Four different sublineages of highly pathogenic avian influenza H5N1 introduced in
Hungary in 2006-2007

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Abstract

Highly pathogenic avian influenza (HPAI) H5N1 viruses were introduced to Hungary during 2006-2007 in three separate waves. This study aimed at determining the full-length genomic coding regions of the index strains from these epizootics in order to; (i) understand the phylogenetic relationship to other European H5N1 isolates, (ii) elucidate the possible connection between the different outbreaks and (iii) determine the putative origin and way of introduction of the different virus variants. Molecular analysis of the HA gene of Hungarian HPAI isolates obtained from wild birds during the first introduction revealed two groups designated Hungarian1 (HUN1) and Hungarian2 (HUN2) within sublineage 2.2B and clade 2.2.1, respectively. Sequencing the whole coding region of the two index viruses A/mute swan/Hungary/3472/2006 and A/mute swan/4571/Hungary/2006 suggests the role of wild birds in the introduction of HUN1 and HUN2 viruses: the most similar isolates to HUN1 and HUN2 group were found in wild avian species in Croatia and Slovakia, respectively. The second introduction of HPAI H5N1 led to the largest epizootic in domestic waterfowl in Europe. The index strain of the epizootic A/goose/Hungary/14756/2006 clustered to sublineage 2.2.A1 forming the Hungarian3 (HUN3) group. A common ancestry of HUN3 isolates with Bavarian strains is suggested as the most likely scenario of origin. Hungarian4 (HUN4) viruses isolated from the third introduction clustered with isolate A/turkey/United Kingdom/750/2007 forming a sublineage 2.2.A2. The origin and way of introduction of HUN4 viruses is still obscure, thus further genetic, phylogenetic, ecological and epidemiological data are required in order to elucidate it.

Keywords: Avian influenza, H5N1, Highly pathogenic, Hungary, Phylogenetic characterisation
1. Introduction

Avian influenza viruses (AIVs) belong to genus *Influenzavirus* within the family *Orthomyxoviridae* (Cox et al., 2000). The enveloped virions contain eight segments of negative single-stranded genomic RNA encoding four structural and seven non-structural viral proteins. Based on the antigenic properties of the envelope glycoproteins, haemagglutinin (HA) and neuraminidase (NA), influenza viruses are classified into 16 HA and nine NA subtypes (Wallensten, 2006). All these subtypes are perpetuated in natural hosts of AIVs, i.e., wild aquatic birds, where the virus and host live normally in relative harmony (Gorman et al., 1992). AIVs are separated into highly pathogenic (HPAIV) and low pathogenic (LPAIV) pathotypes depending on their capability of causing fatal disease in chickens. HPAIVs may develop from their LPAIV H5 or H7 precursor viruses after transmission from wild birds and adaptation to susceptible poultry species. The major molecular determinant of pathogenicity is the presence of multiple basic amino acids at the proteolytic cleavage site of HA rendering it cleavable by ubiquitous furin proteases present in many tissues, whereas LPAIVs carry only two non-consecutive basic amino acid residues at the same position restricting proteolytic activation of HA to the respiratory and intestinal tracts (Swayne and Suarez, 2000).

H5N1 HPAIV virus isolated in Guangdong in southern China in 1996 caused severe disease in geese farms (Xu et al., 1999). Reassortants of this precursor virus caused fatal outbreaks in chicken in 1997, and infected 18 humans, of whom six died. New variants of HPAI H5N1 re-emerged in 2002, and caused lethal disease in wild waterfowl (Sturm-Ramirez et al., 2004). By 2004 it became endemic in several countries of East and Southeast Asia causing large-scale outbreaks in poultry populations (Li et al., 2004). The virus was now also lethal to mammals, especially felids (Keawcharoen et al., 2004). Sporadic human cases also occurred;
until February 2009, 409 people have been infected and 256 died (WHO, 2009). The virus
was transmitted to wild bird populations causing severe mortalities e.g. in migratory
waterfowl at Lake Qinghai North-Western China (Chen et al., 2005). The Qinghai-like HPAI
H5N1 spread intensively through Central Asia to the Black Sea region. Outbreaks were
reported in Turkey, Romania and Croatia in October 2005, in Ukraine in November 2005
(OIE, 2007; WHO, 2007). Quite unexpectedly, within a short period 18 European countries
were infected between January and March 2006, most likely by an unusual migration of
waterfowl caused by the harsh cold winter (Kilpatrick et al., 2006).

