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25 **Abstract**

26

27 Highly pathogenic avian influenza (HPAI) H5N1 viruses were introduced to Hungary during
28 2006-2007 in three separate waves. This study aimed at determining the full-length genomic
29 coding regions of the index strains from these epizootics in order to; (i) understand the
30 phylogenetic relationship to other European H5N1 isolates, (ii) elucidate the possible
31 connection between the different outbreaks and (iii) determine the putative origin and way of
32 introduction of the different virus variants. Molecular analysis of the HA gene of Hungarian
33 HPAI isolates obtained from wild birds during the first introduction revealed two groups
34 designated Hungarian1 (HUN1) and Hungarian2 (HUN2) within sublineage 2.2B and clade
35 2.2.1, respectively. Sequencing the whole coding region of the two index viruses A/mute
36 swan/Hungary/3472/2006 and A/mute swan/4571/Hungary/2006 suggests the role of wild
37 birds in the introduction of HUN1 and HUN2 viruses: the most similar isolates to HUN1 and
38 HUN2 group were found in wild avian species in Croatia and Slovakia, respectively. The
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
41 sublineage 2.2.A1 forming the Hungarian3 (HUN3) group. A common ancestry of HUN3
42 isolates with Bavarian strains is suggested as the most likely scenario of origin. Hungarian4
43 (HUN4) viruses isolated from the third introduction clustered with isolate A/turkey/United
44 Kingdom/750/2007 forming a sublineage 2.2.A2. The origin and way of introduction of
45 HUN4 viruses is still obscure, thus further genetic, phylogenetic, ecological and
46 epidemiological data are required in order to elucidate it.

47

48 **Keywords:** Avian influenza, H5N1, Highly pathogenic, Hungary, Phylogenetic
49 characterisation

50 1. Introduction

51

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

68 H5N1 HPAIV virus isolated in Guangdong in southern China in 1996 caused severe disease
69 in geese farms (Xu et al., 1999). Reassortants of this precursor virus caused fatal outbreaks in
70 chicken in 1997, and infected 18 humans, of whom six died. New variants of HPAI H5N1 re-
71 emerged in 2002, and caused lethal disease in wild waterfowl (Sturm-Ramirez et al., 2004).
72 By 2004 it became endemic in several countries of East and Southeast Asia causing large-
73 scale outbreaks in poultry populations (Li et al., 2004). The virus was now also lethal to
74 mammals, especially felids (Keawcharoen et al., 2004). Sporadic human cases also occurred;

75 until February 2009, 409 people have been infected and 256 died (WHO, 2009). The virus
76 was transmitted to wild bird populations causing severe mortalities e.g. in migratory
77 waterfowl at Lake Qinghai North-Western China (Chen et al., 2005). The Qinghai-like HPAI
78 H5N1 spread intensively through Central Asia to the Black Sea region. Outbreaks were
79 reported in Turkey, Romania and Croatia in October 2005, in Ukraine in November 2005
80 (OIE, 2007; WHO, 2007). Quite unexpectedly, within a short period 18 European countries
81 were infected between January and March 2006, most likely by an unusual migration of
82 waterfowl caused by the harsh cold winter (Kilpatrick et al., 2006).

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

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

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

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

111

112 **2. Materials and methods**

113

114 *2.1. Virus selection*

115

116 During the course of the epizootics, all HPAI H5N1 viruses isolated from the 64 wild bird and
117 31 domestic poultry cases were subjected to partial molecular analysis (Pálmai et al., 2007).
118 The H5 proteolytic cleavage site together with the flanking regions as well as the N1 gene
119 were identified with conventional RT-PCR using J3/B2a primers described in Diagnostic
120 Manual for Avian Influenza (Anonymous, 2006) and N1-1/N1-2 primers recommended by
121 WHO (WHO, 2005), respectively. The obtained preliminary sequence data showed that the
122 wild bird isolates can be divided into two distinct sublineages designated Hungarian1 (HUN1)
123 and Hungarian2 (HUN2) groups. In the present study 12 wild bird H5N1 isolates were

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

139

140 2.2. Virus isolation

141

142 The viruses were isolated from dead wild waterfowl, domestic goose, duck and guinea fowl
143 according to European standardised protocols (Alexander, 2005). Briefly, 10% w/v organ
144 suspension (trachea, lung, brain, liver and intestines) prepared in PBS containing antibiotics
145 and antimycotics was inoculated into the allantoic cavity of five 9-11-day-old specific
146 pathogen-free (SPF) embryonated chicken eggs. The eggs were incubated in a humidified
147 atmosphere at 37°C for six days and were candled daily. The allantoic fluid originated from
148 dead embryos and the end of the passage period were examined by hemagglutination assay.

149 Since in most cases the volume of the original isolates was not appropriate for genetic
150 analysis, a second passage was performed, but in some cases a third passage was applied to
151 increase the viral titre (Table 1).

