

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

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25 Abstract

26

Highly pathogenic avian influenza (HPAI) H5N1 viruses were introduced to Hungary during 27 2006-2007 in three separate waves. This study aimed at determining the full-length genomic 28 coding regions of the index strains from these epizootics in order to; (i) understand the 29 phylogenetic relationship to other European H5N1 isolates, (ii) elucidate the possible 30 connection between the different outbreaks and (iii) determine the putative origin and way of 31 introduction of the different virus variants. Molecular analysis of the HA gene of Hungarian 32 HPAI isolates obtained from wild birds during the first introduction revealed two groups 33 designated Hungarian1 (HUN1) and Hungarian2 (HUN2) within sublineage 2.2B and clade 34 2.2.1, respectively. Sequencing the whole coding region of the two index viruses A/mute 35 swan/Hungary/3472/2006 and A/mute swan/4571/Hungary/2006 suggests the role of wild 36 37 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 38 39 second introduction of HPAI H5N1 led to the largest epizootic in domestic waterfowl in 40 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 41 isolates with Bavarian strains is suggested as the most likely scenario of origin. Hungarian4 42 (HUN4) viruses isolated from the third introduction clustered with isolate A/turkey/United 43 Kingdom/750/2007 forming a sublineage 2.2.A2. The origin and way of introduction of 44 HUN4 viruses is still obscure, thus further genetic, phylogenetic, ecological and 45 epidemiological data are required in order to elucidate it. 46

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48 Keywords: Avian influenza, H5N1, Highly pathogenic, Hungary, Phylogenetic49 characterisation

50 1. Introduction

51

Avian influenza viruses (AIVs) belong to genus Influenzavirus within the family 52 Orthomyxoviridae (Cox et al., 2000). The enveloped virions contain eight segments of 53 negative single-stranded genomic RNA encoding four structural and seven non-structural 54 viral proteins. Based on the antigenic properties of the envelope glycoproteins, 55 haemagglutinin (HA) and neuraminidase (NA), influenza viruses are classified into 16 HA 56 and nine NA subtypes (Wallensten, 2006). All these subtypes are perpetuated in natural hosts 57 of AIVs, i.e., wild aquatic birds, where the virus and host live normally in relative harmony 58 (Gorman et al., 1992). AIVs are separated into highly pathogenic (HPAIV) and low 59 pathogenic (LPAIV) pathotypes depending on their capability of causing fatal disease in 60 chickens. HPAIVs may develop from their LPAIV H5 or H7 precursor viruses after 61 transmission from wild birds and adaptation to susceptible poultry species. The major 62 molecular determinant of pathogenicity is the presence of multiple basic amino acids at the 63 proteolytic cleavage site of HA rendering it cleavable by ubiquitous furin proteases present in 64 many tissues, whereas LPAIVs carry only two non-consecutive basic amino acid residues at 65 the same position restricting proteolytic activation of HA to the respiratory and intestinal 66 tracts (Swayne and Suarez, 2000). 67

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 reemerged 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 largescale 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 75 was transmitted to wild bird populations causing severe mortalities e.g. in migratory 76 waterfowl at Lake Qinghai North-Western China (Chen et al., 2005). The Qinghai-like HPAI 77 H5N1 spread intensively through Central Asia to the Black Sea region. Outbreaks were 78 reported in Turkey, Romania and Croatia in October 2005, in Ukraine in November 2005 79 (OIE, 2007; WHO, 2007). Quite unexpectedly, within a short period 18 European countries 80 were infected between January and March 2006, most likely by an unusual migration of 81 waterfowl caused by the harsh cold winter (Kilpatrick et al., 2006). 82

Detailed phylogenetic analyses revealed that H5N1 viruses evolved to form nine major clades 83 (H5N1 Evolution Working Group WHO/FAO/OIE, 2007). Clade 2 is the most diversified, 84 and within this clade, the Qinghai-like viruses form a separate clade designated 2.2. Clade 2.2 85 HPAI H5N1 strains of European, Middle East and Asian origin were further clustered to 86 87 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 88 three putative branches, branch 2.2.1.1-3 were defined (Nagy et al., 2008). According to the 89 present official nomenclature, branch 2.2.1.1 was designated a new third order clade 2.2.1. 90 (H5N1 Evolution Working Group WHO/FAO/OIE, 2008), and "sub-clade" 2.2.3 was 91 proposed a possible additional third order clade. Due to the inconsistency of nomenclature, 92 the authors designate the remaining former "sub-clades" as sublineages 2.2A and 2.2B, while 93 branches are defined as sublineages 2.2A1 and 2.2A2. As a result of recent extensive, large-94 scale sequence analysis, the number of partial and whole genomes of European HPAI H5N1 95 isolates expanded in the past months. Detailed sequence analysis studies were reported from 96 Italy, Croatia, Slovenia (Salzberg et al., 2007), Denmark (Bragstad et al., 2007), Germany 97 (Rinder et al., 2007; Weber et al., 2007; Starick et al., 2008), France (Gall-Reculé, 2008), 98

99 Switzerland (Hofmann et al., 2008), Czech Republic and Slovakia (Nagy et al., 2008) as well
100 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 fulllength 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.

