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Concurrent Acquisition of a Single Nucleotide Polymorphism in Diverse Influenza H5N1 Clade 2.2 Sub-clades

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Running Title: Concurrent Influenza SNP Acquisitions

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Highly pathogenic Influenza A H5N1 was first identified in Guangdong Province in 1996, followed by human cases in Hong Kong in 1997 [1,2]. The number of confirmed human cases now exceeds 300 and the associated Case Fatality Rate exceeds 60% [3]. The genetic diversity of the serotype continues to increase. Four distinct clades or sub-clades have been linked to human cases [4-7]. The gradual genetic changes identified in the sub-clades have been attributed to copy errors by viral encoded polymerases that lack an editing function, thereby resulting in antigenic drift [8]. We report here the concurrent acquisition of the same polymorphism by multiple, genetically distinct, clade 2.2 sub-clades in Egypt, Russia, Kuwait, and Ghana. These changes are not easily explained by the current theory of "random mutation" through copy error, and are more easily explained by recombination with a common source. The recombination role is further supported by the high fidelity replication in swine influenza [9] and aggregation of single nucleotide polymorphisms in H5N1 clade 2.2 hemagglutinin [10].

The study of influenza evolution in nature has been aided by the emergence of a new strain (clade 2.2) first identified at Qinghai Lake in central China in the spring of 2005. Sequencing of all eight genes [11,12] showed that isolates from migratory waterfowl were easily distinguishable from previous isolates linked to poultry and human infections in eastern and southeastern Asia [13,14]. The new strain was subsequently found in outbreaks in Russia, Kazakhstan and Mongolia [15,16]. Prior to these clade 2.2 outbreaks, the highly pathogenic Asian version of H5N1 had never been reported west of China. The detection of H5N1 in migratory waterfowl in the summer of 2005 in Russian and Mongolian bird sanctuaries signaled the start of a major geographical expansion of H5N1. In the following 12 months, almost 50 countries west of China reported H5N1 for the first time. All

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infections were clade 2.2. The geographical reach included Europe, the Middle East and Africa. This expansion offered a unique opportunity to study the evolution of H5N1 as it migrated into new regions, producing human cases in Turkey, Iraq, Azerbaijan, Egypt, Djibouti in 2006 and Nigeria and Pakistan in 2007. Sequence analysis indicated all cases were due to clade 2.2 infections. The outbreaks were due to multiple introductions and isolates had region specific polymorphisms. The sequences also allowed for the monitoring of new acquisitions of discriminating polymorphisms. These new acquisitions created additional sub-clades defined in phylogenetic analysis. Moreover, close monitoring of these changes could provide insight into the mechanisms underlying the rapid evolution of H5N1 in general, and sub-clade 2.2 in particular. The single nucleotide polymorphism distribution pathways could then be used to predict vaccine targets.

We isolated H5N1 from patients and poultry [17] in Egypt. The first poultry isolates were collected February 2006, and the first human cases developed symptoms in March 2006. Analysis of the H5N1 isolates collected between February and May 2006 defined a series of HA and NA regional markers. These markers from Egypt were also found in the human case from Djibouti, as well as in poultry isolates from Israel and Gaza. A subset of these markers was also identified upstream in European isolates and downstream in west African isolates. These isolates are listed in table S1.

After a lull in reported infections over the summer, H5N1 re-emerged in Egypt in September 2006. The more recent isolates had the same regional markers seen in the previous season. However, both poultry and human isolates had acquired a series of new polymorphisms. Non-synonymous polymorphisms were identified in samples collected from

a cluster of three family members from the Gharbiya governorate in the Nile Delta. HA gene polymorphisms were identified in or near the receptor binding domain, including V223I and M230I (H3 numbering),, as well as the oseltamivir resistance polymorphism, N294S, in the NA gene. The patients first developed symptoms in December 2006 and all three infections were fatal (a detailed report on patients and polymorphism tracing will be described elsewhere).

Additional cases in early 2007 included HA sequences with a 3 BP deletion of the nucleotides encoding Ser at position 133, as well as a case with a novel HA cleavage site, GERRRRKR. The changes were found in multiple patients in central and southern Egypt. The above non-synonymous changes were associated with additional synonymous and non-synonymous changes in the HA and NA sequences that created additional sub-clades of sub-clade 2.2. However, the isolates from the 2006/2007 season maintained the regional markers seen in early 2006 in isolates from Egypt, Djibouti, Israel and Gaza.

Chicken isolates from Gharbiya samples collected on February 15, 2007 included one sequence that was closely related to the sequences from the human Gharbiya cluster. The sequence from this isolate, A/chicken/1892N3-HK49/2007 (HK49), had the regional markers previously seen in the 2006 and 2007 isolates, as well as HA non-synonymous changes, V223I and M230I. Additionally, it also had an NA synonymous polymorphism, G743A, appended onto the genetic background of the human Gharbiya cluster, as seen in the NA cladogram in figure 1 (or the corresponding HA cladogram in figure 1S). The location of the isolate on the tip of the branch indicated it was a recent acquisition. Indeed, there were only two additions when compared to the most closely related human isolate.

