Geographical Spread of Highly Pathogenic Avian Influenza Virus H5N1 during the 2006 Outbreak in Austria

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In spring 2006, highly pathogenic avian influenza virus (HPAIV) of subtype H5N1 was detected in Austria in 119 dead wild birds. The hemagglutinin cleavage site showed that the amino acid sequence motif was identical to that of the Qinghai lineage. For detailed analysis, the hemagglutinin (HA) and neuraminidase (NA) genes of 27 selected Austrian H5N1 viruses originating from different regions and wild bird species were analyzed phylogenetically, which revealed two clearly separated Austrian subclusters, both belonging to European cluster EMA-1. Subcluster South (SCS) contains virus isolates from the south of Austria as well as from Slovenia, Turkey, Egypt, and Nigeria. The second subcluster, Northwest (SCN), covered a larger group of viruses originating from different locations and wild bird species in the northern and very western parts of Austria, as well as from Bavaria and Switzerland. Surprisingly, virus isolates originating from two mute swans and one wild duck found on the north side of the Alps did not cluster with SCN but with SCS. Together with isolates from Bavarian, the Czech Republic, Italy, and Slovakia, they form a genuine subgroup named subgroup Bavaria (SGB). This subgroup forms a link to SCN, indicating a spread of the virus from south to north. There has been a general assumption that the generic HPAI introduction route into Europe was from Russia to north Germany, introducing cluster EMA-2 into Europe. Interestingly, our findings support the assumption of an alternative introduction of the HPAI H5N1 virus from Turkey to central Europe, where it spread as cluster EMA-1 during the outbreak of 2006.

Highly pathogenic H5N1 viruses have been recognized in Asia since 1996, when the first Asian H5N1 virus (A/Goose/Guangdong/1/96) was isolated from sick geese in southern China (25). Since then, this virus has caused endemic infections in poultry in many southeast Asian countries (13, 18). Although influenza viruses in wild aquatic birds occasionally are transmitted to chickens and turkeys, where they may produce outbreaks of severe disease, they do not appear to have entered the wild bird populations to a substantial extent until late April to June 2005, when a large outbreak of H5N1 infection occurred at Qinghai Lake in western China, a major breeding site of migratory birds (2). Subsequently to the outbreak at Qinghai Lake from April to June 2005, H5N1 viruses have continued to cause outbreaks in Asia and Europe (http://www.who.int).

A major molecular determinant for the pathogenicity of H5 and H7 viruses is the amino acid sequence specifying the proteolytic cleavage site of hemagglutinin (HA). In lowly pathogenic avian influenza virus (LPAIV), single basic residues at the cleavage site restrict the proteolytic activation of HA to the respiratory and intestinal tracts. In contrast, insertion mutations at the genomic locus encoding the endoproteolytic cleavage site resulting in the presence of a polybasic site render it accessible for ubiquitous protease, resulting in severe, systemic infections (17). All analyzed viruses originating from Qinghai Lake showed the series of basic amino acids at the HA cleavage site PQGERRKKRGLF, which is characteristic of high pathogenicity in chickens. They also exhibited a 20-amino-acid deletion of the neuraminidase (NA) stalk (residues 49 to 68) that is characteristic of the NA of the A/Goose/Guangdong/1/96 virus (2).

Salzberg et al. analyzed 36 isolates of highly pathogenic avian influenza (HPAI) H5N1 viruses collected from Europe, northern Africa, the Middle East, and Asia and described the genetic relationships among these isolates, which affect birds and humans (16). He grouped the isolates into three distinct lineages, one encompassing all known non-Asian isolates, and hypothesized that this Europe-African lineage has been introduced into the European-African region at least three times and has split into three distinct, independently evolving sublineages: EMA-1, EMA-2, and EMA-3. These three clades possibly represent either separate introductions or a single introduction from Asia via Russia into Europe or any other western site, which then has subsequently evolved into three lineages, EMA-1, EMA-2, and EMA-3 (16). EMA-2 contains the first German H5N1-positive swan found at the beginning of February 2006 on the Baltic island Ruegen (A/Cygnus cygnus/Germany/R65/06). This suggests a single introduction route for this cluster, because a phylogenetic analysis of the HA and the NA nucleotide sequences revealed that the closest genetic relative was an isolate from Astrakhan (A/Cygnus olor/Astrakhan/Ast05-2-3/2005). From Astrakhan, located in southern Russia, a westward movement of wild birds to central Europe in late January/early February 2006 is suggested (24).