152

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

154

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

170

171 *2.4. Genetic and phylogenetic analysis*

172

173 Sequences were assembled and edited using the BioEdit v.7.0.7. (Hall, 1999) and the
174 DNASTAR 7 (Lasergene, WI, USA) software package. Identification of all gene segments
175 were performed with BLAST search on sequences available at the National Center for
176 Biotechnology Information, Bethesda, Maryland, USA (<http://www.ncbi.nlm.nih.gov>).
177 Genotyping of the gene segments were carried out with Influenza A Virus Genotype Tool (Lu
178 et al., 2007). Distance based neighbour-joining and character based maximum parsimony
179 phylogenetic trees were generated using the Molecular Evolutionary Genetics Analysis
180 (MEGA) software v.4.0. (Tamura et al., 2007). The neighbour-joining algorithm was
181 implemented with the Kimura-2 parameters model using a transition-to-transversion ratio of
182 2.0. Other models were also tested, which showed similar topologies. The topology of trees
183 was confirmed by 1000 bootstrap replicates. Maximum likelihood phylogenetic trees were
184 generated by the PHYLIP version 3.68 software (Felsenstein, 2004). Trees were edited with
185 Tree Explorer program of MEGA v.4.0. For phylogenetic analysis, in addition to the four full-
186 length coding sequences of the Hungarian isolates, a set of H5N1 strains from Europe,
187 Middle-East, Africa and Asia were selected from the Influenza Virus Resource at NCBI (Bao
188 et al., 2008).

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

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

193

194

195 **3. Results**

196

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

198

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

208 No further H5N1 cases were identified during the next two months. However, in June 2006,
209 the largest H5N1 epizootic of Europe broke out in Hungary. The first suspicion (neurological
210 signs) was reported on June 3rd 2006 from a free-range goose farm of 3000 birds located in
211 county Bács-Kiskun, about 90 km north-east of the first confirmed mute swan case. The
212 presence of the virus was confirmed on June 9th 2006 by the NRL. In the next month and a
213 half, 29 HPAI H5N1 outbreaks were confirmed at seven localities (Figure 1). Out of the 29
214 affected holdings, 13 were goose farms, 16 duck farms and one mixed holding (duck, goose,
215 guinea fowl). The population of the holdings ranged from 110 to 33768 birds. During the
216 course of the epizootic 8374 cloacal/tracheal swabs and 800 carcasses were submitted for
217 virus detection. Out of these samples, 155 duck and 230 goose swabs as well as carcasses of
218 66 ducks, 60 geese and one guinea fowl were positive for H5N1 HPAIV. Immediate
219 emergency (e.g. as stamping out of suspected flocks) as well as pre-emptive measures
220 (depopulation in 1 km zone around the outbreaks) were successfully implemented, to prevent
221 the spread of the virus to other counties of Hungary and the surrounding countries. During the

222 epizootic, 675 premises were affected, one million birds were culled and the total damage
223 caused by the epizootic reached 7.3 million Euros.

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

235

236 *3.2. Phylogenetic analysis*

237 *3.2.1. Phylogenetic analysis of hemagglutinin coding sequences*

238

239 Sequence analysis of the complete hemagglutinin coding sequence of the 12 selected wild
240 bird H5N1 isolates confirmed the previous HUN1 and HUN2 clustering. The four isolates
241 belonging to HUN1 group (Table 1) were almost identical in the examined genomic region;
242 only A/mute swan/Hungary/4912/2006 isolate contained an A1017T substitution that proved
243 to be silent. The hemagglutinin sequences of the eight HUN2 isolates (Table 1) were identical
244 with the exception of A/mute swan/4571/2006, which contained a C942A substitution also
245 resulting in no amino acid change.

246 Comparison of the Hungarian H5N1 index strains with the available European, Middle East
247 and African HA genes by maximum parsimony method revealed that they belong to four
248 different groups (neighbour-joining and maximum likelihood methods gave similar topology).
249 The HUN1 isolates clustered with Croatian H5N1 strain A/cygnus olor/Croatia/1/2005
250 (bootstrap value: 63) in sublineage 2.2B that comprises of Czech, German “Northern group”,
251 Danish, Swedish, United Kingdom, Russian, and Nigerian “African sublineage A” (Ducatez
252 et al., 2006, 2007) isolates (Figure 2).

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

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

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

268

269 *3.2.2. Phylogenetic analysis of full genomes of the index strains*

270

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

275 Phylogenetic trees of the NA gene and the six internal genes showed the same topology as
276 was observed in the case of the HA tree, but their resolution was lower due to the reduced
277 number of sequence data available. The PB2 maximum parsimony phylogenetic tree in Figure
278 3 represents the phylogenetic relationship between European, Middle East and African H5N1
279 isolates in the internal gene segments. The only remarkable difference compared with the HA
280 tree is the lack of available whole genome sequence in sublineage 2.2A1, therefore the HUN3
281 index strain is the first candidate in this group.

