H7 subtypes of AIVs have occasionally become highly pathogenic viruses that caused disease with mortality as high as 100 % in poultry [2, 3]. In wild birds, all 16 HA and 9 NA subtypes have been detected [4], a primary reason why wild migratory water birds are considered reservoirs of AIV in nature [5] where they usually get infected, shed and carry virus without showing clinical signs [5–7]. The movement of wild birds creates favorable conditions for dispersal of influenza viruses along their migratory routes [5, 8–10] and contributes to the rapid emergence of novel strains both in wild bird and other hosts. Therefore, it is important to continuously conduct surveillance of AIVs in the wild bird population.

In Japan, current surveillance programs for AIVs, especially those of HPAI viruses, focus on the monitoring of avian populations for deaths and the sampling of live wild birds at several wetlands [11–17]. These surveillance activities not only provide data on the extent of HPAI H5N1 virus circulation in wild birds, but also certainly improve our knowledge of the ecology of LPAI viruses in wild migratory birds. As a result of surveillance, several specific subtypes of AIVs such as H3, H4, and H6 are frequently detected among wild birds, whereas H1, H2 H7, H10, and H11 are less frequently detected. On the other hand, H12 and the other HA subtypes are rarely detected [10]. In this study, we provide the first report of an H12N3 virus in Japan isolated from an Eurasian green-winged teal in eastern Hokkaido and describe a likely scenario for its genesis.

During AIV surveillance of wild birds in 2009, an AIV subtype H12N3 was detected in the cloacal sample of a

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green-winged teal, which was captured at Lake Komuke in Hokkaido, Japan (44° 16′ 30″ N, 143° 29′ 26″ E). The cloacal swab was collected by licensed bird banders, who also identified bird species. The swab was placed in sterile tubes containing a virus transport medium (VTM) (M4RT; Remel, Inc., Lenexa, KS) and stored at −80 °C until use. The virus was successfully recovered from the cloacal swab of the green-winged teal using embryonated chicken eggs for virus isolation and the hemagglutination test for viral detection from allantoic fluids of the eggs obtained 72 h post inoculation of the sample. The virus was identified as influenza A virus of H12N3 subtype by virological and genetic analyses performed as previously described [11]. Hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests were conducted to confirm the subtype of recovered virus using antisera of A/duck/Albeta/60/76(H12N5) and A/duck/Hong Kong/820/80(H5N3), respectively. The recovered virus showed reaction with antisera of the H12N5 at HI titer of 1:80 and it was inhibited 91 % by antisera of A/duck/Hong Kong/820/80(H5N3). After confirming the subtype by serological tests, the virus was designated as A/green-winged teal/Japan/9KS0643/2009(H12N3).

The entire genome sequence of A/green-winged teal/Japan/9KS0643/2009(H12N3) was obtained by Sanger sequencing using universal primer sets [18, 19], and additional primers designed in our laboratory to amplify the full length of influenza A virus (primer information available upon the request). The sequencing was performed using a BigDye terminator ver. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed with an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems). Viral sequences were deposited in GenBank under accession numbers KM668060–KM668067. Genetic analysis of HA amino acid sequence of the A/green-winged teal/Japan/9KS0643/2009 (H12N3) indicated that the virus has an HA cleavage site sequence, VPQVQDR/GLF, indicating characteristic of LPAI virus. The virus maintains the avian receptor-binding specificity at positions Q-222 and G-224 (H5 numbering) [20]. The PB2 gene of this virus contains E-158, E-627, and D-701, also suggesting features of low pathogenicity in mammals [21, 22].

All gene segments of the A/green-winged teal/Japan/9KS0643/2009 (H12N3) were compared with those of other AIV sequences available in GenBank by BLASTN using GENETYX ver.10 software (GENETYX Corp., Tokyo Japan). As shown in Table 1, the HA gene of A/green-winged teal/Japan/9KS0643/2009 (H12N3) was closely related to that of A/duck/Tsukuba/212/2006 (H12N5), sharing 98.93 % nucleotide identity. The NA gene of the isolate was similar to that of A/duck/Hokkaido/327/2009 (H1N3) with 99.43 % nucleotide identity. Nucleotide identity comparisons for internal genes revealed that the M gene sequence of the isolate was completely identical to that of A/wild duck/Korea/A323/2009 (H10N1). The NS gene of the isolate was closely related to that of A/ruddy shelduck/Mongolian/894V/2009(H4N3), sharing 99.30 % nucleotide identity. The other internal genes were similar to those of Japanese AIV strains. Among those, PB1, PA, and NP genes of the A/green-winged teal/Japan/9KS0643/2009(H12N3) were most closely related to the corresponding genes of A/avian/Japan/8KI0040/2008(H3N5) with nucleotide identities of 99.56, 99.22, and 99.26 %, respectively. The PB2 gene showed 99.25 % homology between A/green-winged teal/Japan/9KS0643/2009(H12N3) and A/duck/Chiba/1/2007(H9N2). These results indicated that various subtypes of influenza A viruses comprised a gene pool out of which a novel reassortant A/green-winged teal/Japan/9KS0643/2009(H12N3) has emerged.