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TABLE 1. Forward (F) and reverse (R) PCR primers used for amplification and sequencing of the HA and NA genes

<table>
<thead>
<tr>
<th>Gene (fragment)</th>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA (1st)</td>
<td>atH5-1F</td>
<td>AGC AAA AGC AGG GGT ATA AT</td>
</tr>
<tr>
<td></td>
<td>atH5-535R</td>
<td>TGT CCT TTT TGA TAA GCA ATTA</td>
</tr>
<tr>
<td>HA (2nd)</td>
<td>atH5-420F</td>
<td>AGA TCA TCC CCA AAA GTC C</td>
</tr>
<tr>
<td></td>
<td>atH5-1043R</td>
<td>CCT TGA GGG GTA TTA TTT CGA</td>
</tr>
<tr>
<td>HA (3rd)</td>
<td>atH5-72F</td>
<td>ATG AGT CAA TAA ACT TGG AGA GT</td>
</tr>
<tr>
<td></td>
<td>atH5-1344R</td>
<td>AGT CCA GCA ACT TAG GAA TCC</td>
</tr>
<tr>
<td>HA (4th)</td>
<td>atH5-1230F</td>
<td>CAT TGA CAA AAT GAA CAC TCA</td>
</tr>
<tr>
<td></td>
<td>atH5-1765R</td>
<td>GTG TTT TTA ACC ACA CTG AAC T</td>
</tr>
<tr>
<td>NA (1st)</td>
<td>atN1-MINUS20</td>
<td>AGC AAA AGC AGG AGT TCA A</td>
</tr>
<tr>
<td></td>
<td>atN1-760R</td>
<td>ATA TGA TCC GTG CCC AT</td>
</tr>
<tr>
<td>NA (2nd)</td>
<td>atN1-619F</td>
<td>TGA ART ACA ATG GCA TAA C</td>
</tr>
<tr>
<td></td>
<td>atN1-1132R</td>
<td>GGR TCC CAA ATC ATT TCA AA</td>
</tr>
<tr>
<td>NA (3rd)</td>
<td>atN1-916F</td>
<td>TTT CAA TCA RAA TTY GGA GTA GC</td>
</tr>
<tr>
<td></td>
<td>atN1-1406R</td>
<td>ACA AAC TAC TTG TCA ATG GTG A</td>
</tr>
</tbody>
</table>

* Locations of primer positions are indicated in the primer name and are relative to the start of the coding regions of the hemagglutinin gene (reference sequence DQ90035) and the neuraminidase gene (reference sequence AJ367074), respectively.

The aim of this study was to perform a phylogenetic analysis of Austrian HPAI H5N1 isolates from the outbreak of 2006 to determine their linkage to the European clusters EMA-1, EMA-2, and EMA-3 and to identify possible implications for H5N1 introduction routes into Austria.

MATERIALS AND METHODS

Sample material. Between the first case in February 2006 and the last H5N1 detection in May 2006, about 3,151 dead birds were screened for avian influenza virus and 119 samples were found to be positive. These 119 avian influenza virus-positive samples were subtyped using an H5-specific real-time reverse transcription-PCR (RT-PCR) and a conventional N1 RT-PCR (5). The pathotyping of H5-positive samples was performed by sequencing the HA cleavage site (5).