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

This polymorphism was also found in two additional chicken isolates, A/chicken/1890N3-HK45/2007 and A/chicken/1891N3-CLEVB/2007, collected the same week in the Gharbiya governorate, but these two isolates fell onto a separate branch of the tree, indicating the same polymorphism had been appended onto two distinct sub-clades. These two isolates had three new polymorphisms relative to isolates collected in June, 2006. Plaque-purified sub-clones of HK49 were isolated because the original sequence had mixed signals in the NA and HA sequences. These HK49 sub-clones fell into two major groups. The NA consensus sequences for the two groups had 11 differences that matched the 11 differences between the two sets of chicken sequences. The major species were closely related to the sequences from the human cluster, while the minor species were closely related to the two additional chicken sequences describe above. However, all sequenced, plaque-purified clones had G743A (data not shown), confirming the acquisition of G743A onto two distinct genetic backgrounds in Egypt.

G743A was subsequently found in human isolates from patients who developed symptoms in April 2007. Included were siblings with HA sequences that had the 3 BP deletion seen in earlier patients from central Egypt. Like the chicken sequences above, the G743A polymorphism was appended onto sequences identified earlier in Egypt. Similarly, distinct sequences from another patient, A/Egypt/2630-NAMRU3/2007, that acquired G743A, also fell onto a separate branch.

The distinct branches displayed in the NA cladogram were also seen in the HA cladogram in supplemental figure 1. The isolates with G743A are also located at the tips of the branches, supporting a recent acquisition of the polymorphism.

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

In February 2007 an H5N1 clade 2.2 outbreak was reported in Kuwait. The sequences in Kuwait traced back to a June, 2006 massive wild bird outbreak at Uvs Lake in Mongolia, which affected wild birds in northern Mongolia and southern Siberia. Related sequences were subsequently isolated in the outbreak in South Korea in November, 2006. These sequences have been designated clade 2.2.3 and are represented in an NA cladogram in figure 2 or an HA cladogram in supplemental figure 2. The 2006 sequences did not have G743A. However, G743A was present in all reported NA sequences from Kuwait. Related sequences were subsequently reported in German outbreaks in Bavaria, Saxony, and Thuringen during the summer of 2007, outbreaks in Krasnodar in the fall of 2007, and outbreaks in Saudi Arabia in the winter of 2007. All reported NA sequences had G743A.

In February 2007, a distinct H5N1 clade 2.2.3 outbreak occurred near Moscow, Russia. Isolates from infected chickens were most closely related to 2006 sequences from Azerbaijan. The sequences formed a separate branch which is also seen in Figure 2. Although these clade 2.2.3 isolates were collected from a wide geographical area, G743A was not found in any of the 2006 isolates. The isolates from Moscow map onto the tip of the branch formed by the Azerbaijan isolates. Subsequently, sequences related to the Moscow sequences were reported from Rostov, Russia. As seen in figure 2, these sequences had G743A. (see supplement figure 2 for HA phylogenetic tree or supplement Table 1 for partial NA sequences with G743A).

Similarly, in April, 2007, an H5N1 clade 2.2 outbreak occurred near Tema, Ghana. Sequences from three chickens were most closely related to turkey isolates collected in December 2006 in the Ivory Coast. Figure 3 contains the isolates from western

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

Africa, which include isolates from Nigeria collected in 2006 and 2007. Like the Egypt, Russian, and German isolates above, the 2007 isolates extended branches rooted with 2006 isolates which did not have G743A. 2007 isolates from Ghana and Nigeria have G743A appended onto the 2006 genetic backgrounds.

Although G743A was not present in the root sequences from the 2007 isolates which had G743A, it was present in a subset of clade 2.2 sequences from Europe, which are shown in Figure 4. G743A was found in a series of closely related sequences from southern Germany, Switzerland, and France (additional partial sequences in Table 1).

The NA G743A polymorphism can be traced through public H5N1 sequences. Isolates with full NA sequences are listed in the NA phylogram in figure 5. The polymorphism was identified in the first reported sequences linked to the spread of H5N1 in Asia in 2003/2004 in South Korea [18] and Japan [19]. The polymorphism was subsequently identified in clade 1 isolates in southeast Asia, as well as clade 2.1 isolates in Indonesia and clade 2.3 isolates in China. The first reported clade 2.2 isolates were in wild birds in Germany [20] (see figure 4) collected in February 2006. The isolates in Germany formed distinct HA and NA branches due to a series of regional markers in these isolates.

The concurrent acquisition of the same polymorphism by multiple sub-clades challenges the current theory of influenza evolution that invokes random mutations as the mechanism for the generation of antigenic drift. The isolates with the newly acquired polymorphisms map to the tips of the branches of the phylogenetic trees, indicating the acquisitions were recent. The number of new acquisitions for the examples described was 2-6. Thus, in each case the acquisition of G743A was included in a small number of new acquisitions,

which would be inconsistent with random mutations. All discussed isolates on the tips of the branches were collected in 2007, and in five geographically distinct regions representing 11 clade 2.2 genetic backgrounds. These data do not support a common progenitor sequence because the most closely related sequences to each of the respective recent isolates do not have this change. Similarly, concurrent mutation / selection by eleven isolates that map to six branches in four countries and were collected over a short time frame is also unlikely.

An alternative explanation for the appending of these sequences is through homologous recombination between closely related sequences. The newly acquired polymorphisms in the recent isolates in Egypt are readily found in recent H5N1 isolates, as noted in the three HA examples previously described above. Moreover, the G743A polymorphism is found in these genetically distinct sequences collected over a short time frame.

