

# Phylogenetic analysis of the surface proteins of influenza A (H5N1) viruses isolated in asian and african populations

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## SUMMARY

Highly pathogenic H5N1 virus can infect a variety of animals and continually poses a threat to animal and human health. Here, phylogenetic analysis of the hemagglutinin and neuraminidase genes indicated that the hemagglutinin gene of all human isolates, although very similar to each other, fell within different clades corresponding to antigenically distinguishable variants. Likewise, the N1 neuraminidase gene forms a clade that is evolutionarily distinct from previously characterized N1 neuraminidases. So, although all H5N1 viruses were derived from ancestors circulating in south-east Asia more than ten years ago, since 2003 they have evolved into geographically distinct groups within each country.

**KEY WORDS:** H5N1 virus, Phylogenetic analysis, Flu pandemic, Genetic evolution, H5N1 surface antigens

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Until 1997, avian influenza viruses of any subtypes were not considered capable of infecting humans directly.

In May 1997, influenza A subtype H5N1 virus was isolated from an infected child in the Special Administrative Region of Hong Kong, followed by another 17 human cases, 5 of which fatal (Babakir-Mina *et al.*, 2007).

Recent studies showed that the severity of infection in humans depends on the presence of a highly pathogenic HA (Shinya *et al.*, 2006; Stevens *et al.*, 2006; Yamada *et al.*, 2006) and the ability of the virus to spread from the initial site of infection to the lower respiratory tract where avian-like influenza receptors are found (Riel *et al.*, 2006).

In 2003, new cases of H5N1 infection in poultry

(including ducks) were reported in South East Asia with concurrent human cases. Since then, outbreaks in birds have swept throughout Asia, Europe and Africa, and in some countries such as Indonesia the infection is now considered endemic.

As a consequence the likelihood of a new human influenza pandemic has increased over the past few years, as numerous cases of infection have been reported in Asia and Africa.

In this paper, 145 sequences of hemagglutinin (HA) and 113 neuraminidase (NA) sequences of the human H5N1 isolates were randomly downloaded from GenBank and phylogenetic analysis carried out. The NA and HA sequences of human H5N1 virus from 1997 to 2007 were downloaded from the NCBI influenza virus resource (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>). GenBank accession numbers are reported in Table 1.

Two different data sets were built up for both HA and NA gene segments. Multiple sequence alignments of HA and NA segments were obtained us-

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TABLE 1 - GenBank accession numbers of the NA and HA genes used in this study.