There were 24 outbreaks at 16 distinct Austrian wetland locations concerning six different species. At some locations only one single bird was affected, while other outbreaks encompassed up to 20 animals and several species. For our phylogenetic studies, we focused on those 27 birds signifying each outbreak and location that exhibited the highest genetic signal in our generic real-time PCR analyses. These 27 viruses represent all geographically different regions, their temporal occurrence, and as many different species as possible. In addition, a virus isolate from a cat (EF395844) found in an animal shelter in Mellach also was included in the study (12).

RT-PCR. Viral RNA was isolated with the RNeasy Mini kit (Qiagen, Austria) from an organ pool containing trachea, lung, intestine, liver, and pancreas. After the reverse transcription of the RNA, a PCR amplification of the HA and NA gene in a two-step RT-PCR protocol was performed. The reverse transcription of 8 μL RNA was performed with the SuperScript II kit (Invitrogen, Austria) using the random primer Uni12 according to the manufacturer’s instructions. The reaction was carried out at 42°C for 30 min. The complete HA and NA open reading frames were PCR amplified with the AmpliTaqGold Master Mix kit (Applied Biosystems, Austria), 0.4 μM each primer (Table 1), and 3 μL of the cDNA. The 1.8-kb HA gene was amplified in two overlapping fragments with primer pairs H5-1F and H5-1043R and H5-72F and H5-1765R, resulting in a 1,043- and a 983-bp fragment, respectively (Table 1). The 1.4-kb NA gene was amplified with the primer pair N1-MINUS20 and N1-1406R (Table 1). The MgCl₂ concentration was adjusted to 2 mM. The PCR profile for amplification was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 50°C to 52°C (depending on the primer) for 1 min, and elongation at 72°C for 1 min. The final elongation was performed for 10 min at 72°C.

The two overlapping PCR products of the HA gene (1,043 and 983 bp) and the NA fragment (1.4 kb) were separated by electrophoresis in an ethidium-stained gel. Fragments were visualized using a UV transilluminator. DNA bands of the correct size were excised and recovered using a QIAquick Gel Extraction kit (Qiagen, Austria) according to the manufacturer’s instructions.

Nucleotide sequencing. Sequencing reactions were performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Five μL of purified DNA was added to 5 μL of the sequencing reaction mix. To generate full-length sequences of the HA and NA genes, a standard direct sequencing reaction was carried out using the PCR primers mentioned above together with additional HA (H5-420F, H5-1230F, H5-535R, H5-1344R)- and NA (N1-619F, N1-916F, N1-760R, N1-1132R)-specific primers, resulting in four and three overlapping amplicons, respectively (Table 1). These newly developed primers were directly used for sequencing the PCR products without subcloning. Unincorporated dye terminators were removed using the DyeEx 2.0 Spin kit (Qiagen) by following the protocol of the manufacturer. Each PCR product was sequenced from the 5’ and the 3’ ends by capillary electrophoresis on a 3130xl Genetic Analyzer (Applied Biosystems).

Computer-assisted analysis. In our analysis, additional sequences of representative European and non-European isolates of the HPAI H5N1 virus were included from the NCBI (National Center for Biotechnology Information) GenBank. Nucleotide sequences were aligned using the ClustalX2 program (23) and edited with BioEdit v7.0.9 (9). All gaps were removed from the HA and NA alignments, resulting in alignments of 1,653 and 1,180 nucleotides (nt), respectively. Phylogenetic relationships were estimated with MEGA 4 (21) and MrBayes 3.2.1 (10) using the neighbor-joining method employing the substitution model of Tamura-Nei et al. (22). A total of 2,000 bootstrap replicates were performed to assess the statistical significance of the observed clustering. Furthermore, trees were constructed by Bayesian inference, as implemented in the program MrBayes 3.2.1 (10). Markov chain Monte Carlo sampling was performed for one million generations in two simultaneous runs. A/Goose/Guangdong/1/96 was used as the outgroup for both the HA (AF144305) and NA (AF144304) genes.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers that have been deposited in GenBank are listed in Table 2.