The example of G743A is dramatic, but not unique. Movement of three HA polymorphisms are depicted in Figure 6. These three polymorphisms are found in the same set of 2006 isolates with G743A. One polymorphism is in all of the isolates with G743A. The HA polymorphism, C689T, was initially reported in the first isolates from the outbreak in Siberia in the summer of 2005. In 2007, the polymorphism is also in Egyptian isolates in one of the branches which formed in the 2006/2007 and includes two of the first isolates from Gharbiya which had NA G743A. This polymorphism has been stable and was present in the 2008 isolate on the branch. A second example is the non synonymous change G754A, which encodes the receptor binding domain change, M230I. This polymorphism is in one of the German isolates and the isolate in the branch that links back to the other initial

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

isolate with G743A. G754A is also on additional 2007 isolates in Egypt. The third polymorphism, C1614T, is in another 2006 isolate from Germany, but is found on two Egyptian branches. One of the branches is a subset of the isolates with G754A. C1614T is a recent acquisition by isolates on this branch. Recently, analysis of human influenza found evidence for homologous recombination over small regions [21]. The majority of these acquisitions were in NA. Detection of additional examples was limited, but the analysis did not extend beyond sequences generated through an influenza sequencing program, which limited parental sequences from early human isolates as well as isolates from other species, including avian and swine.

In this paper the acquisitions that link through Germany represent a small subset of the newly acquired polymorphisms, but help demonstrate mapping of the polymorphisms. A more detailed map will be presented elsewhere. The origin of the acquisitions by clade 2.2 isolates is evolving. The initial regional polymorphisms frequently traced back to H5N1 isolates in Asia. Similar results for human influenza were recently described [22,23]. The phylogenetic analysis identified Asian origins for seasonal flu, which subsequently spread to Europe and North America, followed by South America.

In this paper, more recent acquisitions trace back to other clade 2.2 isolates. Many of the most recent acquisitions are present in isolates in Egypt, or in the newly emerging clade 2.2.3, which has become dominant in Europe

Recombination is further supported by the aggregation of clade 2.2 polymorphisms from Germany, Egypt, and sub-Sahara Africa into a single human hemaglutinnin sequence in Nigeria [10].

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

This shuffling of single nucleotide polymorphisms creates new sequences which define antigenic drift. Mapping of these pathways and associations may be used to develop novel vaccine targets representing rapidly evolving genomes.

Figure 1 NA Phylogram of Egyptian Isolates

NA phylogram of positions 43-1337. Isolates with G743A are annotated. NA regional markers are C150T, C236T, A703G, A740G, T1088C and G1280A. Accession numbers and additional isolates with G743A listed in Table S1.Egyptian isolates, accession numbers, collection date and governorate in Table S1. Trees generated using neighbor joining with 100 bootstrap repetitions. Sequences generated as described previously [24].

Figure 2 NA Phylogram of Clade 2.2.3 Isolates.

NA phylogram of positions 43-1337. Isolates with G743A are annotated. Accession numbers and additional isolates with G743A listed in Table S1. Trees generated using neighbor joining with 100 bootstrap repetitions. Sequences generated as described previously [24].

Figure 3 NA Phylogram of West African Isolates.

NA phylogram of positions 43-1337. Isolates with annotated. Accession numbers and additional isolates with G743A listed in Table S1. Trees generated using neighbor joining with 100 bootstrap repetitions. Sequences generated as described previously [24].

Figure 4 NA Phylogram of European Isolates.

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

NA phylogram of positions 43-1337. Isolates with G743A are annotated. Accession numbers and additional isolates with G743A listed in Table S1. Trees generated using neighbor joining with 100 bootstrap repetitions. Sequences generated as described previously [24]

Figure 5 NA Phylogram of H5N1 Isolates with G743A

Accession numbers and additional isolates with G743A listed in Table S1. Trees generated using neighbor joining with 100 bootstrap repetitions. Sequences generated as described previously [24]

Figure 6 HA Phylogram of Recent Egyptian Isolates

HA phylogram of positions 93-1688 in isolates with C689T, G754A, and C1614T. HA regional markers are G467A, C661T, C727T, A880G, T937C, and G1018T. Egyptian isolates, accession numbers, collection date and governorate in Table S1. Trees generated using neighbor joining with 100 bootstrap repetitions. Sequences generated as described previously [24].

Figure S1 HA Phylograms of Egyptian Isolates

HA phylogram of positions 93-1688. Isolates with G743A are annotated. HA regional markers are G467A, C661T, C727T, A880G, T937C, and G1018T. Egyptian isolates, accession numbers, collection date and governorate in Table S1. Trees generated using neighbor joining with 100 bootstrap repetitions. Sequences generated as described previously [24].

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

Figure S2 HA Phylogram of Clade 2.2.3 Isolates

HA phylogram of positions 93-1688. Isolates with G743A are annotated. Accession numbers and additional isolates with HA polymorphisms listed in Table S1. Trees generated using neighbor joining with 100 bootstrap repetitions. Sequences generated as described previously [24].

Figure S3 HA Phylogram of West African Isolates.

HA phylogram of positions 93-1688. Isolates with G743A are annotated. Accession numbers listed in Table S. Trees generated using neighbor joining with 100 bootstrap repetitions. Sequences generated as described previously [24].

Figure S4 HA Phylogram of European Isolates.

HA phylogram of positions 93-1688. Isolates with G743A are annotated. Accession numbers listed in Table S. Trees generated using neighbor joining with 100 bootstrap repetitions. Sequences generated as described previously [24].

Table S1 Isolates and Accession Numbers

Names and accession numbers of HA and NA sequences used in figures 1-6. Partial sequences [25-32] with polymorphisms in figures 1-5 are listed.

