行政院國家科學委員會專題研究計畫　成果報告

禽流感病毒 H6N1 亞型基因定序及其與病原性的關係

計畫類別：整合型計畫
計畫編號：NSC93-2317-B-002-002-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：國立臺灣大學獸醫學系暨研究所

計畫主持人：王金和

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流感病毒預防及控制-禽流感病毒 H6N1 亞型基因定序及其與病原性的關係

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ABSTRACT

The current concern of influenza is that avian influenza viruses transmit or provide to humans. Although the genomic information of human influenza A viruses is increasing, little of this type of data is available for viruses circulating in poultry farms. This study presented the genetic characterization of 9 H6N1 viruses isolated from chicken farms from 2000 to 2003. The whole complete eight genes of 3 avian isolates and the six genes of the rest 6 avian isolates were sequenced and compared. The phylogenetic trees of those genes demonstrate that influenza H6N1 viruses circulating in chickens in Taiwan are quite unique and different from those of other countries. *Corresponding author TEL: 02-23680628, Fax: 02-23631542, E-mail: chingho@ntu.edu.tw

Key words: Avian influenza, H6N1, gene analysis

INTRODUCTION

There are 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of influenza A viruses. Most of the subtypes have been recognized in avian species, especial wild birds [6], but only three HAs and two NAs (H1, H2, H3, N1, and N2) have been recognized in human influenza viruses. The appearance of new subtypes of influenza A viruses can lead to pandemics in humans because humans have no antibodies against the new subtype viruses. The genes responsible for the emergence of a new virus are thought to have been introduced from avian species [7,15].

Avian influenza viruses, H6N1 and H9N2 have been enzootic in Asia during the past decade and have been isolated from different types of terrestrial poultry worldwide [2]. The H6N1 associated with H9N2 have been involved in the genesis of the pathogenic H5N1 influenza viruses of 1997, which caused the death of human beings [4].

The first H6N1 avian influenza outbreak in Taiwan was reported in 1972 [8]. Since then, the surveillance has been continuous performed [3]. Recently, more and

more H6N1 viruses were isolated from the field around Taiwan [14]. Some of them caused mortality and egg production drop in chickens. Several H6N1 avian influenza viruses were isolated from chickens in this laboratory during 2000 and 2003. The present study was to investigate the epidemiology of those viruses and whether their genes emerge to human or other viruses.

MATERIALS AND METHODS

Viruses.

The viruses used in this study were isolated from the field in Taiwan during 2000 and 2003 (Table 1). Viruses were propagated in 11-day-old SPF chicken embryos (Animal Health Research Institute, Chidin, Taiwan). The aliquots were kept in -70 C for gene analysis.

Cloning and sequencing of virus gene fragments.

The eight gene fragments of both strains were extracted with TriSolution (Genmedika Biotechnol. Corp., Taipei) and amplified with specific primers by reverse transcription–polymerase chain reaction (RT-PCR) for different fragments (Table 2-9). Different primers were used for the same gene fragment from different virus isolates. Because some primers didn't amplify some isolates, we designed new primers for those isolates.

The RT-PCR products were cloned into yT&A cloning kit (Yeastern Biotech. Co., Taipei) and sequenced using ABI prism cycle sequencing kit (Perkin Elmer, Banchburg, NJ). Forward and reverse sequences were performed.

Sequence analysis.

The nucleotide sequences of the AIV we isolated and the sequences we gathered from the GenBank were aligned using the Vector NTI Suite 6 software (InforMax, Inc., Bethesda, MD). Phylogenetic trees were generated based on the alignment of the complete or partial nucleotide sequences of each viral gene by using the Neighbor Joining method of Saitou and Nei. The regions of each gene used in analysis included: PB2, nt position 1-2277; PB1, 1-2271; PA, 1-2148; HA, 1-1701; NP, 7-1479; NA, 28-1402 ; M, 7-909 and NS, 1-836. Accession numbers of virus sequences from the GenBank used in this study were as the followings.