RESULTS

HPAI H5N1 virus isolates were detected in 119 wild birds. No domestic bird from an Austrian poultry farm was inf ected by H5N1 virus during the Austrian influenza outbreak in 2006, with the one exception of six domestic chickens from a local animal shelter in Mellach. The HPAI H5N1 virus-positive wild birds found in 2006 included six different species: 82 mute swans, 28 wild ducks, 1 wild goose, 2 coots, 1 crested grebe, 1 egret, 4 nondetermined wild birds, and 3 cats from the animal shelter in Mellach (12). In contrast to many other European countries, in Austria no HPAI H5N1 virus-positive bird was registered in the year 2007 (20). Chronologically, the Austrian outbreak started with the detection of two positive mute swans (A/swan/Mellach/215/2006 and A/swan/Mellach/216/2006) from the storage lake in Mellach on 13 February 2006 and ended with the last detected HPAI H5N1 virus-positive swan (A/swan/Laakirchen/2703/2006), which was found at the rapids of the river Traun in upper Austria on 26 April 2006. Geographically, we could discern several main areas of reported dead wild birds, namely, (i) the storage lake in Mellach (district Graz Umgebung), (ii) the storage lake in Gralla (district Leibnitz), (iii) the animal shelter in Graz (district Graz Stadt), and certain locations on the north side of the Alps, including (iv) along the river Danube between Perg (province Upper Austria) and Hainburg (province Lower Austria), (v) in the region Salzkammergut with lakes Attersee and Traunsee, and (vi) Lake Constance, on the border with Germany (Bavaria) and Switzerland.

All 119 positive wild birds were found to be positive in a generic real-time influenza A virus RT-PCR assay specific for...
The virus isolates classified into SCS originated entirely from dead birds found in the regions south of the Alps. It comprises isolates from two wild ducks and two swans found in the storage lakes in Mellach (A/duck/Mellach/335/2006 and see above) and Gralla (A/duck/Leibnitz/243/2006), which clustered together with the swan isolate 760 from Slovenia (A/swan/Slovenia/760/2006) and turkey isolate 1 from Turkey (A/turkey/Turkey/1/2005) and are genetically closely related to poultry isolates from Egypt and Nigeria (Fig. 1 and 2). The only mammalian isolate, from a cat (EF395844), grouped together with the swan isolate 403 (A/swan/ArcheNoah/403/2006), thus suggesting that host-specific mutation did not occur (Fig. 1 and 2) (12). Within SCN, no such homogeneous geographical origin could be identified, because it contains isolates from such geographically diverse regions as Lake Constance, the region Salzkammergut, and different locations along the river Danube and the river Inn, which clustered together with the first detected virus in Bavaria (A/duck/Bavaria1/2006) and an isolate from Switzerland (A/coot/Switzerland/V544/2006) as well as with other isolates from Bavaria and south Germany (Fig. 1, 2, and 3). Surprisingly, one wild duck and two swan isolates (A/swan/Perg/1358/2006, A/swan/Ybbs/1106/2006, and A/swan/ArcheNoah/403/2006), all three found on the north side of the Alps, genetically belong to the subgroup of Bavaria 2, which were called subgroup South (SCS) and subgroup Northwest (SCN) (Fig. 1 and 2).

To sequence the complete HA and NA genes and consequently perform phylogenetic analysis, we carefully selected a sample of 27 H5N1-positive isolates from wild birds representing an unbiased sample of all positively analyzed animals in the diagnostic routine (Table 2). The phylogenetic characterization of the HA and NA genes revealed that all Austrian isolates clustered within EMA-1, or according to the most recent nomenclature, within subclade 2.2.1. (http://www.who.int/esr/disease/influenza/en). No viruses of cluster EMA-2 or EMA-3, or subclade 2.2.2 or 2.2.3, were found in our study (Fig. 1 and 2). We could identify two Austrian subclusters within EMA-1, which were called subcluster South (SCS) and subcluster Northwest (SCN) (Fig. 1 and 2).