Acknowledgements

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Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

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Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

Figure 1 NA Phylogram of Egyptian Isolates

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

Figure 2 NA Phylogram of Clade 2.2.3 Isolates

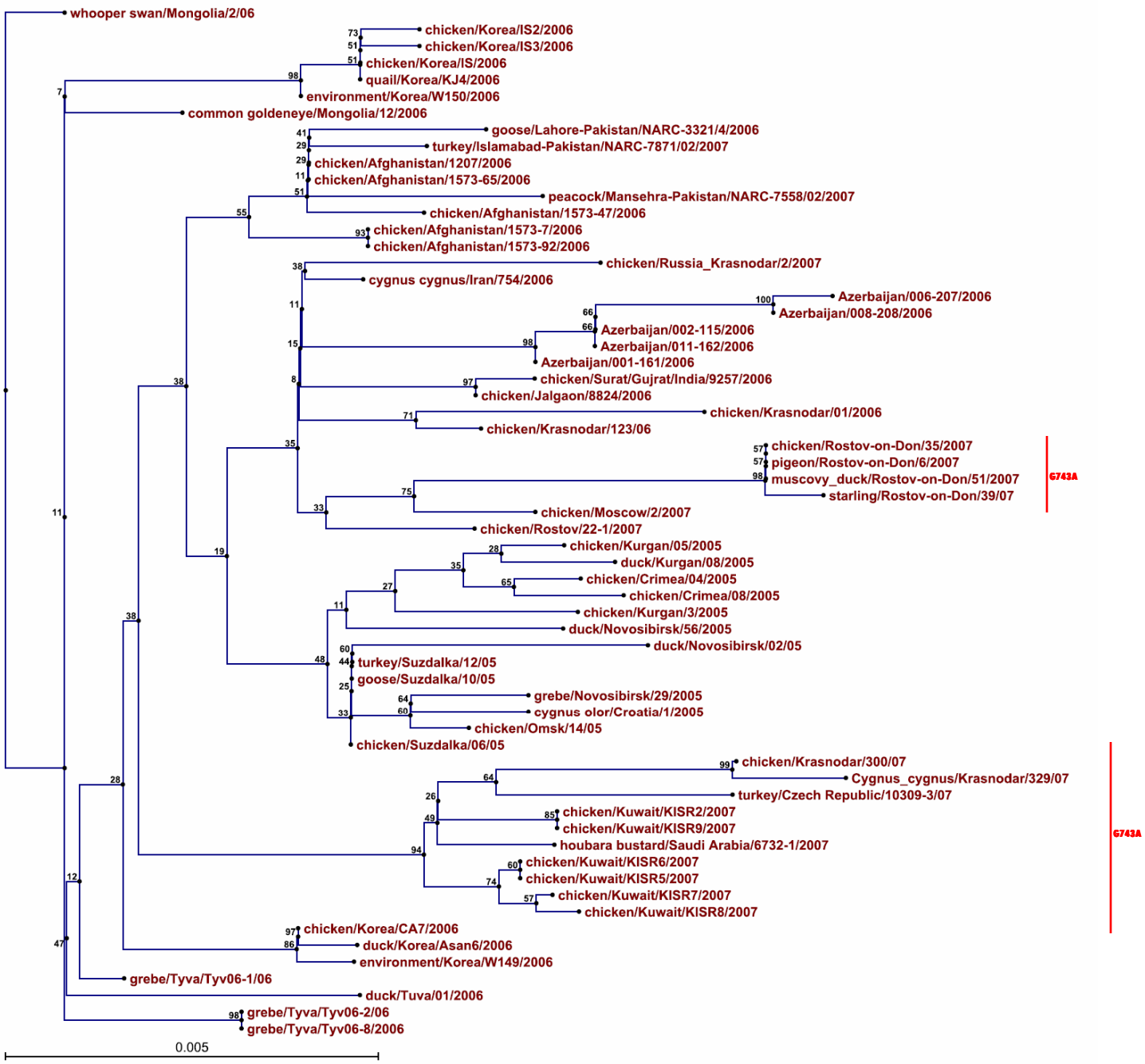

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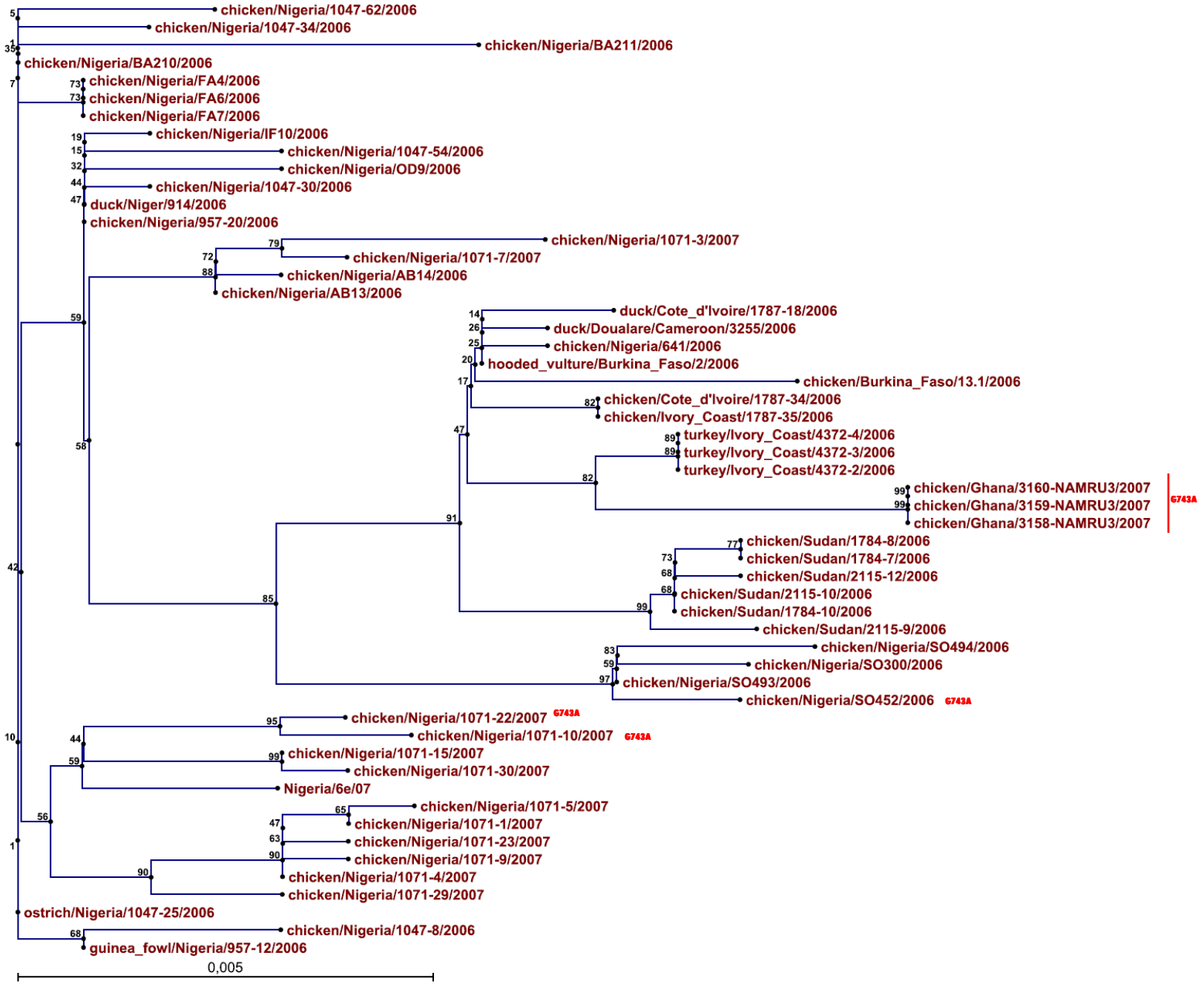
Figure 3 NA Phylogram of West African Isolates



Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

Figure 4 NA Phylogram of European Isolates

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Figure 5 NA Phylogram of H5N1 Isolates With G743A