| <i>H5N1 types</i>              | <i>HA gene</i> | <i>H5N1 types</i>        | <i>HA gene</i> |
|--------------------------------|----------------|--------------------------|----------------|
| A/Viet Nam/JP178/2004          | EF456795       | A/Indonesia/341H/2006    | EU146729.      |
| A/Viet Nam/JP4207/2005         | EF456798.      | A/Indonesia/534H/2006    | EU146737.      |
| A/Viet Nam/JP14/2005           | EF456799.      | A/Indonesia/535H/2006    | EU146753.      |
| A/Viet Nam/JPHN30321/2005      | EF456802.      | A/Indonesia/546H/2006    | EU146755.      |
| A/Viet Nam/HN30408/2005        | EF456803.      | A/Indonesia/542H/2006(   | EU146777.      |
| A/Viet Nam/1203/2004           | AY818135.      | A/Indonesia/560H/2006    | EU146785.      |
| A/Viet Nam/HN31242/2007        | EU294369.      | A/Indonesia/567H/2006    | EU146801.      |
| A/Viet Nam/1194/2004           | AY651333.      | A/Indonesia/5/2005       | EU146622.      |
| A/Viet Nam/3046/2004           | AY651335.      | A/Indonesia/7/2005       | EU146632.      |
| A/Viet Nam/3062/2004           | AY651336.      | A/Indonesia/175H/2005    | EU146640.      |
| A/Vietnam/CL01/2004            | DQ497719.      | A/Indonesia/160H/2005    | EU146648.      |
| A/Vietnam/CL02/2004            | DQ497720.      | (A/Indonesia/195H/2005   | EU146656.      |
| A/Vietnam/CL17/2004            | DQ497721.      | (A/Indonesia/286H/2006   | EU146688.      |
| A/Vietnam/CL36/2004            | DQ497724.      | A/Indonesia/CDC194P/2005 | CY014168.      |
| A/Vietnam/CL100/2004           | DQ497725.      | A/Indonesia/CDC7/2005    | CY014177.      |
| A/Vietnam/PEV16T/2005          | DQ535724.      | A/Indonesia/CDC184/2005  | CY014197.      |
| A/Viet Nam/3212/2004           | EF451059.      | A/Indonesia/CDC287E/2005 | CY014198.      |
| A/Viet Nam/1204/2004           | EF541404.      | A/Indonesia/CDC292N/2005 | CY014200.      |
| A/Vietnam/CL20/2004            | DQ497722.      | A/Indonesia/CDC329/2006  | CY014206.      |
| A/Vietnam/CL26/2004            | DQ497723.      | A/Indonesia/CDC357/2006  | CY014207.      |
| A/Vietnam/CL105/2005           | DQ497726.      | A/Indonesia/CDC370/2006  | CY014209.      |
| A/Vietnam/CL115/2005           | DQ497727.      | A/Indonesia/CDC390/2006  | CY014213.      |
| (A/Vietnam/CL119/2005          | DQ497728.      | A/Indonesia/CDC594/2006  | CY014272.      |
| A/Vietnam/CL2009/2005          | DQ497729.      | A/Indonesia/CDC595/2006  | CY014280.      |
| A/Turkey/12/2006               | EF619982.      | A/Indonesia/CDC599/2006  | CY014303.      |
| A/Turkey/15/2006               | EF619989.      | A/Indonesia/CDC523/2006  | CY014311.      |
| A/Turkey/65596/2006            | EF619998.      | A/Indonesia/CDC582/2006  | CY014384.      |
| A/Thailand/16/2004             | EF541408.      | A/Indonesia/CDC610/2006  | CY014393.      |
| A/Thailand/Chaiyaphum/622/2004 | EF541417.      | A/Indonesia/CDC623/2006  | CY014401.      |
| A/Thailand/LFPN-2004/2004      | AY679514.      | A/Indonesia/CDC624/2006  | CY014417.      |
| A/Thailand/1(KAN-1)/2004       | AY555150.      | A/Indonesia/CDC625/2006  | CY014433.      |
| A/Thailand/2(SP-33)/2004       | AY555153.      | A/Indonesia/CDC634/2006  | CY014441.      |
| A/Thailand/SP83/2004           | EF541410.      | A/Indonesia/CDC625L/2006 | CY014465.      |
| A/Thailand/676/2005            | DQ360835.      | A/Indonesia/CDC669P/2006 | CY014489.      |
| (A/Thailand/NK165/2005         | DQ372591.      | A/Indonesia/CDC669/2006  | CY014481.      |
| A/Thailand/NKFE/2005           | DQ885610.      | A/Indonesia/CDC644T/2006 | CY014510.      |
| A/Thailand/NKNP/2005           | DQ885612.      | A/Indonesia/CDC739/2006  | CY014529.      |
| A/Thailand/RPNP/2005           | DQ885616.      | A/Indonesia/CDC742/2006  | CY014537.      |
| A/Thailand/HA20/2005           | DQ885618.      | A/Indonesia/CDC759/2006  | CY014543.      |
| A/Thailand/4(SP-528)/2004      | AY626143.      | A/Indonesia/CDC938E/2006 | CY017646.      |
| A/Thailand/5(KK-494)/2004      | AY627885.      | A/Indonesia/CDC835/2006  | CY017662.      |
| A/Prachinburi/6231/2004        | EF541392.      | A/Indonesia/CDC836/2006  | CY017670.      |
| A/Thailand/Kan353/2004         | EF541411       | A/Indonesia/CDC887/2006  | CY017688.      |
| A/Thailand/1(KAN-1A)/2004      | EF107522.      | A/Indonesia/TLL001/2006  | EU015403.      |
| (A/Iraq/1/2006                 | EU146870.      | A/Indonesia/TLL002/2006  | EU015404.      |
| A/Iraq/754/2006                | EU146877.      | A/Indonesia/TLL003/2006  | EU015405.      |
| A/Iraq/756/2006                | EU146878.      | A/Indonesia/TLL004/2006  | EU015406.      |
| A/Indonesia/CDC1031/2007       | CY019352.      | A/Indonesia/TLL007/2006  | EU015409.      |
| A/Indonesia/CDC1032/2007       | CY019384.      | A/Indonesia/TLL012/2006  | EU015414.      |
| A/Indonesia/CDC1046/2007       | CY019408.      | A/Indonesia/TLL009/2006  | EU015411.      |
| A/Indonesia/298H/2006          | EU146697.      | A/Indonesia/TLL008/2006  | EU015410.      |
| A/Indonesia/292H/2006          | EU146713.      | A/Hong Kong/213/2003     | EF541401.      |

follow

TABLE 1 - GenBank accession numbers of the NA and HA genes used in this study.