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This classification into two subclusters and one additional sub-
group was further confirmed by our phylogenetic analysis of the NA gene (Fig. 2). As the NA gene of four isolates (A/grebe/Bregenz/916/2006, A/duck/Bregenz/907/2006, A/swan/Hainburg/2172/2006, and A/swan/ArcheNoah/403/2006) could only partially be sequenced, they were excluded from the phylogenetic study.

This classification into two subclusters and one subgroup is mirrored by the HA and NA sequences. Contrary to the HA.
FIG. 2. Phylogenetic relationships of NA nucleotide sequences estimated with the neighbor-joining method employing the Tamura-Nei substitution model. Numbers next to the branches indicate the percentages of 2,000 bootstrap replicates (values above the branches) or posterior probability values (below the branches) from a corresponding Bayesian analysis. Branch lengths are drawn to the scale at the bottom of each tree. Austrian isolates are shown in boldface letters.
gene, which showed only one nucleotide substitution, the NA gene showed five. All mutations are nonsynonymous and characteristic for the two subclusters and the one subgroup. SCN is defined by two mutations within the NA [49C(17I) and 385A(129V)] and one within the HA gene [13A(5V)]. SCN is locality specific, because it was found...
only in isolates from Krems, Hoeflein, Wien, Schwechat, and Hainburg. SGB is characterized by the one mutation, which is shared with SCS, and two additional mutations within the NA gene [130T (44R→I) and 124A (414S→N)].

**DISCUSSION**

We analyzed Austrian H5N1 HPAIV isolates from the outbreak in the year 2006 to study introduction routes or possible sources of infection and, if possible, to characterize species-specific differences between different wild birds and between wild birds and mammals. All 27 selected and analyzed isolates of different geographic origins and of different animal species clustered with EMA-1, confirming the findings reported by Salzberg et al. (16). Our results refer to both sequenced genes, the HA and NA genes. Within EMA-1 we could observe two genotypically distinct, nonoverlapping groups of H5N1 isolates. In relation to their geographic origin, we termed these two subclusters SCN and SCS, the latter containing a further subgroup, named SGB.

SCN contains 24 Austrian isolates of five different wild bird species originating from a string of locations, ranging from the very eastern part to the very western part of Austria (Hainburg to Bregenz), and they clustered together with the first detected HPAI H5N1 case in Bavaria (A/mallard/Bavaria/1/2006) and an isolate from Switzerland (A/coot/Switzerland/V544/2006) (Fig. 1, 2, and 3). With the exception of three isolates that were found within SCN (A/swan/Perg/1358/2006, A/swan/Ybbs/1106/2006, and A/Scharding/1398/2006), it comprises all analyzed and sequenced species on the north side of the Austrian Alps. The SCN showed one nonsynonymous mutation within the HA gene [13A (49V→M)] and two within the NA gene [100A (32T→C) and 36A (129V→I)]; one of the latter two [100A (32T→C)] mirrors its geographic location in the very eastern part of Austria (Krems, Hoeflein, Wien, Schwechat, and Hainburg).

Isolates from two swans and one wild duck from a storage lake in Mellach, one wild duck isolate from a storage lake in Gralla, and one cat isolate from an animal shelter (EF395844) form subcluster SCS, excepting the isolates of the SCS subgroup SGB, which is extensively described in the ensuing paragraph (12).

SGB isolates appear to be genetically related to SCS but are found in regions north of the Alps. SCS is characterized by one nonsynonymous mutation within the NA gene [130T (44R→C)]. The Austrian isolates of SCS cluster together with the first Slovenian H5N1-positive isolate (A/swan/Slovenia/760/2006) and the first Turkish H5N1-positive isolate (A/turkey/Turkey/1/2005), as well as with poultry isolates from Egypt and Nigeria (A/duck/Egypt/2253/2006 and A/chicken/Nigeria/SA454) (4). This close phylogenetic relationship becomes apparent in both of the analyzed genes, HA and NA, indicating a virus introduction from Nigeria and Egypt via Turkey and Slovenia into Austria. Our phylogenetic analysis therefore supports the hypothesis that EMA-1 originated in Africa (4) and subsequently spread from Africa to the Middle East and the European southeast (Egypt and Turkey) and then on via Slovenia into central Europe (Austria), following the avian migratory routes further north. This hypothesis also is supported by the timeline of the various registration dates of positive HPAI H5N1 cases in Europe: turkey1 from Turkey on 13 October 2005 (OIE notification date), the first European case of HPAI H5N1, swan760 from Slovenia, on 12 February 2006 (OIE notification date), and the two Austrian swans 215 and 216 from Mellach on 13 February 2006 (Austrian notification date; OIE notification, 20 February) (http://www.oie.int/Eng/info_ev/en_AI_factoids_H5N1_Timeline.htm).