Nature Precedings : hdl:10101/npre.2008.459.4 : Posted 7 May 2008

Figure 6 HA Phylogram of Recent Egyptian Isolates

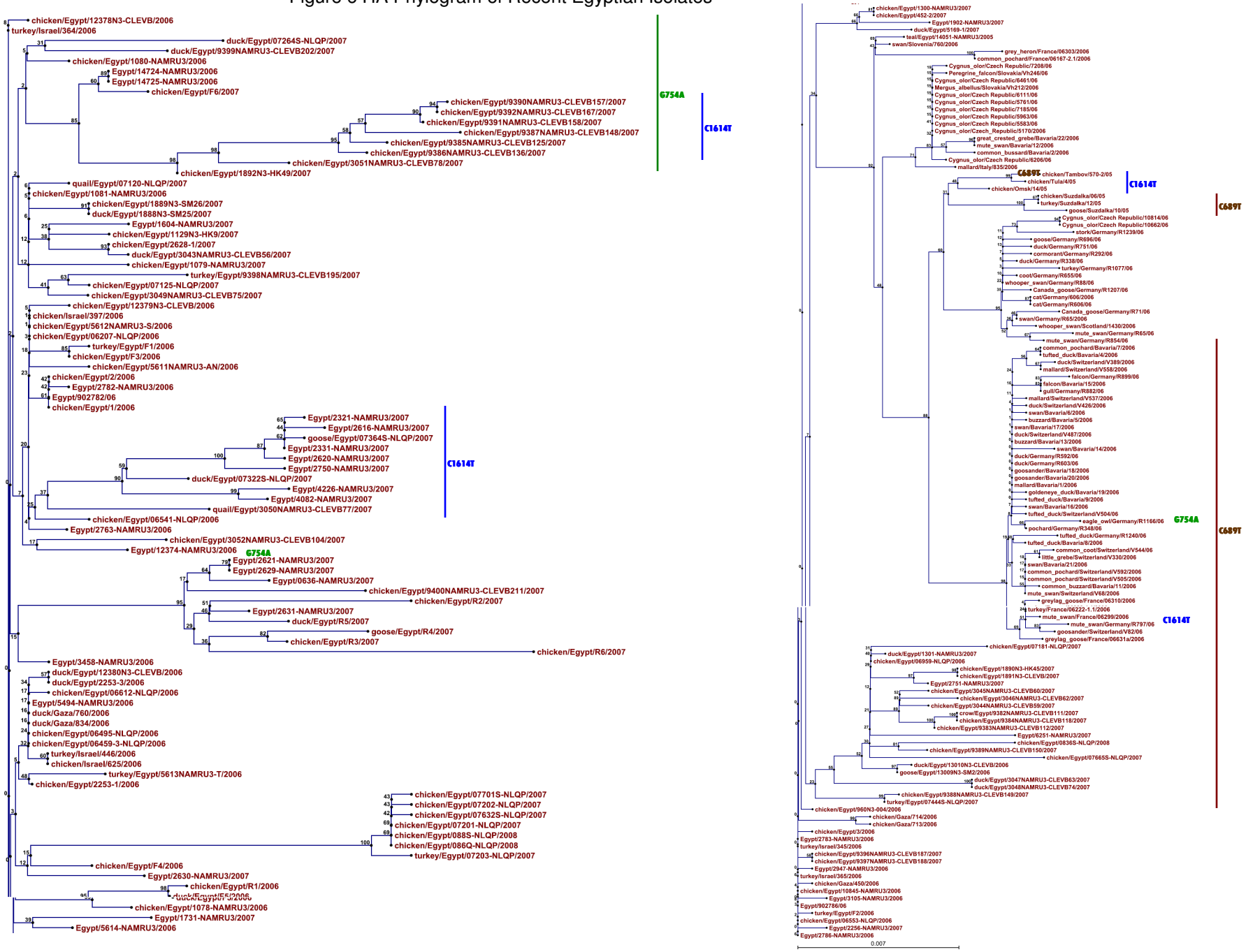

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Figure S1 HA Phylogram of Egyptian Isolates

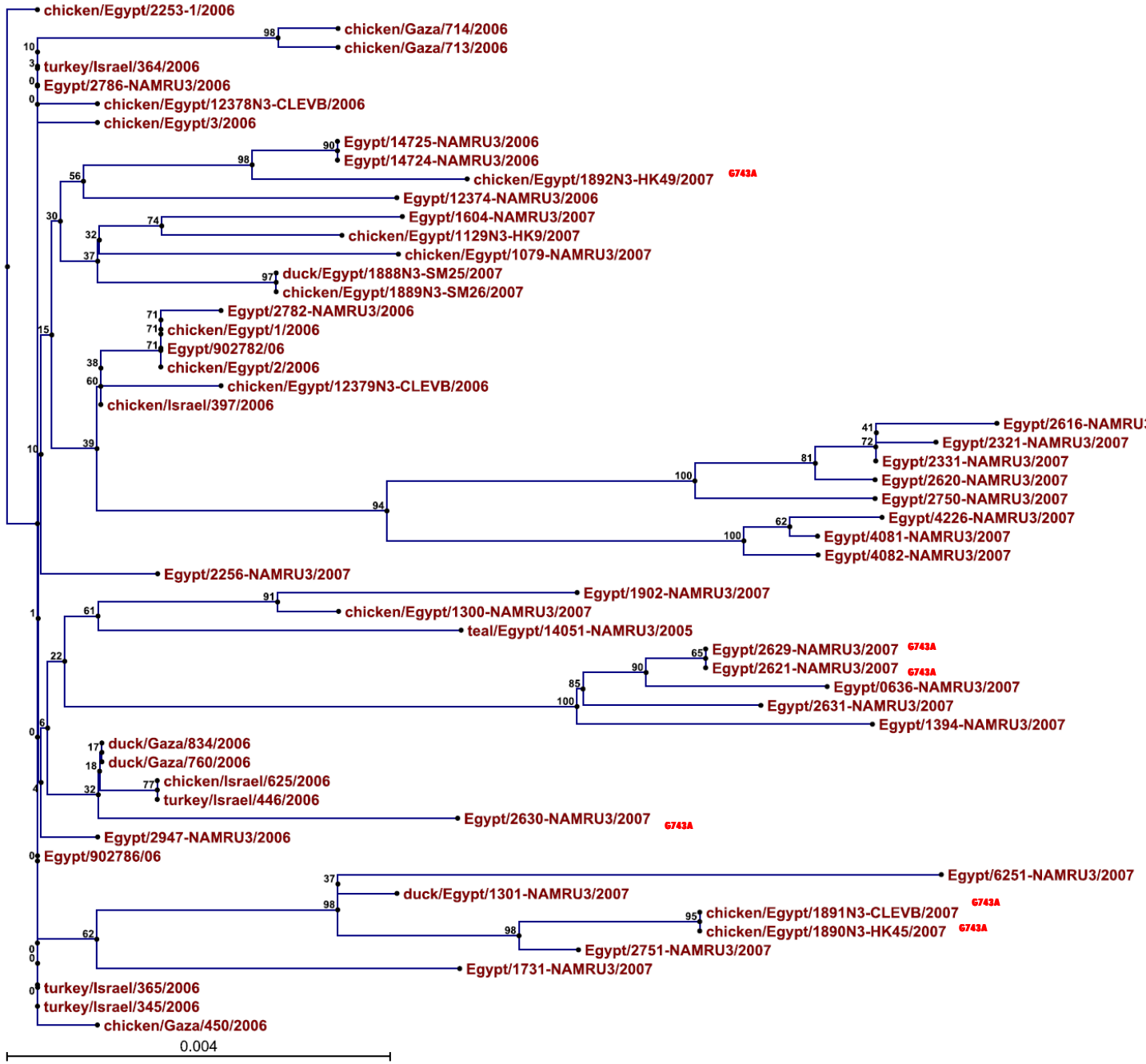

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Figure S2 HA Phylogram of Clade 2.2.3 Isolates