| <i>H5N1</i> types         | <i>HA</i> gene | <i>H5N1</i> types         | <i>HA</i> gene |
|---------------------------|----------------|---------------------------|----------------|
| A/China/2006              | EF624256.      | A/Vietnam/CL105/2005      | DQ493075       |
| A/China/GD01/2006         | DQ835313.      | A/Viet Nam/HN31242/2007   | EU294372       |
| A/Jiangsu/1/2007          | EU434686.      | A/Vietnam/CL01/2004       | DQ250159       |
| A/Beijing/01/2003         | EF587277.      | A/Vietnam/CL36/2004       | DQ493073       |
| A/human/China/GD02/2006   | EU263981.      | A/Vietnam/CL100/2004      | DQ493074       |
| A/Anhui/1/2005            | DQ371928.      | A/Vietnam/CL115/2005      | DQ493076       |
| A/Anhui/2/2005            | DQ371929.      | A/Vietnam/CL119/2005      | DQ493077       |
| A/Guangxi/1/2005          | DQ371930.      | A/Vietnam/CL2009/2005     | DQ493078       |
| A/Cambodia/JP52a/2005     | EF456805.      | A/Viet Nam/1194/2004      | EF541466       |
| A/Azerbaijan/001-161/2006 | EU146874.      | A/Turkey/12/2006          | EF619981       |
| A/Azerbaijan/002-115/2006 | EU146875.      | A/Turkey/651242/2006      | EF619992       |
| A/Egypt/902786/2006       | EU146868.      | A/Thailand/LFPN-2004/2004 | AY679513       |
| A/Egypt/1394-NAMRU3/2007  | EF535817.      | A/Thailand/1(KAN-1)/2004  | AY555151       |
| A/Egypt/1604-NAMRU3/2007  | EF535818.      | A/Thailand/2(SP-33)/2004  | AY555152       |
| A/Egypt/1731-NAMRU3/2007  | EF535819.      | A/Thailand/SP83/2004      | EF541472       |
| A/Egypt/1902-NAMRU3/2007  | EF535820.      | A/Thailand/4(SP-528)/2004 | AY577316       |
| A/Egypt/2256-NAMRU3/2007  | EF535821.      | A/Thailand/16/2004        | EU268221       |
| A/Egypt/14725-NAMRU3/2006 | EF200513.      | A/Thailand/NKFE/2005      | DQ885611       |
| A/Egypt/14724-NAMRU3/2006 | EF200512.      | A/Thailand/NKNP/2005      | DQ885613       |
| A/Egypt/2620-NAMRU3/2007  | EF535825.      | A/Thailand/RPNP/2005      | DQ885617       |
| A/Nigeria/6e/07           | EU146920.      | A/Thailand/NA60/2005      | DQ885620       |
| A/Egypt/902782/2006       | EU146867.      | A/Prachinburi/6231/2004   | EF512562       |
| A/Egypt/2321-NAMRU3/2007  | EF535822.      | A/Thailand/676/2005       | DQ360836       |
| A/Egypt/2616-NAMRU3/2007  | EF535824.      | A/Thailand/NK165/2005     | DQ372593       |
| A/Egypt/2621-NAMRU3/2007  | EF535826.      | A/Thailand/5(KK-494)/2004 | AY627886       |
| A/Egypt/0636-NAMRU3/2007  | EF382359.      | A/Indonesia/CDC938/2006   | CY017640       |
| A/Hong Kong/485/97        | AF102681       | A/Indonesia/CDC940/2006   | CY017656       |
| (A/Hong Kong/514/97       | AF102682       | A/Indonesia/CDC835/2006   | CY017664       |
| (A/Hong Kong/491/97       | AF102677.      | A/Indonesia/CDC887/2006   | CY017690       |
| (A/Hong Kong/542/97       | AF102678.      | A/Indonesia/321H/2006     | EU146723       |
| (A/Hong Kong/503/97       | AF102679       | A/Indonesia/341H/2006     | EU146731       |
| (A/Hong Kong/532/1997     | AF102680.      | A/Indonesia/536H/2006     | EU146760       |
| (A/Hong Kong/538/97       | AF102674.      | A/Indonesia/546H/2006     | EU146761       |
| (A/HongKong/97/98         | AF102676       | A/Indonesia/560H/2006     | EU146786       |
| (A/HongKong/156/97        | AF036356       | A/Indonesia/583H/2006     | EU146819       |
| (A/Hong Kong/516/97       | AF102673       | A/Indonesia/604H/2006     | EU146827       |
| A/HongKong/481/97         | AF046096       | A/Indonesia/CDC7/2005     | CY014179       |
| A/Hong Kong/482/97        | AF046098       | A/Indonesia/CDC292N/2005  | CY014227       |
| (A/Hong Kong/483/97       | AF046097       | A/Indonesia/CDC326T/2006  | CY014229       |
| H5N1 types                | NA gene        | A/Indonesia/CDC357/2006   | CY014230       |
| A/Viet Nam/1194/2004      | EF541466       | A/Indonesia/CDC370/2006   | CY014231       |
| A/Viet Nam/3046/2004      | AY651446       | A/Indonesia/CDC390/2006   | CY014233       |
| A/Viet Nam/1203/2004      | AY651447       | A/Indonesia/CDC287E/2005  | CY014238       |
| A/Viet Nam/3062/2004      | AY651448       | A/Indonesia/CDC194P/2005  | CY014240       |
| A/Viet Nam/JP178/2004     | EF456796       | A/Indonesia/CDC594/2006   | CY014274       |
| A/Viet Nam/JP4207/2005    | EF456800       | A/Indonesia/CDC523/2006   | CY014313       |
| A/Viet Nam/HN30408/2005   | EF456804       | A/Indonesia/CDC610/2006   | CY014398       |
| A/Viet Nam/HG-178/2004    | DQ094286       | A/Indonesia/CDC623/2006   | CY014406       |
| A/Viet Nam/BL-014/2005    | DQ094290       | A/Indonesia/CDC624/2006   | CY014422       |
| A/Viet Nam/DT-036/2005    | DQ094291       | A/Indonesia/CDC634/2006   | CY014446       |
| A/Viet Nam/HG-207/2005    | DQ094292       | A/Indonesia/CDC669        | CY014486       |
| A/Vietnam/PEV16T/2005     | DQ535726       | A/Indonesia/CDC644T/2006  | CY014507       |
| A/Viet Nam/HN30408/2005   | EF456804       | A/Indonesia/CDC739/2006   | CY014531       |
| A/Vietnam/CL26/2004       | DQ493072       | A/Indonesia/CDC742/2006   | CY014539       |