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112 2. Materials and methods

- 113
- 114 2.1. Virus selection

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During the course of the epizootics, all HPAI H5N1 viruses isolated from the 64 wild bird and 116 31 domestic poultry cases were subjected to partial molecular analysis (Pálmai et al., 2007). 117 The H5 proteolytic cleavage site together with the flanking regions as well as the N1 gene 118 were identified with conventional RT-PCR using J3/B2a primers described in Diagnostic 119 Manual for Avian Influenza (Anonymous, 2006) and N1-1/N1-2 primers recommended by 120 121 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) 122 and Hungarian2 (HUN2) groups. In the present study 12 wild bird H5N1 isolates were 123

selected for further genetic analysis based on their geographical origin (Table 1). The HUN1 124 HUN2 index 125 and strains. A/mute swan/Hungary/3472/2006 and A/mute/swanl/Hungary/4571/2006 were subjected to complete genome analysis, whereas the 126 HA coding sequences were determined from the remaining ten strains. The 29 isolates 127 originated from the domestic poultry epizootic in 2006 showed no sequence difference in the 128 targeted HA and N1 regions, and were designated Hungarian3 (HUN3) group. Index strain 129 A/goose/Hungary/14756/2006 (Table 1) was selected for complete genome sequence analysis. 130 Isolates originated from the 2007 outbreaks showed no sequence differences in the targeted 131 regions and were designated Hungarian4 (HUN4) group. The partial coding sequences of each 132 segment of the two isolates, A/goose/Hungary/2823/2007 (GenBank accession numbers: 133 EF446768-446775) and A/goose/Hungary/3413/2007 (GenBank accession numbers: 134 EF446776-446783) were determined by the Community Reference Laboratory for Avian 135 influenza and Newcastle Disease (VLA, Weybridge, UK). The missing coding sequences of 136 the coding regions of the index strain A/goose/Hungary/2823/2007 were completed in this 137 138 study.

139

140 2.2. Virus isolation

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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).

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153 *2.3. RNA extraction, RT-PCR and sequencing*

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Viral RNA was extracted from infective allantoic fluid by High Pure Viral RNA Extraction 155 Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's 156 recommendations. For amplification of the whole coding sequence of the polymerase 157 complex a protocol described by Li et al. (2007) was applied, whereas the whole coding 158 regions of the remaining genes were amplified by a modified protocol published by Hoffmann 159 et al. (2001) and Kiss et al. (2008). Two different RNA batches from each isolate were 160 subjected to sequencing. The amplicons were generated with the Qiagen OneStep RT-PCR 161 Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The 162 parameters of the thermoprofile were as follows: 50° 30', 94° 15'; 35X (94° 30''; 50° 30"; 163 72° 2'); 72° 7'; hold. The RT-PCR products were gel-purified using the QIAquick Gel 164 Extraction Kit (QIAGEN). The amplicons were sequenced with the same primers that were 165 used for amplification with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit 166 167 (Applied Biosystems, Foster City, CA, USA) on an automatic ABI PRISM 3130 genetic analyser (Applied Biosystems). Where sequence discrepancies were found between the two 168 RNA batches, a third sequencing reaction was performed. 169

170

171 2.4. Genetic and phylogenetic analysis

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Sequences were assembled and edited using the BioEdit v.7.0.7. (Hall, 1999) and the 173 DNASTAR 7 (Lasergene, WI, USA) software package. Identification of all gene segments 174 were performed with BLAST search on sequences available at the National Center for 175 Biotechnology Information, Bethesda, Maryland, USA (http://www.ncbi.nlm.nih.gov). 176 Genotyping of the gene segments were carried out with Influenza A Virus Genotype Tool (Lu 177 et al., 2007). Distance based neighbour-joining and character based maximum parsimony 178 phylogenetic trees were generated using the Molecular Evolutionary Genetics Analysis 179 180 (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 181 2.0. Other models were also tested, which showed similar topologies. The topology of trees 182 was confirmed by 1000 bootstrap replicates. Maximum likelihood phylogenetic trees were 183 generated by the PHYLIP version 3.68 software (Felsenstein, 2004). Trees were edited with 184 185 Tree Explorer program of MEGA v.4.0. For phylogenetis analysis, in addition to the four fulllength coding sequences of the Hungarian isolates, a set of H5N1 strains from Europe, 186 187 Middle-East, Africa and Asia were selected from the Influenza Virus Resource at NCBI (Bao et al., 2008). 188