Three Austrian isolates, one from a wild duck from Scharding/Inn, one from a swan from Ybbs-Persenbeug (district Scheibbs), and one from a swan from Perg, grouped together with two Bavarian isolates from a buzzard and a grebe (A/common buzzard/Bavaria/2/2006 and A/great crested grebe/Bavaria/22/2006). In our analysis, this group also encompasses the swan isolate from the Czech Republic (A/Cygnus olor/Czech Republic/5170/2006), the peregrine falcon isolate from Slovakia (A/peregrine falcon/Slovakia/vh246/2006), and two Italian isolates (A/Cygnus olor/Italy/808/2006 and A/mallard/Italy/835/2006). The virus isolates from these birds form a genuine subgroup within SCS and therefore were named SGB. One nonsynonymous mutation [130T (44R→C)] is shared with SCS, demonstrating that SGB belongs to this specific subcluster. In contrast to SCS, however, SGB is characterized by two additional nonsynonymous mutations within the NA gene [100A (32V→I) and 124A (414S→N)]. As all three SGB-specific Austrian isolates originating from wild birds were found at locations together with other SCN-specific isolates, SGB might be the link between SCN and SCS, suggesting an origin of the virus in the south (Slovenia) and a spread from south to north (Austria, Bavaria, Slovakia, and the Czech Republic). We hypothesize that H5N1 entered Austria via Slovenia, first forming SCS and then spreading further north, crossing the Alps and forming SGB, and then spreading further east and west, forming SCN. H5N1 might then have spread from Austria to Germany (Bavaria), the Czech Republic, and Slovakia.

In our phylogenetic study, only EMA-1-like viruses were found in Austria (Fig. 1, 2, and 3), and no viruses of cluster EMA-2 and EMA-3 could be found. In Germany, a geographical and temporal separation of cluster EMA-1 and EMA-2 was evident, as EMA-2-like viruses were found in the north of Germany as well as in Denmark and Sweden, while EMA-1-like isolates were found in the south of Germany, also including the genotypes Bavaria 1 and Bavaria 2 (1, 11, 20). A phylogenetic analysis of the HA and NA genes showed that an isolate from Astrakhan (A/Cygnus olor/Astrakhan/Ast05-2-3/2005), located in southern Russia and belonging to EMA-2, was the closest genetic relative to the German EMA-2-like H5N1 isolates, suggesting its viral ancestry. EMA-2 viruses most probably were carried from Russia into Germany by migrating wild birds in late January/early February 2006 (24). From the island of Ruegen, where the first German H5N1-positive swan (A/Cygnus cygnus/Germany/R65/06) was found, the outbreak spread south and west (20). In summary, it appears that EMA-2 approached central Europe on an east-to-west route and later turned south, but according to our analysis, it never reached Austrian territory; EMA-1 arrived on a south-to-north route, causing the Austrian H5N1 outbreaks in 2006 (15, 20, 24).

Austrian H5N1 viruses were found first in the south, forming SCS (without SGB), and it persisted for the short period of a few days. All subsequent cases were found on the north side of the Alps, forming SGB and SCN, and persisted for a much
longer period of approximately 3 months (from February to May). However, a longer persistence of the infection also would mean a greater possibility of mutations accumulating, thus creating a greater genetic diversity among the isolates, which can be seen in *SCN*, where the geographic location and mutation rate correlates. A repeated introduction of closely related EMA-1-like viruses, however, also would explain this genetic diversity, but this is not supported by our study, which indicates a single virus introduction from the south (Slovenia) and then a continuous spread northwards across the Alps (Fig. 3).