follow

TABLE 1 - GenBank accession numbers of the NA and HA genes used in this study.

| <i>H5N1 types</i>         | <i>HA gene</i> | <i>H5N1 types</i>               | <i>HA gene</i> |
|---------------------------|----------------|---------------------------------|----------------|
| A/Indonesia/CDC759/2006   | CY014545       | A/Egypt/902786/2006             | EU146883       |
| A/Indonesia/CDC1032/2007  | CY019386       | A/Nigeria/6e/07                 | EU146921       |
| A/Indonesia/CDC1046/2007  | CY019410       | A/Egypt/14725-NAMRU3/2006       | EF222322       |
| A/Indonesia/5/2005        | EU146623       | A/Egypt/14724-NAMRU3/2006       | EF222323       |
| A/Indonesia/6/2005        | EU146625       | A/Egypt/12374-NAMRU3/2006       | EF222324       |
| A/Indonesia/175H/2005     | EU146642       | A/Egypt/0636-NAMRU3/2007        | EF382360       |
| A/Indonesia/195H/2005     | EU146658       | A/HongKong/481/97               | AF084271       |
| A/Indonesia/245H/2005     | EU146674       | A/Hong Kong/483/1997            | AF102668       |
| A/Indonesia/283H/2006     | EU146682       | A/Hong Kong/485/97              | AF102664       |
| A/Indonesia/538H/2006     | EU146686       | A/HongKong/486/97               | AF084275       |
| A/Indonesia/286H/2006     | EU146689       | A/Hong Kong/482/97              | AF102656       |
| A/HK/212/03               | AY575881       | A/Hong Kong/488/97              | AF102657       |
| A/HK/213/03               | AY575882       | A/Hong Kong/507/97              | AF102659       |
| A/human/Zhejiang/16/2006  | DQ643810       | A/Hong Kong/516/97              | AF102660       |
| A/China/GD01/2006         | DQ835315       | A/Hong Kong/538/97              | AF102662       |
| A/Beijing/01/2003         | EF587279       | A/Hong Kong/491/97              | AF102665       |
| A/human/China/GD02/2006   | EU263982       | A/Hong Kong/503/97              | AF102666       |
| A/Anhui/1/2005            | EU128239       | A/Hong Kong/532/1997            | AF102667       |
| A/Jiangsu/2/2007          | EU434696       | A/Hong Kong/514/97              | AF102669       |
| A/Cambodia/JP52a/2005     | EF456793       | A/Hong Kong/542/97              | AF102670       |
| A/Azerbaijan/006-207/2006 | EU146886       | A/Puerto Rico/8/1934(Cambridge) | J02146         |
| A/Azerbaijan/008-208/2006 | EU146887       | A/Brisbane/59/2007              | CY030233       |
| A/Azerbaijan/011-162/2006 | EU146888       | A/Brevig_Mission/1/18           | AF250356       |
| A/Azerbaijan/002-115/2006 | EU146890       |                                 |                |