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

191 The sequences were deposited in GenBank under the following accession numbers:192 FJ445226-FJ445249.

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194

195 **3. Results**

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197 *3.1. Epidemiological data of HPAI H5N1 outbreaks in Hungary in 2006-2007*

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In Hungary, the first HPAI H5N1 outbreak suspicion was reported on February 6th 2006, and 199 presence of the virus was confirmed by standard international methods (Alexander, 2005) on 200 February 17th in three mute swans found dead in the county of Bács-Kiskun in Southern 201 Hungary. During the following two months, 2109 dead birds (including wild and farmed 202 waterfowl, other wild bird and poultry species) were sent to the Central Veterinary Institute of 203 204 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 205 and one mallard (Pálmai et al., 2007). The implemented control measures prevented the 206 spread of the infection to other wild and domestic birds. 207

No further H5N1 cases were identified during the next two months. However, in June 2006, 208 the largest H5N1 epizootic of Europe broke out in Hungary. The first suspicion (neurological 209 signs) was reported on June 3rd 2006 from a free-range goose farm of 3000 birds located in 210 county Bács-Kiskun, about 90 km north-east of the first confirmed mute swan case. The 211 presence of the virus was confirmed on June 9th 2006 by the NRL. In the next month and a 212 half, 29 HPAI H5N1 outbreaks were confirmed at seven localities (Figure 1). Out of the 29 213 affected holdings, 13 were goose farms, 16 duck farms and one mixed holding (duck, goose, 214 215 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 216 virus detection. Out of these samples, 155 duck and 230 goose swabs as well as carcasses of 217 66 ducks, 60 geese and one guinea fowl were positive for H5N1 HPAIV. Immediate 218 emergency (e.g. as stamping out of suspected flocks) as well as pre-emptive measures 219 (depopulation in 1 km zone around the outbreaks) were successfully implemented, to prevent 220 the spread of the virus to other counties of Hungary and the surrounding countries. During the 221

epizootic, 675 premises were affected, one million birds were culled and the total damagecaused by the epizootic reached 7.3 million Euros.

The third wave of H5N1 infections arrived in January 2007. HPAI suspicion (neurological 224 signs) were reported on January 21st 2007 from a free-range goose farm of 3300 animals 225 located in the county of Csongrád, approximately 60 km east of the site of the previous 226 domestic poultry epizootic (Figure 1). Presence of the virus was confirmed by the NRL on 227 January 24th. This outbreak was followed by a second one reported from a goose farm located 228 9 km from the first outbreak on January 25th 2007. Appropriate control measures were 229 implemented, the infection was stamped, and no other H5N1 cases occurred on the territory of 230 Hungary. During the epidemiological surveillance, 4055 cloacal/tracheal swabs and 208 231 carcasses were subjected to virus detection, but only the ten samples originated from the 232 above-mentioned two holdings proved to be positive. To date no further HPAI presence was 233 234 detected in Hungary.

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236 *3.2. Phylogenetic analysis*

237 3.2.1. Phylogenetic analysis of hemagglutinin coding sequences

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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).

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269 *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 (<u>www.flugenome.org</u>) 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.

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283 *3.3. Molecular characterisation*

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Results of the molecular characterisation of the four different Hungarian HPAI H5N1 strains 285 are summarised in Table 2. The hemagglutinin genes of each virus possessed the multiple 286 basic amino acids characteristic of HPAI H5N1. Markers of receptor binding preference to 287 2,3-NeuAc-Gal linkages of avian cell-surface receptors (Ha et al., 2001) were found at amino 288 289 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 290 clade 2.2 viruses (Chen et al., 2006) were identified in all Hungarian strains. The S145L and 291 A172T mutation leading to increased virulence in terrestrial poultry (Perdue and Suarez, 292 2000) were not found even in the genomes of the goose isolates. Analysis of the translated 293 HA1 protein sequence with NetNglyc 1.0 server, six potential N-linked glycosylation sites 294

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 at 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 proteinsof 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.

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327 4. Discussion

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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 336 the first introduction wave revealed two groups, HUN1 and HUN2, corresponding to 337 sublineage 2.2B and clade 2.2.1, respectively. HA sequences of the Hungarian isolates within 338 the groups were in most cases identical, which phenomenon can be explained by the restricted 339 geographical area and time course of these outbreaks. Sequencing of the whole coding 340 sequence of the geographically distinct two index viruses A/mute swan/Hungary/3472/2006 341 and A/mute swan/4571/Hungary/2006 revealed that each segment clustered with the 342 corresponding clusters indicating the absence of segment reassortment. 343

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 348 Astrakhan and Kurgan (Salzberg et al., 2007) where H5N1 viruses disseminated from and 349 reached Europe along two different routes (Rinder et al., 2007) possibly corresponding to the 350 351 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 352 a separate group within sublineage 2.2B, whereas other European 2.2B viruses which also 353 emerged at the beginning of February 2006 in Northern Germany (OIE, 2007) and later in the 354 neighbouring countries form a separate group (Figure 2). 355

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, 360 2007) and spread to Central Europe (Slovenia, Austria, France, Slovakia, Czech Republic and 361 Southern Germany) almost exactly at the same time as in Hungary possibly via the main 362 South-North migratory route of wild birds from Africa to Europe. This assumption is further 363 supported by detecting HPAIV H5N1 of this cluster in Nigeria, in January 2006. On the other 364 hand, analysis of partial HA sequences from the neighbouring countries of Hungary revealed 365 an interesting phenomenon; both sublineage 2.2B and clade 2.2.1 viruses emerged and co-366 367 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 368

was not determined yet, but the fact that Turkish and Romanian isolates from 2005 alsobelong 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.