282

283 *3.3. Molecular characterisation*

284

285 Results of the molecular characterisation of the four different Hungarian HPAI H5N1 strains
286 are summarised in Table 2. The hemagglutinin genes of each virus possessed the multiple
287 basic amino acids characteristic of HPAI H5N1. Markers of receptor binding preference to
288 2,3-NeuAc-Gal linkages of avian cell-surface receptors (Ha et al., 2001) were found at amino
289 acid residues Q222 and R224 (amino acid positions 226 and 228 in H3 numbering, 238 and
290 240 at H5N1 HA gene). The three amino acid residues (99I, 240G and 268N) characteristic of
291 clade 2.2 viruses (Chen et al., 2006) were identified in all Hungarian strains. The S145L and
292 A172T mutation leading to increased virulence in terrestrial poultry (Perdue and Suarez,
293 2000) were not found even in the genomes of the goose isolates. Analysis of the translated
294 HA1 protein sequence with NetNglyc 1.0 server, six potential N-linked glycosylation sites

295 were predicted in the Hungarian isolates at amino acid positions 27, 39, 181, 302, 500 and
296 559.

297 Analysis of the NA gene revealed the presence of the 20-amino-acid deletion (amino acids
298 49-68) and the arginine at position 110 characteristic of clade 2.2 viruses (Chen et al., 2006).
299 Amino acid 275 is H for all Hungarian AI isolates, indicating full susceptibility to oseltamivir
300 (Smith et al., 2006). Three potential N-linked glycosylation sites were predicted at amino acid
301 positions 68, 126 and 215.

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

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

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

320 involved in host cell signalling pathways (Jackson et al., 2007), were found in the NS proteins
321 of all sequenced H5N1 isolates.

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

326

327 **4. Discussion**

328

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

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

344 Sublineage 2.2B HUN1 viruses emerged at the beginning of February 2006 in Southern
345 Hungary in the wetland areas along river Danube. Interestingly, during the course of the wild
346 bird (mute swan) outbreaks, the HUN1 viruses remained restricted to this small geographical
347 area.

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

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

360 Clade 2.2.1 HPAI H5N1 strains first emerged in Italy at the beginning of February (OIE,
361 2007) and spread to Central Europe (Slovenia, Austria, France, Slovakia, Czech Republic and
362 Southern Germany) almost exactly at the same time as in Hungary possibly via the main
363 South-North migratory route of wild birds from Africa to Europe. This assumption is further
364 supported by detecting HPAIV H5N1 of this cluster in Nigeria, in January 2006. On the other
365 hand, analysis of partial HA sequences from the neighbouring countries of Hungary revealed
366 an interesting phenomenon; both sublineage 2.2B and clade 2.2.1 viruses emerged and co-
367 circulated in Romania in 2005 (data not shown), which means that clade 2.2.1 viruses had
368 been present in Europe far before they were detected in Italy. The ancestor of these strains

369 was not determined yet, but the fact that Turkish and Romanian isolates from 2005 also
370 belong to this clade raises several questions about the exact origin of these strains.

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

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

381 The third introduction wave of HPAI H5N1 into the territory of Hungary occurred in January
382 2007, when no presence of HPAI was reported in the country. These HUN4 strains form a
383 distinct, monophyletic cluster, sublineage 2.2A2 with isolate A/turkey/United
384 Kingdom/750/2007 within clade 2.2A. A direct epidemiological link was established between
385 the Hungarian and United Kingdom outbreaks (Irvine et al., 2007). The common ancestor,
386 origin and way of introduction of HUN4 viruses are still obscure.

387 Molecular analysis of each segment of the Hungarian HPAI H5N1 isolates revealed identical
388 molecular markers regarding proteolytic cleavage site, receptor-binding preference, and
389 glycosylation sites of HA, stalk deletion and glycosylation pattern of NA. The pathogenicity
390 markers described in European H5N1 viruses (Bragstad et al., 2007; Gal Reculé et al., 2007)
391 regarding the polymerase complex, NP, M and NS genes were present in the examined
392 Hungarian strains. Heterogeneity was found in PB2 627 position: the HUN2 strain possessed
393 glutamic acid, while the other representative strains contained lysine. This result is in

394 accordance with findings in Sweden (Kiss et al, 2008) and the Czech Republic (Nagy et al.,
395 2008). The other difference, the EPKV motif found at the N-terminus of NS1 protein is
396 uncommon among European H5N1 strains, the closest viruses possessing this motif are
397 originated from Astrakhan. Sequencing of additional HUN3 strains is necessary in order to
398 elucidate this finding.