ing CLUSTAL X program (Thompson *et al.*, 1997). For the NA sequence data set, the first 230 nucleotides (spanning the stalk region of the molecule) were removed from the analysis, due to their high variability. Then, all alignments were manually edited using the Bioedit program (Hall, 1999). The evolutionary model was chosen as the best-fitting nucleotide-substitution model for each data set, according to the hierarchical likelihood-ratio test (LRT) implemented in the MODELTEST v. 3.0 software (Posada and Crandall, 1998). The statistical robustness and reliability of the branching order within the phylogenetic tree for each data set were confirmed with a bootstrap analysis using 1000 replicates for the NJ tree and with the Zero Branch Length Test for the ML tree. All calculations were carried out with Phylogenetic Analysis Using Parsimony (PAUP\*) software package version 4.0b10 (Swofford and Sullivan, 2003).

The sequences of the first avian virus transmitted to humans (HongKong/156/97 H5N1) were used as the out-group in the HA data set whereas the three H1N1 viruses were considered as the out-group for the NA data set.

Molecular analysis of the HA sequences disclosed two major clades in the H5N1 viruses isolated since 2003, clearly distinguishable from the 1997 Hong Kong strains (clade 0), even though the HA gene of this latter group is considered to be the direct precursor of the more recent isolates (Fig. 1). These data suggest that the HA of the recent human isolates has undergone significant antigenic drift (Guan *et al.*, 2004).

According to the isolation year, clade 1 viruses emerged early in 2003 (i.e. A/Hong Kong/213/03) and were detected throughout 2005. Clade 2 viruses were first isolated in Asia in 2004 and since then they continued circulating in the Asian continent and spread to Europe and Africa.

Within clade 2, three further sub-clades among human isolates were identified: 2.1, 2.2 and 2.3.4. With regard to the geographical distribution of the isolates, clade 1 comprises viruses isolated from Vietnamese and Thai patients and shows some intermixing with Thai and Vietnamese strains.

Viruses within sub-clade 2.1 were all from Indonesia, whereas strains located in sub-clade 2.2 were from African and Middle-East patients.