381 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 382 distinct, monophyletic cluster, sublineage 2.2A2 with isolate A/turkey/United 383 Kingdom/750/2007 within clade 2.2A. A direct epidemiological link was established between 384 the Hungarian and United Kingdom outbreaks (Irwine et al., 2007). The common ancestor, 385 origin and way of introduction of HUN4 viruses are still obscure. 386

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 399 isolated in 2006-2007 revealed that the most probable source of the wild bird epizootics were 400 wild bird species carrying two distinct variants HPAI H5N1 viruses identified as HUN1 and 401 HUN2 groups, as the most similar isolates were found in Croatia and Slovakia, respectively. 402 In contrast, many questions remained unanswered regarding the origin and introduction of 403 HUN3 and HUN4 strains. Hopefully, the expanding epidemiological and genetic data will 404 contribute to the better understandig of the dissemination and distribution of HPAI H5N1 in 405 406 Europe in 2006-2007.

407

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409

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416 **Conflict of interest statement**

417

418 All authors disclose any financial and personal relationships with other people or419 organisations that could inappropriately influence (bias) their work.

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420	References
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421

Alexander, D.J., 2005. Chapter 2.07.12 avian influenza. Manual for Diagnostic Tests and
Vaccines for Terrestrial Animals 5th edn. Paris: World Organisation for Animal Health.
Available online at: <u>http://www.oie.int/eng/normes/MMANUAL/A_00037.htm</u>.
Anonymous, 2006. Diagnostic Manual for Avian Influenza 2006. Official Journal of the

European Union. Document number C(2006), 3477. Available at: <u>http://forums.flu-lab-</u>
<u>net.eu/files/</u> with methods available at:
<u>http://www.defra.gov.uk/corporate/vla/science/scienceviral-ai-reflab-prot.htm.</u>

430

Bao Y., Bolotov, P., Dernovoy, D., Kiryutin, B., Zaslavsky, L., Tatusova, T., Ostell, J.,
Lipman, D., 2008. The Influenza Virus Resource at the National Center for Biotechnology
Information. J. Virol. 82, 596-601.

434

435 Bragstad, K., Jørgensen, P.H., Handberg, K., Hammer, A.S., Kabell, S., Fomsgaard, A., 2007.

First introduction of highly pathogenic H5N1 avian influenza A viruses in wild and domestic
birds in Denmark, Northern Europe. Virol. J. 4, 43.

438

Chen, H., Smith, G.J., Zhang, S.Y., Qin, K., Wang, J., Li, K.S., Webster, R.G., Peiris, J.S.,
Guan, Y., 2005. Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature 436, 191192.

442

443 Chen, H., Smith, G.J.D., Li, K.S., Wang, J., Fan, X.F., Rayner, J.M., Vijaykrishna, D., Zhang,

444 J.X., Zhang, L.J., Guo, C.T., Cheung, C.L., Xu, K.M., Duan, L., Huang, K., Qin, K., Leung,

- 445 Y.H., Wu, W.L.,Lu, H.R., Chen, Y., Xia, N.S., Naipospos, T.S., Yuen, K.Y., Hassan,S.S.,
- Bahri, S., Nguyen, T.D., Webster, R.G., Peiris, J.S., Guan, Y., 2006. Establishment of
 multiple sublineages of H5N1 influenzavirus in Asia: implications for pandemic control.
 Proc.Natl. Acad. Sci. USA 103, 2845-2850.
- 449
- Claas, E.C., de Jong, J.C., van Beek. R., Rimmelzwaan, G.F., Osterhaus, A.D., 1998. Human
 influenza virus A/HongKong/156/97 (H5N1) infection. Vaccine 16, 977-978.
- 452
- Conenello, G.M., Zamarin, D., Perrone, L.A., Tumpey, T., Palese, P., 2007. A Single
 Mutation in the PB1-F2 of H5N1 (HK/97) and 1918 Influenza A Viruses Contributes to
 Increased Virulence. PLoS Pathog 3(10): e141. doi:10.1371/journal.ppat.0030141.
- 456
- Cox, N.J., Fuller, F., Kaverin, N., Klenk, H.D., Lamb, R.A., Wahy, B.W., McCauley, J.W.,
 Nakamura, K., Palese, P., Webster, R. G. 2000. *Orthomyxoviridae*. In Van Regenmortel,
 M.H., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J.,
 Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (eds.). Virus taxonomy. Seventh
 report of the international committee on taxonomy of viruses. Academic Press: San Diego,
 585-597.
- 463
- 464 Ducatez, M.F., Olinger, C.M., Owoade, A.A., Landtsheer, S., Ammerlaan, W., Niesters,
 465 H.G.M., Osterhaus, A.D., Fouchier, R.A., Muller, C.P., 2006. Avian flu: multiple
 466 introductions of H5N1 in Nigeria. Nature 442, 37.
- 467
- 468 Ducatez, M.F., Olinger, C.M., Owoade, A.A., Tarnagda, Z., Tahita, M.C., Sow, A., De
- 469 Landtsheer, S., Ammerlaan, W., Ouedraogo, J.B., Osterhaus, A.D., Fouchier, R.A., Muller,