Detailed phylogenetic analyses revealed that H5N1 viruses evolved to form nine major clades
(H5N1 Evolution Working Group WHO/FAO/OIE, 2007). Clade 2 is the most diversified,
and within this clade, the Qinghai-like viruses form a separate clade designated 2.2. Clade 2.2
HPAI H5N1 strains of European, Middle East and Asian origin were further clustered to
EMA1-3 “clades” (Salzberg et al., 2007). This nomenclature has recently been proposed to be
changed to 2.2.1, 2.2.2 and 2.2.3 “sub-clades” (Starick et al., 2008). Within “sub-clade” 2.2.1
three putative branches, branch 2.2.1.1-3 were defined (Nagy et al., 2008). According to the
present official nomenclature, branch 2.2.1.1 was designated a new third order clade 2.2.1.
(H5N1 Evolution Working Group WHO/FAO/OIE, 2008), and “sub-clade” 2.2.3 was
proposed a possible additional third order clade. Due to the inconsistency of nomenclature,
the authors designate the remaining former “sub-clades” as sublineages 2.2A and 2.2B, while
branches are defined as sublineages 2.2A1 and 2.2A2. As a result of recent extensive, large-
scale sequence analysis, the number of partial and whole genomes of European HPAI H5N1
isolates expanded in the past months. Detailed sequence analysis studies were reported from
Italy, Croatia, Slovenia (Salzberg et al., 2007), Denmark (Bragstad et al., 2007), Germany
(Rinder et al, 2007; Weber et al., 2007; Starick et al., 2008), France (Gall-Reculé, 2008),
Switzerland (Hofmann et al., 2008), Czech Republic and Slovakia (Nagy et al., 2008) as well as Sweden (Kiss et al., 2008).

In Hungary, the first HPAI H5N1 outbreaks occurred in wild birds in February-March 2006 (Pálmai et al., 2007). The virus temporarily disappeared from the territory of Hungary, but unexpectedly re-emerged in June 2006 and caused the largest domestic waterfowl epizootic in Europe (Ivanics et al., 2007). The last outbreak of HPAI H5N1 in domestic geese was reported in January 2007.

Since H5N1 affected Hungary in three separate waves in 2006/07, this study presents the full-length genomic coding regions of the index strains of these epizootics in order to; (i) understand the phylogenetic relationship to other European H5N1 isolates, (ii) elucidate the possible connection between the different outbreaks and (iii) determine the putative origin and the way of route of introduction of the different virus variants.

2. Materials and methods

2.1. Virus selection

During the course of the epizootics, all HPAI H5N1 viruses isolated from the 64 wild bird and 31 domestic poultry cases were subjected to partial molecular analysis (Pálmai et al., 2007). The H5 proteolytic cleavage site together with the flanking regions as well as the N1 gene were identified with conventional RT-PCR using J3/B2a primers described in Diagnostic Manual for Avian Influenza (Anonymous, 2006) and N1-1/N1-2 primers recommended by WHO (WHO, 2005), respectively. The obtained preliminary sequence data showed that the wild bird isolates can be divided into two distinct sublineages designated Hungarian1 (HUN1) and Hungarian2 (HUN2) groups. In the present study 12 wild bird H5N1 isolates were
selected for further genetic analysis based on their geographical origin (Table 1). The HUN1 and HUN2 index strains, A/mute swan/Hungary/3472/2006 and A/mute/swanl/Hungary/4571/2006 were subjected to complete genome analysis, whereas the HA coding sequences were determined from the remaining ten strains. The 29 isolates originated from the domestic poultry epizootic in 2006 showed no sequence difference in the targeted HA and N1 regions, and were designated Hungarian3 (HUN3) group. Index strain A/goose/Hungary/14756/2006 (Table 1) was selected for complete genome sequence analysis. Isolates originated from the 2007 outbreaks showed no sequence differences in the targeted regions and were designated Hungarian4 (HUN4) group. The partial coding sequences of each segment of the two isolates, A/goose/Hungary/2823/2007 (GenBank accession numbers: EF446768-446775) and A/goose/Hungary/3413/2007 (GenBank accession numbers: EF446776-446783) were determined by the Community Reference Laboratory for Avian influenza and Newcastle Disease (VLA, Weybridge, UK). The missing coding sequences of the coding regions of the index strain A/goose/Hungary/2823/2007 were completed in this study.