Phylogenetic analyses were conducted to examine the relationship of A/green-winged teal/Japan/9KS0643/2009(H12N3) compared to other influenza isolates available in GenBank. Maximum Likelihood analyses were implemented in MEGA6.06 [23], using the Tamura-Nei substitution model with 500 bootstrap replicates. Analysis of the H12 gene indicated that A/green-winged teal/Japan/9KS0643/2009(H12N3) and H12N3 viruses isolated in Mongolia in 2005, and other H12N5 Japanese isolates clustered within the Eurasian lineage. A/green-winged teal/Japan/9KS0643/2009(H12N3) was closely related to A/duck/Tsukuba/212/2006(H12N5) (Fig. 1a). The A/green-winged teal/Japan/9KS0643/2009(H12N3) virus also falls in the Eurasian lineage for NA, but is separated from a group including H12N3 viruses isolated in Mongolia in 2005. The NA gene of A/green-winged teal/Japan/9KS0643/2009(H12N3) was highly related to A/duck/Hokkaido/327/2009(H1N3) (Fig. 1b).

To further explore the evolutionary origin and to date the introduction of H12N3 virus into Japan, molecular dating was performed using the time to most recent common ancestor (TMRCA). Influenza A virus sequences from avian hosts collected globally were downloaded from Influenza Research Database (IRD) on 18 July 2014, aligned using MUSCLE [24] and any incomplete sequences were removed. The final number of sequences in each dataset included PB2 ($n = 272$), PB1 ($n = 280$), PA ($n = 278$), H12 ($n = 154$), NP ($n = 256$), N3 ($n = 275$), M ($n = 309$), NS ($n = 289$). Phylogenetic analysis was performed using Bayesian Markov Chain Monte Carlo method implemented in BEAST v1.8.0 [25]. Date of sample collection associated with each taxa was used to age the tips of the tree. The uncorrelated lognormal relaxed molecular clock and HKY85 substitution model were used with a Bayesian skyride coalescent tree prior. We performed four independent analyses of 50 million generations that were combined after appropriate burning ($\sim$10 %) to produce

Table 1 Nucleotide sequence comparison between A/green-winged teal/Japan/9KS0643/2009(H12N3) and other strains available in GenBank

Gene	Position	Virus with highest percentage of nucleotide identity		Accession no.
		Name of strain	Homology (%)	
PB2	1-2288	A/duck/Chiba/1/2007(H9N2)	99.25	AB874672
PB1	1-2294	A/avian/Japan/8KI0040/2008(H3N5)	99.56	CY079281
PA	1-2188	A/avian/Japan/8KI0040/2008(H3N5)	99.22	CY079280
HA	1-1695	A/duck/Tsukuba/212/2006(H12N5)	98.93	AB669140
NP	1-1497	A/avian/Japan/8KI0040/2008(H3N5)	99.33	CY079278
NA	1-1413	A/duck/Hokkaido/327/2009(H1N3)	99.43	AB560966
M	1-975	A/wild bird/Korea/A323/2009(H10N1)	100.00	JN817541
NS	1-864	A/ruddyshelduck/Mongolia/894 V/2009(H4N3)	99.30	KC871499