470	C.P., 2007. Molecular and antigenic evolution and geographical spread of H5N1 highly
471	pathogenic avian influenza viruses in western Africa. J. Gen. Virol. 88(Pt 8), 2297-2306.

472

Felsenstein, J., 2004. PHYLIP (Phylogeny Inference Package), version3.6. Department of
Genome Sciences, University of Washington, Seattle, WA.

475

476 Fouchier, R., Schneeberger, P., Rzendaal, F., Broekman, J., Kemink, S., Munster, V., Kuiken,

477 T., Rimmelzwaan, G., Schutten, M., vanDoornum, G., Guus, K., Arnold, B., Marion, K.,

478 Osterhaus, A.D., 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis

and a fatal case of acute respiratory distress syndrome. Proc. Natl. Acad. Sci. USA 101, 1356-1361.

481

Gabriel, G., Dauber, B., Wolff, T., Planz, O., Klenk, H.D., Stech, J., 2005. The viral
polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc .Natl.
Acad. Sci. USA 102, 18590-18595.

485

Gall-Reculé, G.L., Briand, F.X., Schmitz, A., Guionie, O., Massin, P., Jestin, V., 2008.
Double introduction of highly pathogenic H5N1 avian influenza virus into France in early
2006. Avian Pathol. 37, 15-23.

489

Gorman, O.T., Bean, W.J., Webster, R.G., 1992. Evolutionary processes in influenza viruses:
divergence, rapid evolution and stasis. Curr. Top. Microbiol. Immunol. 176, 75-97.

492

493 Gupta, R., Jung, E., Brunak, S., 2004. Prediction of N-glycosylation sites inhuman proteins.

494 Available online at: <u>http://www.cbs.dtu.dk/services/NetNGlyc/</u>.

495

Ha, Y., Stevens, D.J., Skehel, J.J., Wiley, D.C., 2001. X-ray structures of H5 avian and H9
swine influenza virus hemagglutinins bond to avian and human receptor analogs. Proc. Natl.
Acad. Sci. USA 98, 11181-11186.

499

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis
program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95-98. Available online at:

502 http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

503

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for

the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275-2289.

506

Hofmann, M.A., Renzullo, S., Baumer, A., 2008. Phylogenetic characterization of H5N1
highly pathogenic avian influenza viruses isolated in Switzerland in 2006. Virus Genes doi:
10.1007/s11262-008-0285-2.

510

511 Influenza Virus Resource, 2004. Available online at:
512 <u>http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.hml</u>.

513

Irwine, R.M., Banks, J., Londt, B.Z., Lister, S.A., Manvell, R. J., Outtrim, L., Russell, C.,
Cox, W.J., Ceeraz, W., Shell, W., Landeg, F. J., Wilesmith, W.J., Alexander, D.J., Brown,
I.H., 2007. Outbreak of highly pathogenic avian influenza caused by Asian lineage NH5N1
virus in turkeys in Great Britin in January 2007. Vet. Rec. 161, 100-101.