PB2
Qa/HK/SF595/00(H6N1) AJ410499, Ph/HK/NT261/00(H6N1) AJ410500, Ck/Taiwan/7-5/99(H6N1) AF255624, Qa/HK/1721-20/99(H6N1) AJ410496, Ph/HK/SH39/99(H6N1) AJ410495, Teal/HK/W312/97(H6N1) AF250476, Bird/Thailand/3.1/04(H5N1) AY651715, Ck/Guangdong/174/04(H5N1) AY609309, Ck/Jilin/9/04(H5N1) AY653193, Ck/Vietnam/C58/04(H5N1) AY818127, Ck/HK/31.4/02(H5N1) AY576386, Gs/Guangdong/1/96(H5N1) AF144300, Vietnam/1203/04(H5N1) AY818126, Vietnam/3062/04(H5N1) AY651721, HK/212/03(H5N1) AY576380, HK/156/97(H5N1) AF036363, HK/1774/99(H3N2) AJ293920, Taiwan/3/71(H3N2) AY210146, Taiwan/2/70(H3N2) AY210144, Taiwan/1/69(H3N2) AY210141, HK/1/68(H3N2) AF348170, HK/427/98(H1N1) AF258525, HK/470/97(H1N1) AF258524, Fiji/15899/83(H1N1) AJ564805

PB1

Qa/HK/SF595/00(H6N1) AJ410506, Ph/HK/NT261/00(H6N1) AJ410507,
Ck/Taiwan/7-5/99(H6N1) AF262210, Qa/HK/1721-20/99(H6N1) AJ410503,
Ph/HK/SH39/99(H6N1) AJ410502, Teal/HK/W312/97(H6N1) AF250477,
Bird/Thailand/3.1/04(H5N1) AY651661, Ck/Guangdong/174/04(H5N1) AY609310,
Ck/Jilin/9/04(H5N1) AY653199, Ck/Vietnam/C58/04(H5N1) AY818130,
Ck/HK/31.4/02(H5N1) AY576398, Gs/Guangdong/1/96(H5N1) AF144301,
Vienam/1203/04(H5N1) AY818129, Vietnam/3062/04(H5N1) AY651667,
HK/212/03(H5N1) AY576392, HK/156/97(H5N1) AF036362,
HK/1774/99(H3N2) AJ293921, Taiwan/3/71(H3N2) AY210279, Taiwan/2/70(H3N2)
AY210282, Taiwan/1/69(H3N2) AY210276, HK/1/68(H3N2) AF348172,
HK/427/98(H1N1) AF258526, HK/470/97(H1N1)AF258527, Fiji/15899/83(H1N1)
AJ564807

PA
Qa/HK/SF595/00(H6N1) AJ410515, Ph/HK/NT261/00(H6N1) AJ410516,
Ck/Taiwan/7-5/99(H6N1) AF262211, Qa/HK/1721-20/99(H6N1) AJ410512,
Ph/HK/SH39/99(H6N1) AJ410511, Teal/HK/W312/97(H6N1) AF250478,
Bird/Thailand/3.1/04(H5N1) AY651607, Ck/Guangdong/174/04(H5N1) AY609311,
Ck/Jilin/9/04(H5N1) AY653198, Ck/Vietnam/C58/04(H5N1) AY818133,
Ck/HK/31.4/02(H5N1) AY576410, Gs/Guangdong/1/96(H5N1) AF144302,
Vietnam/1203/04(H5N1) AY818132, Vietnam/3062/04(H5N1) AY651612,
HK/212/03(H5N1) AY576404, HK/156/97(H5N1) AJ289874,
HK/1774/99(H3N2) AJ293922, Taiwan/3/71(H3N2) AY210202, Taiwan/2/70(H3N2)
AY210199, Taiwan/1/69(H3N2) AY210198, HK/1/68(H3N2) AF348174,
HK/427/98(H1N1) AF258519, HK/470/97(H1N1)AF258518, Fiji/15899/83(H1N1)
AJ605762