2.2. Virus isolation

The viruses were isolated from dead wild waterfowl, domestic goose, duck and guinea fowl according to European standardised protocols (Alexander, 2005). Briefly, 10% w/v organ suspension (trachea, lung, brain, liver and intestines) prepared in PBS containing antibiotics and antimycotics was inoculated into the allantoic cavity of five 9-11-day-old specific pathogen-free (SPF) embryonated chicken eggs. The eggs were incubated in a humidified atmosphere at 37°C for six days and were candled daily. The allantoic fluid originated from dead embryos and the end of the passage period were examined by hemagglutination assay.
Since in most cases the volume of the original isolates was not appropriate for genetic analysis, a second passage was performed, but in some cases a third passage was applied to increase the viral titre (Table 1).

2.3. RNA extraction, RT-PCR and sequencing

Viral RNA was extracted from infective allantoic fluid by High Pure Viral RNA Extraction Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s recommendations. For amplification of the whole coding sequence of the polymerase complex a protocol described by Li et al. (2007) was applied, whereas the whole coding regions of the remaining genes were amplified by a modified protocol published by Hoffmann et al. (2001) and Kiss et al. (2008). Two different RNA batches from each isolate were subjected to sequencing. The amplicons were generated with the Qiagen OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The parameters of the thermoprofile were as follows: 50° 30’, 94° 15’; 35X (94° 30”; 50° 30”; 72° 2’); 72° 7’; hold. The RT-PCR products were gel-purified using the QIAquick Gel Extraction Kit (QIAGEN). The amplicons were sequenced with the same primers that were used for amplification with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an automatic ABI PRISM 3130 genetic analyser (Applied Biosystems). Where sequence discrepancies were found between the two RNA batches, a third sequencing reaction was performed.

2.4. Genetic and phylogenetic analysis
Sequences were assembled and edited using the BioEdit v.7.0.7. (Hall, 1999) and the DNASTAR 7 (Lasergene, WI, USA) software package. Identification of all gene segments were performed with BLAST search on sequences available at the National Center for Biotechnology Information, Bethesda, Maryland, USA (http://www.ncbi.nlm.nih.gov). Genotyping of the gene segments were carried out with Influenza A Virus Genotype Tool (Lu et al., 2007). Distance based neighbour-joining and character based maximum parsimony phylogenetic trees were generated using the Molecular Evolutionary Genetics Analysis (MEGA) software v.4.0. (Tamura et al., 2007). The neighbour-joining algorithm was implemented with the Kimura-2 parameters model using a transition-to-translation ratio of 2.0. Other models were also tested, which showed similar topologies. The topology of trees was confirmed by 1000 bootstrap replicates. Maximum likelihood phylogenetic trees were generated by the PHYLIP version 3.68 software (Felsenstein, 2004). Trees were edited with Tree Explorer program of MEGA v.4.0. For phylogenetic analysis, in addition to the four full-length coding sequences of the Hungarian isolates, a set of H5N1 strains from Europe, Middle-East, Africa and Asia were selected from the Influenza Virus Resource at NCBI (Bao et al., 2008).

Potential N-linked glycosylation sites (score >0.5) were predicted by the NetNGlyc 1.0 Server (Gupta et al., 2004).

The sequences were deposited in GenBank under the following accession numbers: FJ445226-FJ445249.

3. Results
3.1. Epidemiological data of HPAI H5N1 outbreaks in Hungary in 2006-2007

In Hungary, the first HPAI H5N1 outbreak suspicion was reported on February 6\textsuperscript{th} 2006, and presence of the virus was confirmed by standard international methods (Alexander, 2005) on February 17\textsuperscript{th} in three mute swans found dead in the county of Bács-Kiskun in Southern Hungary. During the following two months, 2109 dead birds (including wild and farmed waterfowl, other wild bird and poultry species) were sent to the Central Veterinary Institute of Hungary (national reference laboratory (NRL) for avian influenza). H5N1 was identified at 12 localities in 64 wild birds: 60 mute swans, one coot, one black-headed gull, one cormorant and one mallard (Pálmai et al., 2007). The implemented control measures prevented the spread of the infection to other wild and domestic birds.