- 519 Ivanics, É., Bálint, Á., Pálmai, N., Márton, L., Dán, Á., Ursu, K., Szeredi, L., Deim, Z., Rigó,
- D., Tekes, L., Zarka, P., Nagy, E., Dobos-Kovács, M., Glávits, R., 2007. Diseases in
 Hungarian goose and duck flocks caused by highly pathogenic avian influenza strain (H5N1
 subtype). Magy. Áo. Lapja 129, 387-389.
- 523
- Jackson, D., Hosain, J.M., Hickman, D., Perez, D.R., Lamb, R.A., 2007. A new influenza
 virus determinant: The NS protein four C-terminal residues modulate pathogenicity. PNAS
 105, 4381-4385.
- 527
- Jiao, P. Tian, G., Li, Y., Deng, G., Jiang, Y., Liu, C., Liu, W., Bu, Z., Kawaoka, Y., Chen, H.,
 2008. A single-amino-acid substitution in the NS1 protein changes the pathogenicity o H5N1
 avian influena viruses in mice. J. Virol. 82, 1146-1154.
- 531
- Keawcharoen, J., Oraveerakul, K., Kuiken, T., Fouchier, R.A., Amonsin, A., Payungporn, S.,
 Noppornpanth, S., Wattanodorn, S., Theambooniers, A., Tantilertcharoen, R., Pattanarangsan,
 R., Arya, N., Ratanakorn, P., Osterhaus, D.M., Poovorawan, Y., 2004. Avian influenza H5N1
 in tigers and leopards. Emerg. Infect. Dis. 10, 2189-2191.
- 536
- Kilpatrick, A.M., Chmura, A.A., Gibbons, D.W., Fleischer, R.C., Marra, P.P., Daszak, P.,
 2006. From the Cover: Predicting the global spread of H5N1 avian influenza. PNAS 103,
 19368-19373.
- 540
- 541 Kiss, I., Gyarmati, P., Zohari, S., Wilbe Ramsay, K., Metreveli, G., Weiss, E., Brytting, M.,
- 542 Stivers, M., Lindström, S., Lundkvist, A., Nemirov, K., Thorén, P., Berg, M., Czifra, G.,

- Belák, S., 2008. Molecular characterization of highly pathogenic H5N1 avian influenza
 viruses isolated in Sweden in 2006. Virology. J. 5, 113.
- 545
- Li, K.S., Guan, Y., Wang, J., Smith, G.J.D., Xu, K.M., Duan, L., Rahardjo, A.P.,
 Puthavathana, P. Buranathai, C., Nguyen, T.D., Estoepangestie, A.T., Chaisingh, A.,
 Auewarakul, P., Long, H.T., Hanh, N.T., Webby, R.J., Poon, L.L., Chen, H., Shortridge,
 K.F.,Yuen, K.Y., Webster, R.G., Peiris, J.S., 2004. Genesis of a highly pathogenic and
 potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430, 209-213.
- 551
- Li, Z., Chen, H., Jiao, P., Deng, G., Tian, G., Li, Y., Hoffmann, E., Webster, R.G., Matsuoka,
 Y., Yu, K., 2005. Molecular basis of replication of duck H5N1 influenza viruses in a
 mammalian model. J.Virol. 79, 12058-12064.
- 555
- Li, Z., Jiang, Y., Jiao, P., Wang, A., Zhao, F., Tian, G., Wang, X., Yu,K., Bu, Z., Chen, H.,
 2006. The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J. Virol.
 80, 11115-11123.
- 559
- Li, O.T., Barr, I., Leung, C.Y., Chen, H., Guan, Y., Peiris, J.S., Poon, L.L., 2007. Reliable
 universal RT-PCR assays for studying influenza polymerase subunit gene sequences from all
 16 haemagglutinin subtypes. J Virol. Meth. 142, 218-22.
- 563
- Lu, G., Rowley, T., Garten, R., Donis, R.O., 2007. FluGenome: a web tool for genotyping
 influenza A virus. Nucleic Acids Res. 35(Web Server issue), W275-279.
- 566

567	Nagy, A., Vostinakova, V., Pindova, Z., Hornickova, J., Cernikova, L., Sedlak, K., Mojzis,
568	M., Dirbakova, Z., Machova, J., 2008. Molecular and phylogenetic analysis of the H5N1
569	avian influenza virus caused the first highly pathogenic avian influenza outbreak in poultry in
570	the Czech Republic in 2007. Vet Microbiol. doi: 10.1016/j.vetmic.2008.07.013.
571	
572	OIE (The World Organisation for Animal Health), 2007. Update on highly pathogen avian
573	influenza in animals (type H5 and H7). Available online at:
574	http://www.oie.int/downld/AVIAN%20INFLUENZA/A_AI-Asia.htm.
575	
576	Olsen, B., Munster, V.J., Wallensten, A., Waldenström, J., Osterhaus, A.D., Fouchier, R.A.,
577	2006. Global patterns of influenza a virus in wild birds. Science 312, 384-388.
578	
579	Pálmai, N., Erdélyi, K., Bálint, Á., Márton, L., Dán, Á., Deim, Z., Ursu, K., Löndt, B.Z.,
580	Brown, I. H., Glávits, R., 2007. Pathobiology of highly pathogenic avian influenza virus
581	(H5N1) infection in mute swans (Cygnus olor). Avian Pathol. 36, 245-249.
582	
583	Perdue, M.L. and Suarez D.L., 2000. Structural features of the avianinfluenza virus
584	hemagglutinin that influence virulence. Vet. Microbiol. 74, 77-86.
585	
586	Rinder, M., Lang, V., Fuchs, C., Hafner-Marx, A., Bogner, K.H., Neubauer, A., Büttner, M.,
587	Rinder, H., 2007. Genetic evidence for multi-event imports of avian influenza virus A (H5N1)
588	into Bavaria, Germany. J. Vet. Diagn. Invest. 19, 279-282.
589	
590	Salzberg, S.L., Kingsford, C., Cattoli, G., Spiro, D.J., Janies, D.A., Aly, M.M., Brown, I.H.,
591	Couacy-Hymann, E., De Mia, G.M., Dung do, H., Guercio, A., Joannis, T., Maken Ali, A.S.,