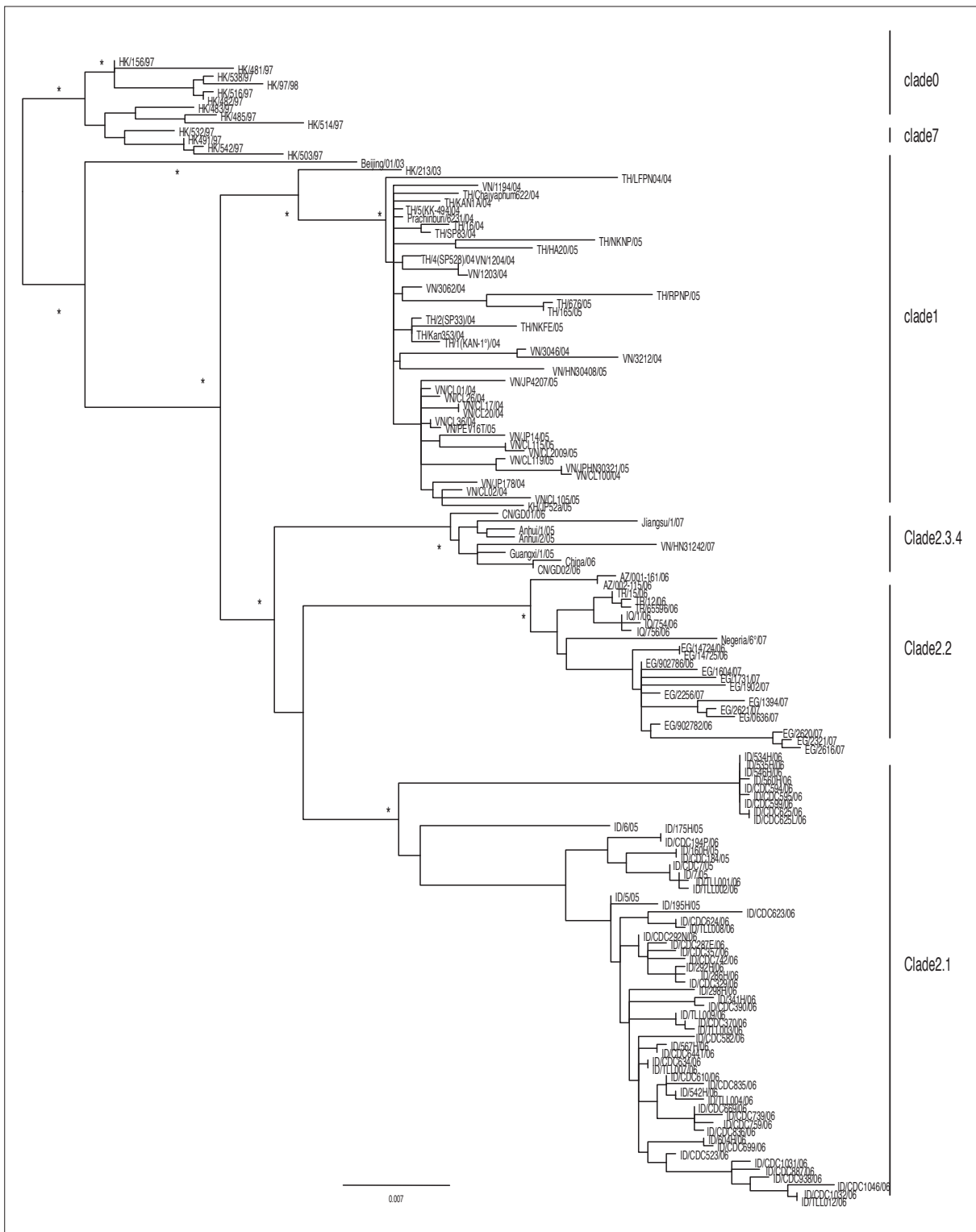


FIGURE 1 - Phylogenetic relationships of the HA gene (1656 nucleotides) of 145 H5N1 human isolates. Tree was constructed based on the NJ method using the GTR+I+G substitution model. The statistical support (bootstrap values for 1000 replicates and branches highly supported by the ML method, (p<0.001).) is indicated as\*.