No further H5N1 cases were identified during the next two months. However, in June 2006, the largest H5N1 epizootic of Europe broke out in Hungary. The first suspicion (neurological signs) was reported on June 3\textsuperscript{rd} 2006 from a free-range goose farm of 3000 birds located in county Bács-Kiskun, about 90 km north-east of the first confirmed mute swan case. The presence of the virus was confirmed on June 9\textsuperscript{th} 2006 by the NRL. In the next month and a half, 29 HPAI H5N1 outbreaks were confirmed at seven localities (Figure 1). Out of the 29 affected holdings, 13 were goose farms, 16 duck farms and one mixed holding (duck, goose, guinea fowl). The population of the holdings ranged from 110 to 33768 birds. During the course of the epizootic 8374 cloacal/tracheal swabs and 800 carcasses were submitted for virus detection. Out of these samples, 155 duck and 230 goose swabs as well as carcasses of 66 ducks, 60 geese and one guinea fowl were positive for H5N1 HPAIV. Immediate emergency (e.g. as stamping out of suspected flocks) as well as pre-emptive measures (depopulation in 1 km zone around the outbreaks) were successfully implemented, to prevent the spread of the virus to other counties of Hungary and the surrounding countries. During the
epizootic, 675 premises were affected, one million birds were culled and the total damage
caused by the epizootic reached 7.3 million Euros.

The third wave of H5N1 infections arrived in January 2007. HPAI suspicion (neurological
signs) were reported on January 21st 2007 from a free-range goose farm of 3300 animals
located in the county of Csongrád, approximately 60 km east of the site of the previous
domestic poultry epizootic (Figure 1). Presence of the virus was confirmed by the NRL on
January 24th. This outbreak was followed by a second one reported from a goose farm located
9 km from the first outbreak on January 25th 2007. Appropriate control measures were
implemented, the infection was stamped, and no other H5N1 cases occurred on the territory of
Hungary. During the epidemiological surveillance, 4055 cloacal/tracheal swabs and 208
carcasses were subjected to virus detection, but only the ten samples originated from the
above-mentioned two holdings proved to be positive. To date no further HPAI presence was
detected in Hungary.

3.2. Phylogenetic analysis

3.2.1. Phylogenetic analysis of hemagglutinin coding sequences

Sequence analysis of the complete hemagglutinin coding sequence of the 12 selected wild
bird H5N1 isolates confirmed the previous HUN1 and HUN2 clustering. The four isolates
belonging to HUN1 group (Table 1) were almost identical in the examined genomic region;
only A/mute swan/Hungary/4912/2006 isolate contained an A1017T substitution that proved
to be silent. The hemagglutinin sequences of the eight HUN2 isolates (Table 1) were identical
with the exception of A/mute swan/4571/2006, which contained a C942A substitution also
resulting in no amino acid change.
Comparison of the Hungarian H5N1 index strains with the available European, Middle East and African HA genes by maximum parsimony method revealed that they belong to four different groups (neighbour-joining and maximum likelihood methods gave similar topology). The HUN1 isolates clustered with Croatian H5N1 strain A/cygnus olor/Croatia/1/2005 (bootstrap value: 63) in sublineage 2.2B that comprises of Czech, German “Northern group”, Danish, Swedish, United Kingdom, Russian, and Nigerian “African sublineage A” (Ducatez et al., 2006, 2007) isolates (Figure 2).

In contrast, HUN2 strains showed the closest nucleotide similarity to the Italian H5N1 isolate A/mute swan/Italy/808/2006 and formed a distinct cluster with high (94) bootstrap value containing strains from Italy, Bavaria “genotype 2”, Czech Republic, Slovakia, and Sweden (Figure 2). The members of this cluster group together with a second cluster including isolates from Austria, Slovenia, France, Turkey Egypt and Nigeria “African sublineage B” forming clade 2.2.1.

The only fully sequenced member of HUN3, A/goose/Hungary/14756/2006 strain belonged to a well-defined group with high (98) bootstrap value that includes of Bavaria “genotype 1”, France “subgroup 2”, German “Southern group” and Swiss isolates forming sublineage 2.2A1 (Figure 2).