European mallards and central European mute swans are mainly sedentary birds or visitants that are infrequently found to migrate, and they do so for short distances only. In the late 1950s at different places in Switzerland, mallards had been banded for studies of bird migration. Most of those birds were found exclusively either at the north or at the south side of the Alps. Most of them were recovered at locations less than 200 km away from their breeding places (8). The maximum flying altitude of mallards or mute swans is approximately 500 m, thus the Alps form a formidable barrier. When Alpine passes are available, the birds might cross the Alps on rare occasions.

The maximum flying altitude of mallards or mute swans is approximately 500 m, thus the Alps form a formidable barrier. When Alpine passes are available, the birds might cross the Alps on rare occasions (G. Spitzer, personal communication). This is confirmed by our findings, discovering *SCN* isolates exclusively on the north side of the Alps, while *SCS* apparently reached the south side of the Alps and, after some dithering, spread northwards, traversing the main Alpine ridge. It then formed *SGB* and fanned out into Bavaria or Slovakia and the Czech Republic.

Curiously, the only swan isolate characterized in Croatia (A/mute swan/Croatia/1/2005) clustered with EMA-2 (16). This dead mute swan (*Cygnus olor*) was found in November 2005. At the same time, there was an outbreak in the region of Astrakhan at the Caspian Sea. Russian mute swans are migratory birds and usually overwinter at the Black or Caspian Sea. Whoooper swans (*Cygnus cygnus*) breeding at the Baltic Sea also are migratory birds and overwinter in the same regions as the Russian mute swans. Mute swans from other European regions, however, do not migrate over longer distances and remain rather stationary (6). It is not known if the mute swan found dead in Croatia was a Russian migratory bird that most probably got lost on its migratory route. According to our phylogenetic analysis, the virus isolate from this Croatian swan is most closely related to an isolate from a mute swan found dead in Astrakhan in November 2005 (A/Cygnus olor/Astrakhan/ Ast05-2-3/2005). Interestingly, isolates from whooper swans found dead at the island of Ruegen during the first German outbreak beginning February 2006 are most closely related to the same isolate from Astrakhan (A/Cygnus olor/Astrakhan/Ast05-2-3/2005) (24). This Croatian swan does not support our hypothesis, but it appears to be an accidental oddball presumably following its own idiosyncratic route. The virus isolate from this swan constitutes the only EMA-2 outbreak in Croatia and has never spread further.

A mute swan found dead in Ragusa (Sicily) in the beginning of February 2006 belongs to EMA-3 (A/Cygnus olor/Italy/742/2006) (16). Russian mute swans, but not European mute swans, migrate over long distances, some of them even reaching Afghanistan (7). This mute swan from Sicily most likely overwintered in Afghanistan, because its virus isolate is most closely related to a virus isolated from a chicken in Afghanistan (A/chicken/Afghanistan/1207/2006) (Fig. 1 and 2) (16). Why this bird did not return to its breeding grounds but was found in Sicily, far away from its customary route, again remains a mystery, like the swan from Croatia mentioned above.

Apart from the study of the potential introduction routes of H5N1, we also investigated potential host-related differences of the virus. Our study comprises isolates from five different wild bird species and one mammalian isolate (EF395844). Swan 403, which had been brought to the animal shelter Arche Noah before its death but apparently had been sick upon its arrival, had infected other birds in the animal shelter. Cat 649 most probably was infected by one of these birds (12). The sequence alignment and phylogenetic analysis of the HA gene revealed no host-related sequence differences between the isolate from swan 403 and the cat isolate, implying that a few viral replication cycles in mammals is not sufficient for a mutation within the HA gene (20, 24).

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**REFERENCES**