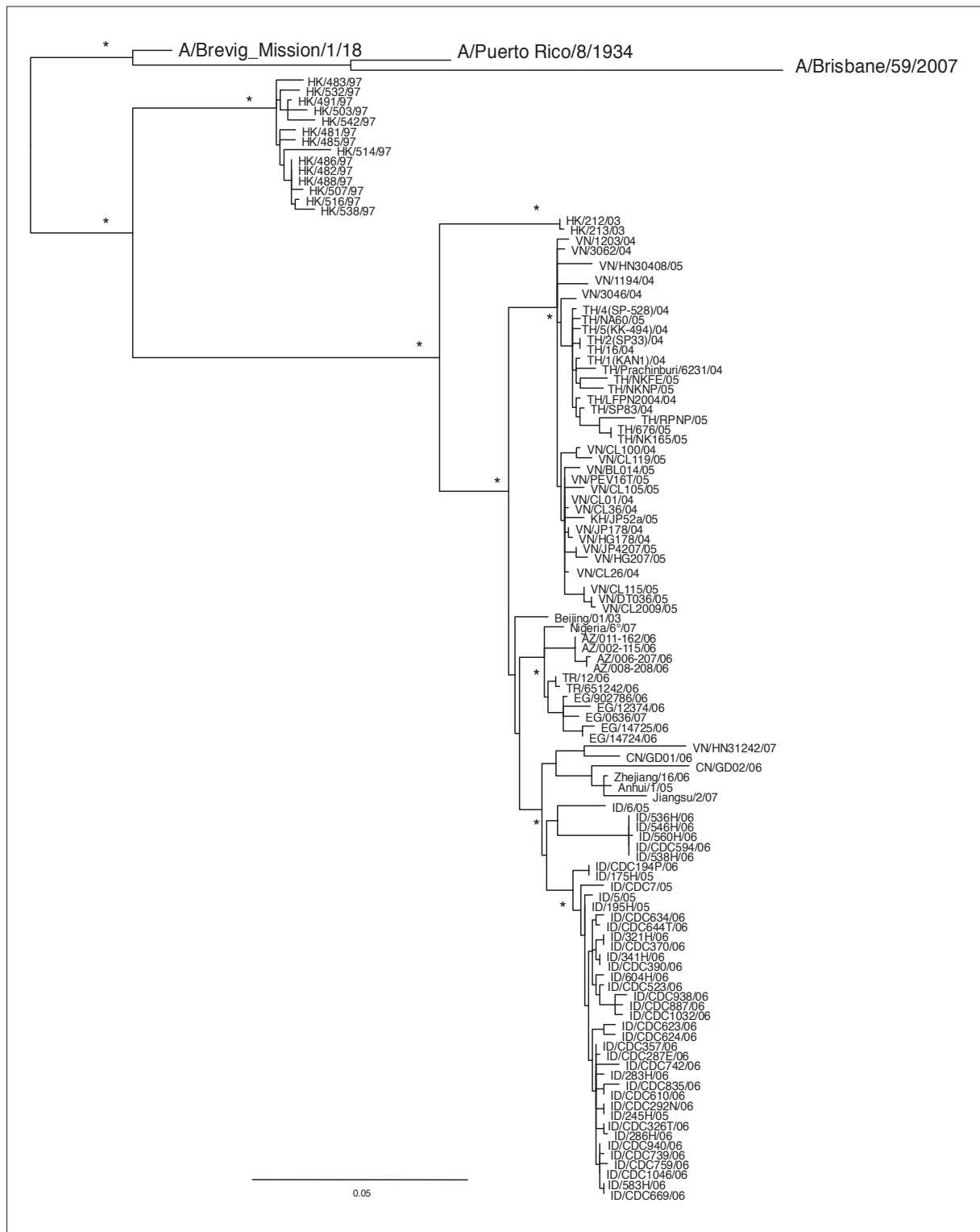


FIGURE 2 - Phylogenetic relationships of the NA gene (1131 nucleotides) of 113 H5N1 human isolates. Tree was constructed based on the NJ method using the GTR+I+G substitution model. The statistical support (bootstrap values for 1000 replicates and branches highly supported by the ML method, ( $p < 0.001$ )) is indicated as\*.

Finally, viruses in sub-clade 2.3.4 were all isolated in China in 2005-2006, except one isolated in Vietnam in 2007 (A/Vietnam/HN31242/07).

All clades and sub-clades were statistically supported.

NA phylogeny of available human sequences showed phylogenetic groupings very similar to those seen in the HA tree, although some of them were not clearly separated (Fig. 2). Two main differences were observed:

- a) A/Hong Kong/212/03 and A/Hong Kong/213/03 formed a group clearly distinguishable from the Vietnamese and Thai isolates in clade 1;
- b) A/Beijing/1/03 NA gene did not constitute a separate lineage and was most closely related to strains from Nigeria, Azerbaijan, Turkey and Egypt.

All H5N1 NA sequences were very distantly related to the human N1 branch, which included two earlier H1N1 human strains, A/Brevig Mission/1/18 and A/PR8/34, as well as a recent isolate, A/Brisbane/59/07.

Although human infection by avian influenza virus A (H5N1) is rare and sporadic, human disease can be severe and lethal.

Therefore, a continuous surveillance of the molecular and genetic features of H5N1 viruses isolated in birds, animals, and humans is essential to identify rapidly virus strains that show a significant antigenic drift and are at greater risk of spreading among humans.

Phylogenetic analysis may help to shed light on the molecular epidemiology of the H5N1 infection in humans.

Interestingly, since 2003 these viruses have evolved into geographically distinct groups within each country.

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