HA
Dk/Hainan/6/04(H6N2) AY773907, Dk/Korea/S17/03(H6N1) AY862613,
Qa/HK/SF595/00(H6N1) AJ410523, Ph/HK/NT261/00(H6N1) AJ410524,
CK/Taiwan/7-5/99(H6N1) AF310983, Qa/HK/1721-20/99(H6N1) AJ410520,
Ph/HK/SH39/99(H6N1) AJ410519, Dk/HK/3461/99(H6N1) AJ410537,
Ck/Taiwan/na3/98(H6N1) AF310984, Teal/HK/W312/97(H6N1) AF250479,
Pintail/Alberta/179/93(H6N1) AY633332, Dk/HK/202/77(H6N1) AJ410544,

NP
Qa/HK/SF595/00(H6N1) AJ410552, Chukka/HK/NT261/00(H6N1) AJ410553,
CK/Taiwan/7-5/99(H6N1) AF261750, Qa/HK/1721-20/99(H6N1) AJ410549,
Ph/HK/SH39/99(H6N1) AJ410548, Teal/HK/W312/97(H6N1) AF250480,
Bird/Thailand/3.1/04(H5N1) AY651495, Ck/Guangdong/174/04(H5N1) AY609313,
Ck/Jilin/9/04(H5N1) AY653196, Ck/Vietnam/C58/04(H5N1) AY818139,
Ck/HK/31.4/02(H5N1) AY575911, Gs/Guangdong/1/96(H5N1) AF144303,
Vietnam/1203/04(H5N1) AY818138, Vietnam/3062/04(H5N1) AY651501,
HK/212/03(H5N1) AY575905, HK/156/97(H5N1) AF036359,
HK/1774/99(H3N2) AJ293924, Taiwan/3/71(H3N2) AY210230, Taiwan/2/70(H3N2)
AY210227, Taiwan/1/69(H3N2) AY210225, HK/1/68(H3N2) AF348180,
HK/427/98(H1N1) AF258516, HK/470/97(H1N1) AF258517, Fiji/15899/83(H1N1)
AJ628066

NA

Qa/HK/SF595/00(H6N1) AJ410561, Ph/HK/NT261/00(H6N1) AJ410562, CK/Taiwan/7-5/99(H6N1) AF208598, Qa/HK/1721-20/99(H6N1) AJ410558, Ph/HK/SH39/99(H6N1) AJ410557, Teal/HK/W312/97(H6N1) AF250481, Bird/Thailand/3.1/04(H5N1) AY651441, Ck/Guangdong/174/04(H5N1) AY609314, Ck/Jilin/9/04(H5N1) AY653195, Ck/Vietnam/C58/04(H5N1) AY818142, Ck/HK/31.4/02(H5N1) AY575887, Gs/Guangdong/1/96(H5N1) AF144304, Vietnam/1203/04(H5N1) AY818141, Vietnam/3062/04(H5N1) AY651448, HK/212/03(H5N1) AY575881, HK/156/97(H5N1) AF036357, HK/1774/99(H3N2) AJ293923, Taiwan/3/71(H3N2) AY210131, Taiwan/2/70(H3N2) AY210126, Taiwan/1/69(H3N2) AY210122, HK/1/68(H3N2) AF348184 Fiji/15899/83A(H1N1) J006954, Dk/Hainan/4/04(H6N2) NA AY706954

MP
Qa/HK/SF595/00(H6N1) AJ410572, Ph/HK/NT261/00(H6N1) AJ410573, CK/Taiwan/7-5/99(H6N1) AF262213, Qa/HK/1721-20/99(H6N1) AJ410569, Ph/HK/SH39/99(H6N1) AJ410568, Teal/HK/W312/97(H6N1) AF250482, Bird/Thailand/3.1/04(H5N1) AY651384, Ck/Guangdong/174/04(H5N1) AY609315, Ck/Jilin/9/04(H5N1) AY653194, Ck/Vietnam/C58/04(H5N1) AY818145, Ck/HK/31.4/02(H5N1) AY575899, Gs/Guangdong/1/96(H5N1) AF144306, Vietnam/1203/04(H5N1) AY818144, Vietnam/3062/04(H5N1) AY651390, HK/212/03(H5N1) AY575893, HK/156/97(H5N1)AF036358, HK/1774/99(H3N2) AJ293925, Taiwan/3/71(H3N2) AY210263, Taiwan/2/70(H3N2) AY210260, Taiwan/1/69(H3N2) AY210256, HK/1/68(H3N2) AF348188, HK/427/98(H1N1) AF258523, HK/470/97(H1N1) AF258522, Fiji/15899/83(H1N1) AJ298947