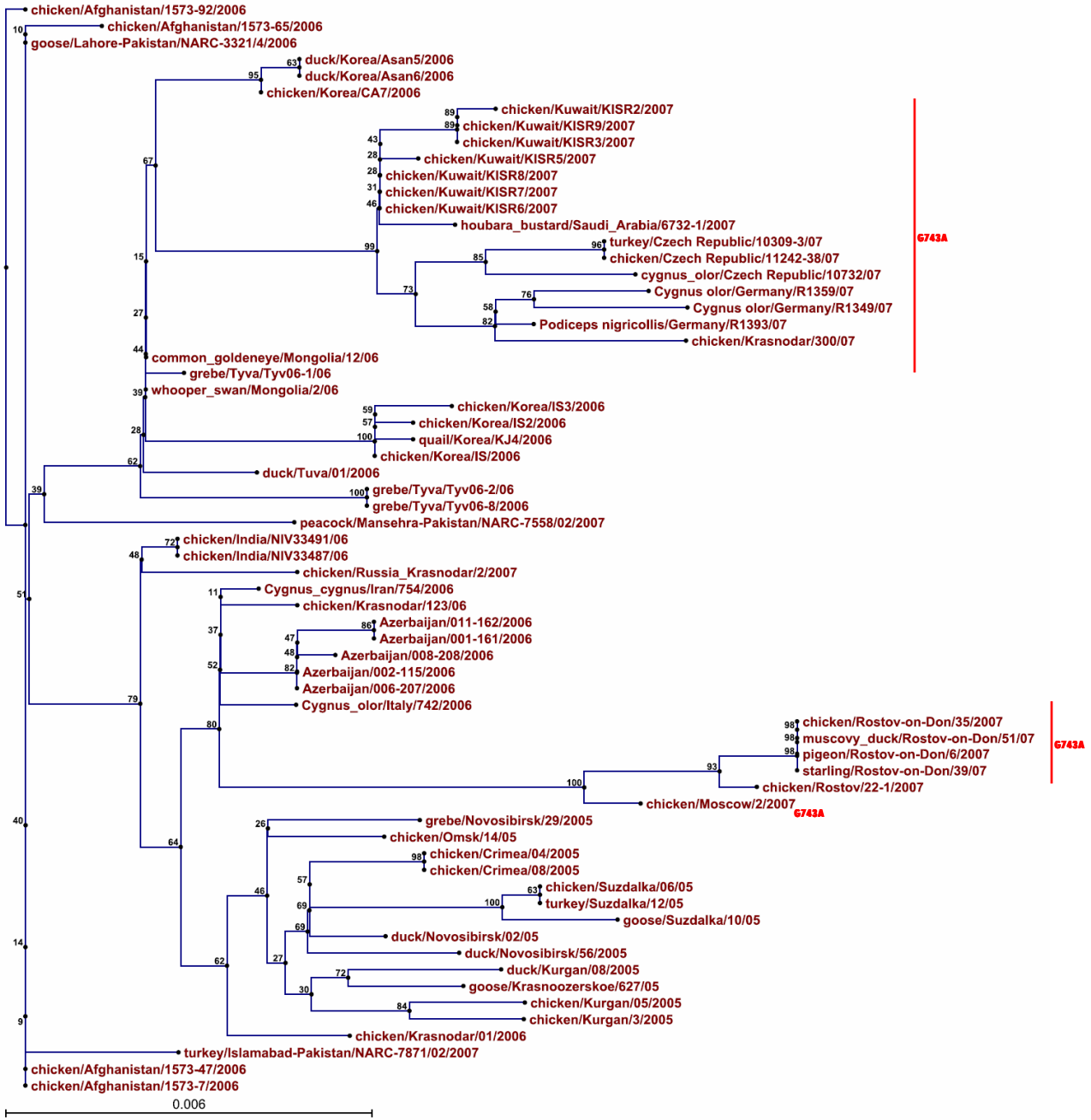

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Figure S3 HA Phylogram of West African Isolates