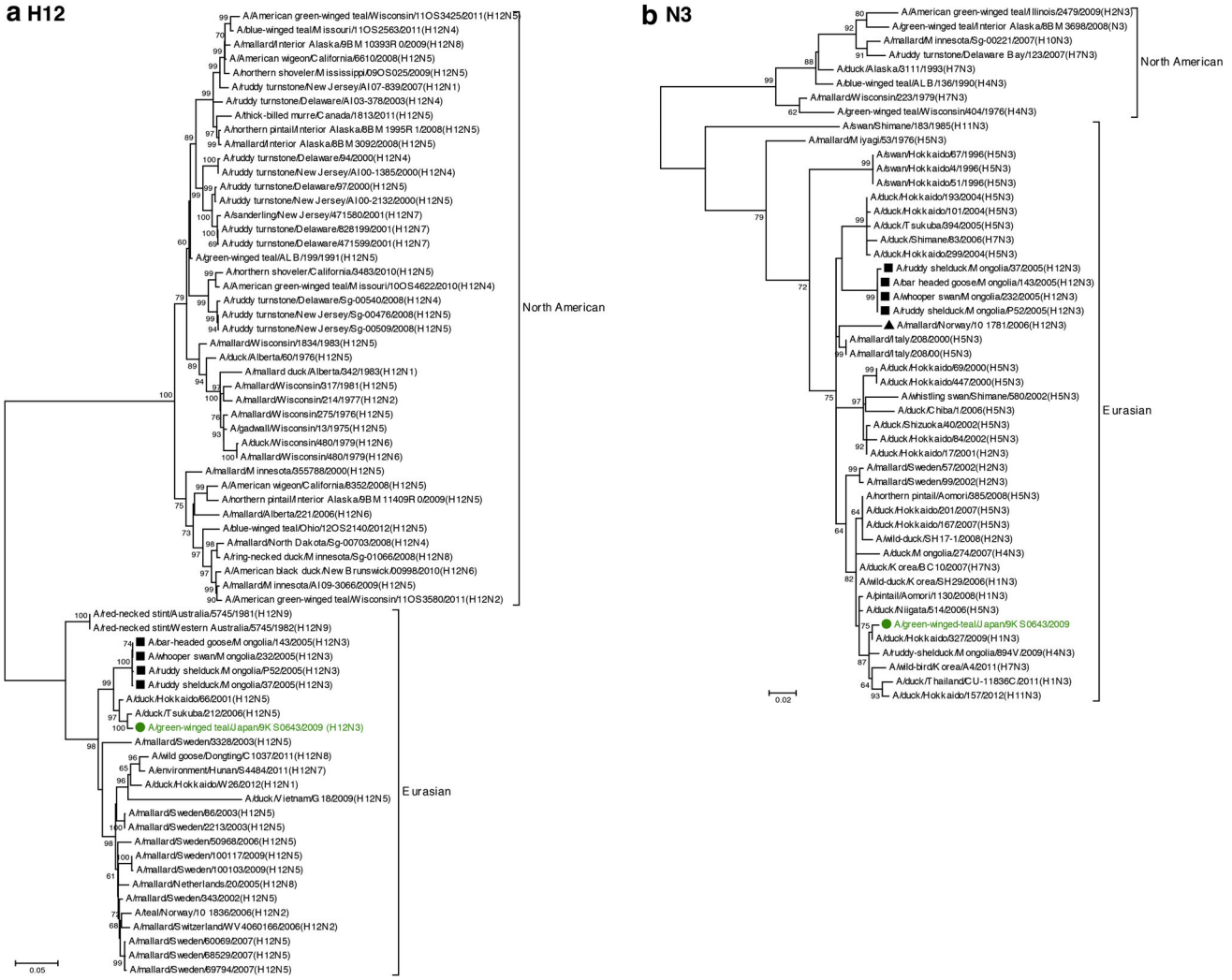

Fig. 1 **a** Phylogenetic tree of H12 genes of A/green-winged teal/Japan/9KS0643/2009(H12N3) (*filled circle*) (highlighted in *green*) together with other H12N3 Mongolian strains isolated in 2005 (*filled square*). **b** Phylogenetic tree of the N3 genes of A/green-winged teal/Japan/9KS0643/2009(H12N3) (*filled circle*) together with other H12N3 Mongolian strains isolated in 2005 (*filled square*). An H12N3 Norway strain isolated in 2006 (*Filled triangle*) and other representative strains of *N3* are shown in the tree. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model. *Numbers* at each branch point indicate bootstrap values ≥60 % in the bootstrap interior branch test. All positions containing gap and missing data were eliminated. Phylogenetic analysis was conducted in MEGA6.06. The *scale bar* indicates 0.05 and 0.02 nucleotide substitutions per site in **a** and **b**, respectively (Color figure online)

10,000 trees. For MRCA analyses, the most recent taxa were used to calibrate the root height of the tree and the nodes were used to estimate the divergence time between two taxa. Differential placement of the H12N3 virus isolate between the eight gene trees suggested that the virus originated as the result of a double reassortment involving an H12N5 virus and an H1N3 virus. The HA and NA tree (Fig. 2) indicated that the H12 HA gene first reassorted with an unsampled NA subtype between 1997 and 2003, and the resultant virus reassorted with an H1N3 virus between 2006 and 2009, giving rise to the H12N3 subtype. Given the promiscuity of H12 and N3 with other NA and HA subtypes respectively [26], we believe this to be a plausible explanation for this virus' emergence. However, owing to the limited number of H12N3 sequences available in the public database, the definitive conclusions on reassortment are difficult to ascertain. Additional extensive surveillance in wild bird populations and resulting sequencing data will help to confirm such hypothesized reassortment events.

It is well known that AIV reassortment has contributed to the emergence of novel influenza strains, some of which have resulted in deadly pandemics [27]. Interestingly, AIVs that were responsible for transferring genes to human pandemic viruses in 1957, 1968, and 2009 were not HPAI viruses [28]. This information highlights the importance of genetic characterization of AIVs in nature, since genes from circulating viruses may be involved as the precursors of future pandemic influenza. In this study, we reported the detailed genetic features of a 2009 LPAIV H12N3 reassortant virus isolated from a cloacal swab of a wild bird in Japan. To our knowledge, this is the first report on the genetic characterization of the H12N3 subtype virus in Japan, which turns out to have a complex and unusual reassortant history.