- 592 Osmani, A., Padalino, I., Saad, M.D., Savić, V., Sengamalay, N.A., Yingst, S., Zaborsky. J.,
- 593 Zorman-Rojs, O., Ghedin, E., Capua, I., 2007. Genome analysis linking recent European and
- African influenza (H5N1) viruses. Emerg Infect Dis. 13:713-718.
- 595
- Seo, S.H., Hoffmann, E., Webster, R.G., 2002. Lethal H5N1influenza viruses escape host
 anti-viral cytokine responses. Nature Medicine, 8, 950-954.
- 598
- Shinya, K., Hamm, S., Hatta, M., Ito, H., Ito, T., Kawaoka, Y., 2004. PB2 amino acid at
 position 627 affects replicative efficiency, but notcell tropism, of Hong Kong H5N1 influenza
 A viruses in mice. Virology 15, 258-266.

602

Smith, G.J.D., Fan, X.H., Wang, J., Li, K.S., Qin, K., et al., 2006. Emergence and
predominance of an H5N1 influenza variant in China. Proc. Natl. Acad. Sci. USA. 103,
16936–16941.

606

- Starick, E., Beer, M., Hoffmann, B., Staubach, C., Werner, O., Globig, A., Strebelow, G.,
 Grund, C., Durban, M., Conraths, F.J., Mettenleiter, T., Harder, T., 2008. Phylogenetic
 analyses of highly pathogenic avian influenza virus isolates from Germany in 2006 and 2007
 suggest at least three separate introductions of H5N1 virus. Vet Microbiol. 128, 243-252.
- Sturm-Ramirez, K.M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Jehg, J.E., Poon, L.,
 Guan, Y., Peiris, M., Webster, R.G., 2004. Reemerging H5N1 influenza A viruses in Hong
 Kong in 2002 are highly pathogenic to ducks. J. Virol. 78, 4892-4901.

- Suzuki, H., Saito, R., Masuda, H., Oshitani, H., Sato, M., Sato, I., 2003. Emergence of
 amantadine-resistant influenza A viruses: epidemiological study. J. Infect. Chemother. 3,
 195–200.
- 619
- Swayne, D.E., Suarez, D.L., 2000. Highly pathogenic avian influenza. Rev. Sci. Tech. 19,
 463-482.
- 622
- Tamura, K., Dudley, J, Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics
- Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.
- 625
- Wallensten, A., 2006. Influenza A virus in Wild Birds PhD thesis Linkoping University
 Sweden, ISBN: 91-85523-15-1.
- 628
- Weber, S., Harder, T., Starick, E., Beer, M., Werner, O., Hoffmann, B., Mettenleiter, T.C.,
 Mundt, E., 2007. Molecular analysis of highly pathogenic avian influenza virus of subtype
 H5N1 isolated from wild birds and mammals in northern Germany. J Gen Virol. 88(Pt 2),
 554-558.
- 633
- WHO (World Health Organization), 2005. Recommended laboratory tests to identify
 influenza A/H5 virus in specimens from patients with an influenza-like illness. Available
 online at: <u>http://www.who.int/csr/disease/avianinfluenza/guidelines/en/avian_labtests1.pdf</u>.
- WHO (World Health Organization), 2007. H5N1 avian influenza. Timeline of major events.
 Available online at:
- 640 <u>http://www.who.int/csr/disease/avian_influenza/ai_timeline/en/index.html.</u>

61	1
04	

WHO/FAO/OIE. H5N1 Evolution Working Group, 2007. Towards a Unified Nomenclature 642 System for the Highly Pathogenic H5N1 Avian Influenza Viruses. Available online at: 643 http://www.who.int/csr/disease/avian influenza/guidelines/nomenclature/en/index.html. 644 645 WHO/FAO/OIE. H5N1 Evolution Working Group, 2008. Towards a Unified Nomenclature 646 System for the Highly Pathogenic H5N1 Avian Influenza Virus (H5N1). Emerg. Infect. Dis., 647 14, e1. Available online at: http://www.cdc.gov/EID/content/14/7/e1.htm. 648 649 WHO (World Health Organization), 2009. Cumulative number of confirmed human cases of 650 influenza avian A/(H5N1) reported WHO. Available online 651 to at: http://www.who.int/csr/disease/avian influenza/country/cases table 2009 03 02/en/index.ht 652 653 ml. 654 Xu, X., Subbarao, K., Cox, N.J., Guo, Y., 1999. Genetic characterization of the pathogenic 655 influenza A/Goose/Guangdong/1/ 96(H5N1) virus: similarity of its hemagglutinin gene to 656 those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology, 216, 15-19. 657 658 Zohari, S., Gyarmati, P., Thorén, P., Czifra, Gy., Bröjer, C., Belák, S., Berg, M., 2008. 659 Genetic characterization of the NS gene indicates co-circulation of two sub-lineages of highly 660 pathogenic avian influenza virus of H5N1 subtype in Northern Europe in 2006. Virus Genes 661

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36, 117-125.