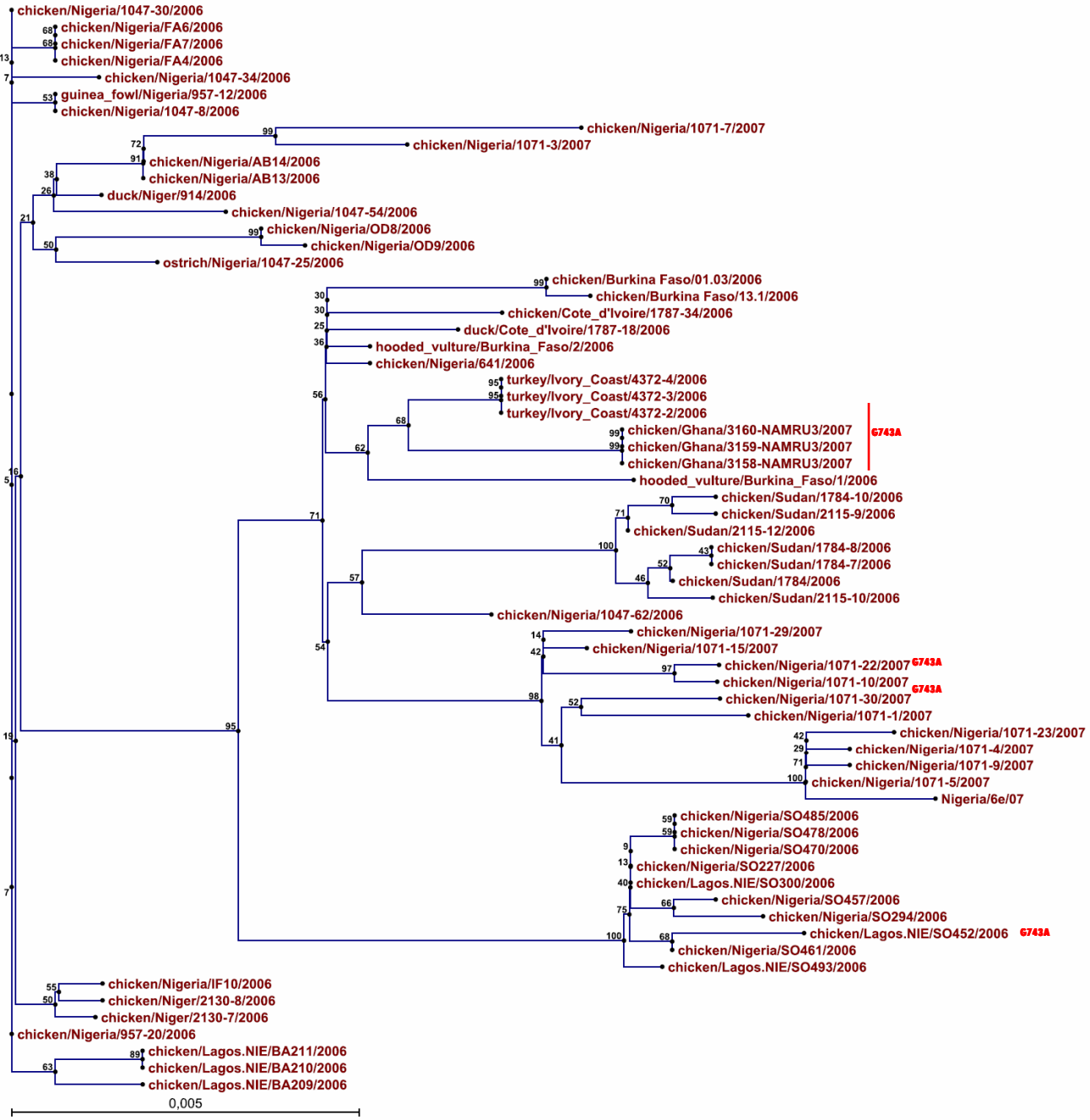

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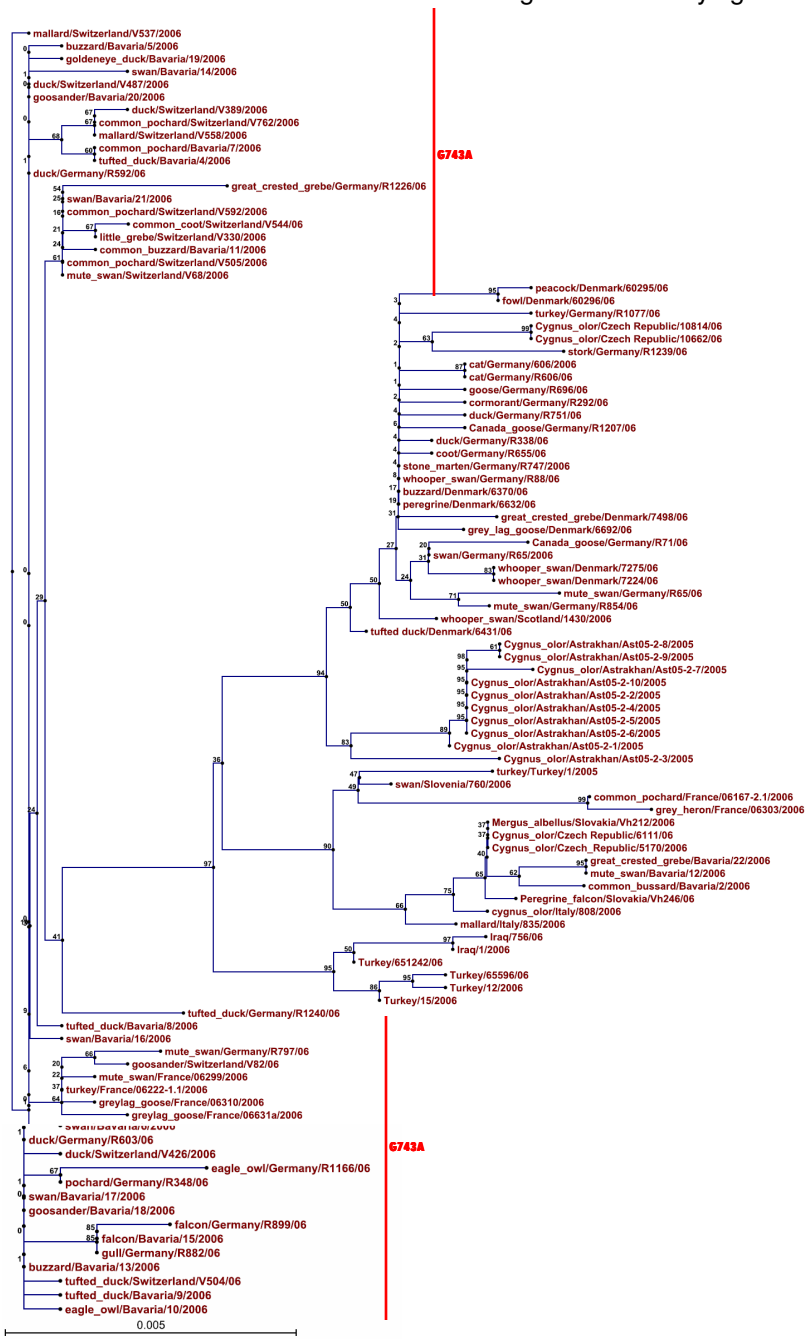
Figure S4 HA Phylogram of European Isolates



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