Members of HUN4 (A/goose/Hungary/2823/2007 and A/goose/Hungary/3413/2007) belonged to a distinct group with high (92) bootstrap value together with a H5N1 strain isolated in the United Kingdom, A/turkey/England/250/2007 forming sublineage 2.2A2. The closest similarity of these strains was found with Russian, Mongolian, Nigerian “African sublineage C”, Sudanese and Ivory Coast isolates. (Figure 2).

3.2.2. Phylogenetic analysis of full genomes of the index strains
The four index strains (Table 1) were subjected to extended phylogenetic analysis of each segment. BLAST search of the 32 gene segments revealed that they were all of avian origin. According to the Influenza A Virus Genotype Tool (www.flugenome.org) the examined Hungarian isolates had the following genotype: KGD5JF1JF1E.

Phylogenetic trees of the NA gene and the six internal genes showed the same topology as was observed in the case of the HA tree, but their resolution was lower due to the reduced number of sequence data available. The PB2 maximum parsimony phylogenetic tree in Figure 3 represents the phylogenetic relationship between European, Middle East and African H5N1 isolates in the internal gene segments. The only remarkable difference compared with the HA tree is the lack of available whole genome sequence in sublineage 2.2A1, therefore the HUN3 index strain is the first candidate in this group.

3.3. Molecular characterisation

Results of the molecular characterisation of the four different Hungarian HPAI H5N1 strains are summarised in Table 2. The hemagglutinin genes of each virus possessed the multiple basic amino acids characteristic of HPAI H5N1. Markers of receptor binding preference to 2,3-NeuAc-Gal linkages of avian cell-surface receptors (Ha et al., 2001) were found at amino acid residues Q222 and R224 (amino acid positions 226 and 228 in H3 numbering, 238 and 240 at H5N1 HA gene). The three amino acid residues (99I, 240G and 268N) characteristic of clade 2.2 viruses (Chen et al., 2006) were identified in all Hungarian strains. The S145L and A172T mutation leading to increased virulence in terrestrial poultry (Perdue and Suarez, 2000) were not found even in the genomes of the goose isolates. Analysis of the translated HA1 protein sequence with NetNglyc 1.0 server, six potential N-linked glycosylation sites
were predicted in the Hungarian isolates at amino acid positions 27, 39, 181, 302, 500 and 559.

Analysis of the NA gene revealed the presence of the 20-amino-acid deletion (amino acids 49-68) and the arginine at position 110 characteristic of clade 2.2 viruses (Chen et al., 2006). Amino acid 275 is H for all Hungarian AI isolates, indicating full susceptibility to oseltamivir (Smith et al., 2006). Three potential N-linked glycolisation sites were predicted at amino acid positions 68, 126 and 215.

Analysis of the genes of the polymerase complex showed that the E627K mutation in PB2 gene, a marker of increased virulence in mice and efficient replication in humans (Shinya et al., 2004; Fouchier et al., 2004) is present in the Hungarian isolates except for A/mute swan/Hungary/4571/2006. The mutations D701N and S714R responsible for efficient replication and virulence in mice (Li et al., 2005) were not found in the genomes of Hungarian H5N1 isolates.

Substitutions in the PB1 (L13P and S678N), PA (K615N) and NP (N319K) genes may contribute to increased virulence in mammalian hosts (Gabriel et al., 2005). Only the PB1 L13P substitution was found in the genomes of the Hungarian isolates. The proapoptotic PB1-F2 protein translated from a second frame in the PB1 gene has been shown to contribute to increased virulence of H5N1 viruses in a mouse model (Conenello et al., 2007). S66 of this protein, which plays key role in virulence, is present in the Hungarian strains.

The NS genes of Hungarian viruses belong to allele A like the majority of H5N1 viruses (Zohari et al., 2008). The five-amino-acid deletion (amino acids 80-84), the P42S, D92E and V144A substitutions associated with antagonising of antiviral effects of interferon and tumour necrosis factor (Seo et al., 2002; Li et al., 2006; Jiao et al, 2008) were present in the Hungarian isolates. The C-terminal four-amino-acid motifs ESKV or EPKV, responsible for pathogenicity increased virulence in mouse model by binding to PDZ domains of proteins.
involved in host cell signalling pathways (Jackson et al., 2007), were found in the NS proteins of all sequenced H5N1 isolates.