665 Legends for figures and tables

- **Table 1.** The Hungarian HPAI H5N1 strains subjected to genetic analysis
- 667 Table 2. Genetic analysis of the whole coding region of each genome segment of the four
- 668 Hungarian HPAI H5N1 index strains
- **Figure 1.** Geographic distribution of the localities affected by HPAI H5N1 in 2006-2007.

670 Outbreaks caused by group HUN1-4 viruses are indicated with dots of different colours.

671 Location of index cases are indicated with triangles

S

Figure 2. Phylogenetic analysis of the HA coding sequences of Hungarian, other European,

673 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,

681 HUN2 strains with blue; HUN3 stains with green and HUN4 strains with yellow.

683 **Table 1.**

684

685

Virus isolate	Locality of isolation	Sampling date	Passage no.	Genetic analysis	Lineage
A/mute swan/Hungary/3472/2006	A/mute swan/Hungary/3472/2006 Nagybaracska		1 st	Whole genome	HUN1
A/mute swan/Hungary/4571/2006	Dunakeszi	2006.02.16.	2^{nd}	Whole genome	HUN2
A/mute swan/Hungary/4912/2006	Csátalja	2006.02.18.	2^{nd}	Hemagglutinin	HUN1
A/mute swan/Hungary/4999/2006	Bátmonostror	2006.02.20.	2^{nd}	Hemagglutinin	HUN1
A/mute swan/Hungary/5203/2006	Dunaszentbenedek	2006.02.21.	1 st	Hemagglutinin	HUN2
A/coot/Hungary/5217/2006	Dávod	2006.02.21.	1 st	Hemagglutinin	HUN2
A/cormorant/Hungary/5237/2006	morant/Hungary/5237/2006 Százhalombatta		2 nd	Hemagglutinin	HUN2
A/mallard/Hungary/5239/2006	Szentendre	2006.02.21.	2 nd	Hemagglutinin	HUN2
A/mute swan/Hungary/6170/2006	Hercegszántó	2006.03.03.	2 nd	Hemagglutinin	HUN1
A/mute swan/Hungary/7054/2006	Mohács	2006.03.07.	2 nd	Hemagglutinin	HUN2
A/mute swan/Hungary/7055/2006	Dunaföldvár	2006.03.07.	3 rd	Hemagglutinin	HUN2
A/mute swan/Hungary/7060/2006	Szalatnak	2006.03.07.	2^{nd}	Hemagglutinin	HUN1
A/goose/Hungary/14756/2006	Bodoglár	2006.06.03.	1 st	Whole genome	HUN3
A/goose/Hungary/2823/2007*	Szentes-Lapistó	2007.01.21.	2^{nd}	Whole genome	HUN4
A/goose/Hungary/3413/2007* Derekegyház-Ördöngős		2007.01.25	3 rd	Whole genome	HUN4

686

687 Bold: the index cases of the different lineages are bolded

688 * These two strains were sequenced by VLA (Weybridge, UK)

Reed

689 **Table 2.**

690

Virus			NA						
			Stalk deletion	Amino acid position					
	99	145	172	238	240	268	Cleavage site	(49-68)	110
3472	Ι	S	А	Q	G	Ν	PQGERRRKKR/G	Yes	R
4571	Ι	S	А	Q	G	Ν	PQGERRRKKR/G	Yes	R
14756	Ι	S	А	Q	G	Ν	PQGERRRKKR/G	Yes	R
2823	Ι	S	А	Q	G	Ν	PQGERRRKKR/G	Yes	R

Virus	s PB2			PB1	PB1-F2			NS1				M2	
	Amino acid position		Amino acid position	Amino acid position	Clade	Deletion (80-84)		An ac pos	nino cid ition]	Amino acid position	n	
	627	701	714	13	66			42	92	144	31	64	66
3472	Κ	D	S	Р	S	Α	Yes	S	Е	А	S	S	Е
4571	Е	D	S	Р	S	А	Yes	S	Е	А	S	S	Е
14756	Κ	D	S	Р	S	А	Yes	S	Е	А	S	S	Е
2823	Κ	D	S	Р	S	А	Yes	S	Е	А	S	S	Е





10

sublineage 2.2A2

sublineage 2.2A1

sublineage 2.2B

possible new third order clade