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

407

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409

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415

416 **Conflict of interest statement**

417

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

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665 **Legends for figures and tables**

666 **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

669 **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

672 **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
674 was confirmed by 1000 bootstrapping steps. Bootstrap values higher than 70 are indicated at
675 nodes. For means of clarity, trees are midpoint rooted. HUN1 strains are marked with red,
676 HUN2 strains with blue; HUN3 stains with green and HUN4 strains with yellow.

677 **Figure 3.** Phylogenetic analysis of the PB2 coding sequences of Hungarian, other European,
678 Middle East and African strains using maximum parsimony method. The topology of the tree
679 was confirmed by 1000 bootstrapping steps. Bootstrap values higher than 70 are indicated at
680 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.

682

683 **Table 1.**

684

685

Virus isolate	Locality of isolation	Sampling date	Passage no.	Genetic analysis	Lineage
A/mute swan/Hungary/3472/2006	Nagybaracska	2006.02.06.	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	Bátmonostor	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	Százhalombatta	2006.02.21	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-Ördögös	2007.01.25	3 rd	Whole genome	HUN4

686

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

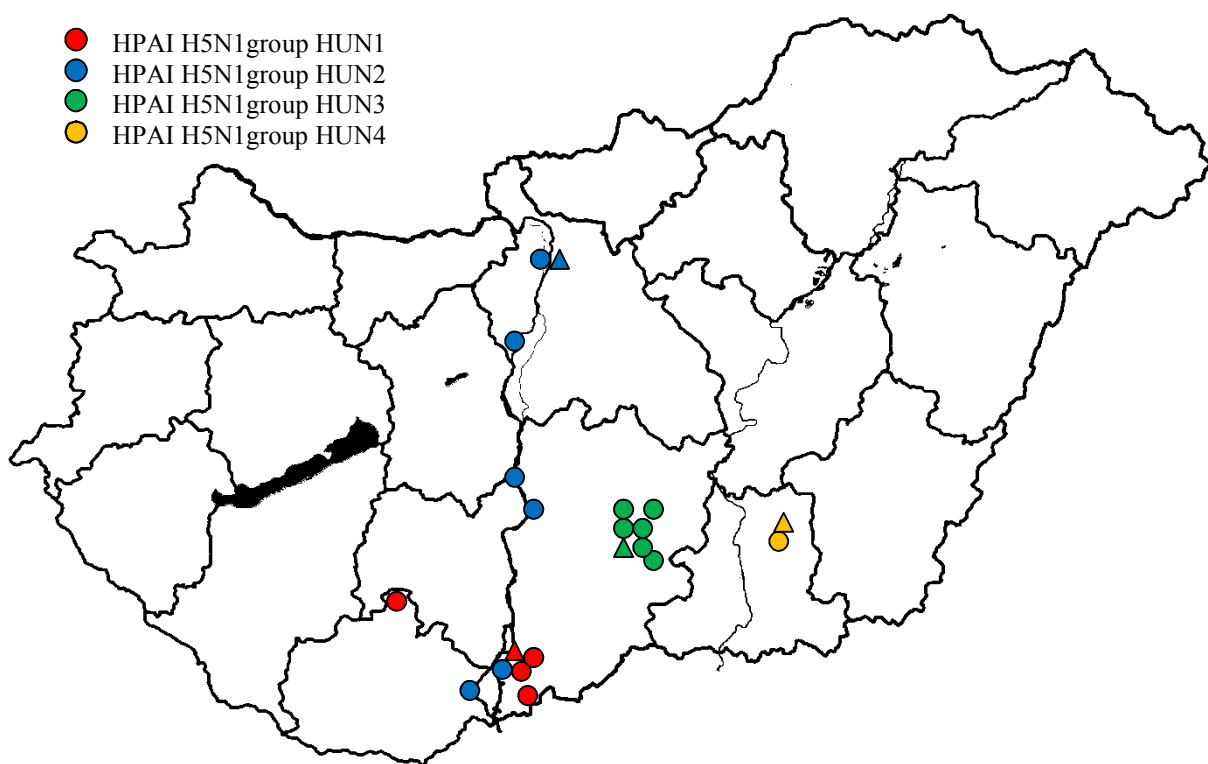
688 * These two strains were sequenced by VLA (Weybridge, UK)

689 **Table 2.**

690

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

Virus	PB2			PB1	PB1-F2		Clade	Deletion (80-84)	NS1			M2		
	Amino acid position			Amino acid position	Amino acid position	Amino acid position			Amino acid position					
	627	701	714	13	66	42			92	144	31	64	66	
3472	K	D	S	P	S	A	Yes	S	E	A	S	S	E	
4571	E	D	S	P	S	A	Yes	S	E	A	S	S	E	
14756	K	D	S	P	S	A	Yes	S	E	A	S	S	E	
2823	K	D	S	P	S	A	Yes	S	E	A	S	S	E	



Accepted