Analysis of the M2 protein showed that the S31N substitution indicating amantadine and rimantadine resistance (Suzuki et al., 2003) is not present in any of the Hungarian strains. S64A and E66A substitutions found in the genomes of several HPAI strains isolated in Hong Kong, Thailand and Vietnam between 2002-2004 (Li et al., 2004) were also absent.

4. Discussion

The present study aimed at the genetic characterisation of the HPAI H5N1 viruses isolated in Hungary during 2006-2007 in order to elucidate their phylogenetic relationship and the possible links between the outbreaks of the three waves of H5N1 infections. Furthermore, since genetic databases of European, Middle East and African H5N1 strains have expanded dramatically in the past months, positioning Hungarian isolates in phylogenetic trees of relatively high resolution is an invaluable genetic tool that can contribute to determining their putative origin and ways of introduction.

Molecular analysis of the HA gene of Hungarian HPAI strains isolated from wild birds during the first introduction wave revealed two groups, HUN1 and HUN2, corresponding to sublineage 2.2B and clade 2.2.1, respectively. HA sequences of the Hungarian isolates within the groups were in most cases identical, which phenomenon can be explained by the restricted geographical area and time course of these outbreaks. Sequencing of the whole coding sequence of the geographically distinct two index viruses A/mute swan/Hungary/3472/2006 and A/mute swan/4571/Hungary/2006 revealed that each segment clustered with the corresponding clusters indicating the absence of segment reassortment.
Sublineage 2.2B HUN1 viruses emerged at the beginning of February 2006 in Southern Hungary in the wetland areas along river Danube. Interestingly, during the course of the wild bird (mute swan) outbreaks, the HUN1 viruses remained restricted to this small geographical area.

Based on previous studies, the ancestor of sublineage 2.2B viruses was suggested in Astrakhan and Kurgan (Salzberg et al., 2007) where H5N1 viruses disseminated from and reached Europe along two different routes (Rinder et al., 2007) possibly corresponding to the two main flyways of migratory birds (Olsen et al, 2006). This hypothesis is in accordance with the results of our phylogenetic analysis, namely the Hungarian and Croatian viruses form a separate group within sublineage 2.2B, whereas other European 2.2B viruses which also emerged at the beginning of February 2006 in Northern Germany (OIE, 2007) and later in the neighbouring countries form a separate group (Figure 2).

Clade 2.2.1 HUN2 viruses were detected ten days after the HUN1 strains in Northern Hungary. This virus variant spread southward along the line of river Danube to the area where HUN1 strains were already present. The different virus variants co-existed in the southern part of Hungary for several weeks providing an opportunity for potential reassortment.

Clade 2.2.1 HPAI H5N1 strains first emerged in Italy at the beginning of February (OIE, 2007) and spread to Central Europe (Slovenia, Austria, France, Slovakia, Czech Republic and Southern Germany) almost exactly at the same time as in Hungary possibly via the main South-North migratory route of wild birds from Africa to Europe. This assumption is further supported by detecting HPAIV H5N1 of this cluster in Nigeria, in January 2006. On the other hand, analysis of partial HA sequences from the neighbouring countries of Hungary revealed an interesting phenomenon; both sublineage 2.2B and clade 2.2.1 viruses emerged and co-circulated in Romania in 2005 (data no shown), which means that clade 2.2.1 viruses had been present in Europe far before they were detected in Italy. The ancestor of these strains
was not determined yet, but the fact that Turkish and Romanian isolates from 2005 also belong to this clade raises several questions about the exact origin of these strains.

The second introduction wave of HPAI H5N1 in Hungary in June 2006 led to the largest domestic waterfowl epizootic in Europe. Based on the data of phylogenetic analysis, this outbreak was caused by a virus different from the previous HUN1 and HUN2 isolates, since the HUN3 index strain A/goose/Hungary/14756/2006 belongs to sublineage 2.2A1.

The first strains of this sublineage emerged in Bavaria one week after those of clade 2.2.1. The ancestor of these strains was not identified, since interestingly, the representatives of this clade were present only in Central Europe. According to the restricted epidemiological and genetic data available regarding this sublineage, very limited conclusion can be drawn; the Bavarian and HUN3 isolates may have a common ancestor. The origin and way of introduction of these strains need further investigation.