Genetic analysis indicated that genes of A/green-winged teal/Japan/9KS0643/2009(H12N3) were highly similar to the corresponding genes of a wide range of AIV subtypes including H1N3, H3N5, H4N3, H9N2, H10N1, and H12N5. Since these influenza A viruses were obtained from wild birds across a broad geographic range in Japan, Korea, and Mongolia (Table 1), this result indicates that the virus has become established in wild bird populations in East Asia. Phylogenetic analysis indicates that H12 viruses are divided into North American and Eurasian lineages. In both lineages, the H12 subtype is most commonly detected in combination with N5, and H12 viruses were first detected from wild birds belonging to American and Eurasian lineages in 1975 and in 1981, respectively. In contrast, H12N3 subtype viruses were only detected in the Eurasian lineage since 2005 in Mongolia [29]. Although A/green-winged teal/Japan/9KS0643/2009(H12N3) appears to be the same subtype with Mongolian strains, it seems to have diverged based on our genetic and phylogenetic analysis. Despite substantial AIV surveillance efforts, only a few H12N3 subtype viruses have been obtained worldwide. This could be because surveillance is not directed and sufficient to have detected the subtype. Alternatively, H12N3 viruses may have only emerged recently. As we demonstrated here, with A/green-winged teal/Japan/9KS0643/2009(H12N3), it is important to

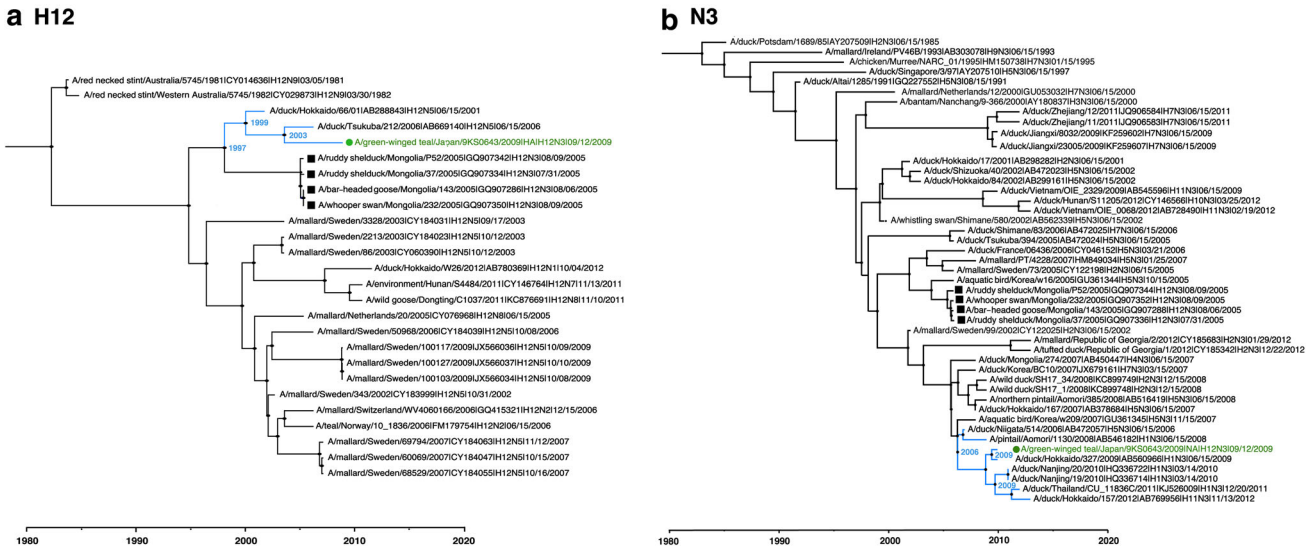

Fig. 2 Dated phylogenies of H12 (**a**) and N3 (**b**) viruses belonging to the Eurasian lineage indicating the time of most recent common ancestor of the A/green-winged teal/Japan/9KS0643/2009 isolate (highlighted in *green* and marked by *filled circle*). *Clades* highlighted in *blue* encompass viruses isolated in Japan that form a monophyletic clade with the H12N3 virus isolated in this study. The *horizontal axis* represents calendar years. Trees were generated using time-stamped sequence data analyzed using Bayesian Markov Chain Monte Carlo method implemented in BEAST v1.8.0 (Color figure online)

continue AIV surveillance in wild birds, but moreover to analyze the origin of emerging strains to improve our knowledge of the viral ecology, the evolution of H12 viruses and other poorly understood viral subtypes, and to explore the epidemiology and pandemic potential of novel AIV.

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