The third introduction wave of HPAI H5N1 into the territory of Hungary occurred in January 2007, when no presence of HPAI was reported in the country. These HUN4 strains form a distinct, monophyletic cluster, sublineage 2.2A2 with isolate A/turkey/United Kingdom/750/2007 within clade 2.2A. A direct epidemiological link was established between the Hungarian and United Kingdom outbreaks (Irwine et al., 2007). The common ancestor, origin and way of introduction of HUN4 viruses are still obscure.

Molecular analysis of each segment of the Hungarian HPAI H5N1 isolates revealed identical molecular markers regarding proteolytic cleavage site, receptor-binding preference, and glycosylation sites of HA, stalk deletion and glycosylation pattern of NA. The pathogenicity markers described in European H5N1 viruses (Bragstad et al., 2007; Gal Reculé et al., 2007) regarding the polymerase complex, NP, M and NS genes were present in the examined Hungarian strains. Heterogeneity was found in PB2 627 position: the HUN2 strain possessed glutamic acid, while the other representative strains contained lysine. This result is in
accordance with findings in Sweden (Kiss et al., 2008) and the Czech Republic (Nagy et al., 2008). The other difference, the EPKV motif found at the N-terminus of NS1 protein is uncommon among European H5N1 strains, the closest viruses possessing this motif are originated from Astrakhan. Sequencing of additional HUN3 strains is necessary in order to elucidate this finding.

In conclusion, epidemiological and phylogenetic analysis of Hungarian HPAI H5N1 strains isolated in 2006-2007 revealed that the most probable source of the wild bird epizootics were wild bird species carrying two distinct variants HPAI H5N1 viruses identified as HUN1 and HUN2 groups, as the most similar isolates were found in Croatia and Slovakia, respectively. In contrast, many questions remained unanswered regarding the origin and introduction of HUN3 and HUN4 strains. Hopefully, the expanding epidemiological and genetic data will contribute to the better understanding of the dissemination and distribution of HPAI H5N1 in Europe in 2006-2007.

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Conflict of interest statement
All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work.
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Legends for figures and tables

Table 1. The Hungarian HPAI H5N1 strains subjected to genetic analysis

Table 2. Genetic analysis of the whole coding region of each genome segment of the four Hungarian HPAI H5N1 index strains

Figure 1. Geographic distribution of the localities affected by HPAI H5N1 in 2006-2007. Outbreaks caused by group HUN1-4 viruses are indicated with dots of different colours. Location of index cases are indicated with triangles

Figure 2. Phylogenetic analysis of the HA coding sequences of Hungarian, other European, Middle East and African strains using maximum parsimony method. The topology of the tree was confirmed by 1000 bootstrapping steps. Bootstrap values higher than 70 are indicated at nodes. For means of clarity, trees are midpoint rooted. HUN1 strains are marked with red, HUN2 strains with blue; HUN3 stains with green and HUN4 strains with yellow.

Figure 3. Phylogenetic analysis of the PB2 coding sequences of Hungarian, other European, Middle East and African strains using maximum parsimony method. The topology of the tree was confirmed by 1000 bootstrapping steps. Bootstrap values higher than 70 are indicated at nodes. For means of clarity, trees are midpoint rooted. HUN1 strains are marked with red, HUN2 strains with blue; HUN3 stains with green and HUN4 strains with yellow.
Table 1.

<table>
<thead>
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<th>Virus isolate</th>
<th>Locality of isolation</th>
<th>Sampling date</th>
<th>Passage no.</th>
<th>Genetic analysis</th>
<th>Lineage</th>
</tr>
</thead>
<tbody>
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<td>A/mute swan/Hungary/3472/2006</td>
<td>Nagybaracska</td>
<td>2006.02.06.</td>
<td>1st</td>
<td>Whole genome</td>
<td>HUN1</td>
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Bold: the index cases of the different lineages are bolded

* These two strains were sequenced by VLA (Weybridge, UK)
Table 2.

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Figure 1

- HPAI H5N1 group HUN1
- HPAI H5N1 group HUN2
- HPAI H5N1 group HUN3
- HPAI H5N1 group HUN4
Figure 3

clade 2.2.1

sublineage 2.2A2

sublineage 2.2A1

sublineage 2.2B

possible new third order clade