NS
Dk/Korea/S17/03(H6N1) AY862677, Qa/HK/SF595/00(H6N1) AJ410581, Ph/HK/NT261/00(H6N1) AJ410582, Ck/Taiwan/7-5/99(H6N1) AF262212, Qa/HK/1721-20/99(H6N1) AJ410578, Ph/HK/SH39/99(H6N1) AJ410577, Teal/HK/W312/97(H6N1) AF 250483, Dk/HK/3461/99(H6N1) AJ410594, Bird/Thailand/3.1/04(H5N1) AY651550, Ck/Guangdong/174/04(H5N1) AY609316, Ck/Jilin/9/04(H5N1) AY653197, Ck/Vietnam/C58/04(H5N1) AY818148, Ck/HK/31.4/02(H5N1) AY576374, Gs/Guangdong/1/96(H5N1) AF144307, Vietnam/1203/04(H5N1) AY818147, Vietnam/3062/04(H5N1) AY651555, HK/212/03(H5N1) AY576368,HK/156/97(H5N1) AF036360, HK/1774/99(H3N2) AJ293941,Taiwan/3/71(H3N2) AY210307, Taiwan/2/70(H3N2) AY210306, Taiwan/1/69(H3N2) AY210302, HK/1/68(H3N2) AF348198, HK/427/98(H1N1) AF258521, HK/470/97(H1N1) AF258520, Fiji/15899/83(H1N1) AJ298950

RESULTS

Clinical cases and viruses.

Nine AIVs were isolated form 9 flocks, including broiler breeder farms, layer breeder farms, and a broiler farm (Table 1). The onsets lasted for 5 to 7 days and then recovered. The weekly mortality ranged from 0.3% to 6.0%. The egg production decreased 0% to 33%.

Necropsy findings.

Hemorrhage was found on the laryngeal, pharyngeal and tracheal mucosa. Air sac showed cloudy and thickening. Papillary hemorrhage was noted in the proventriculus. Kidney was swollen and had urate deposition. *Escherichia coli* was always isolated from the infected chickens.

Genetic sequences.

The PB2 full-length gene segments of 2838N/00, 2838V/00, and 2937/01 were determined. RNA segment 1 contained 2341 nucleotides and coded for a 759-a.a. PB2 protein from MERIK to RMAIN. The nucleotide percent identity among Taiwan H6N1 strains were from 95.4% to 99.6%.

The PB1 full-length gene segments of 2838N/00, 2838V/00, 2896/01, 2937/01, 3072/03, 3115/03, 3127/03 and 3153/03 were determined. This RNA segment 2 contained 2341 nucleotides and coded for a 757-a.a. PB1 protein from MDVNP to LRRQK. The nucleotide percent identity among Taiwan H6N1 strains were from 93.5 to 99.8%.

The PA full-length gene segment of 2838N/00, 2838V/00, and 2937/01 were determined. This RNA segment 3 contained 2233 nucleotides and coded for a 716-a.a. PA protein from MEDFV to THALK. The nucleotide percent identity among Taiwan H6N1 strains were from 98 to 99.6%.

The HA full-length gene segments of 2829/00, 2838N/00, 2838V/00, 2896/01, 2937/01, 3072/03, 3115/03, 3127/03 and 3153/03 were determined. This RNA segment 4 contained 1776 nucleotides. The main HA protein was 551 in length with 329 HA1 and 222 HA2. The mature HA1 was from DKICI to QIETR. The nucleotide percent identity among Taiwan H6N1 strains were from 93.7 to 99.6%. The connecting sequences consisted of a single R, IETR↓GIFG in the present isolates (arrow means cleavage site) except 2838N/00, which was IETR↓GILR. The sequence after the cleavage site in most influenza viruses is GLF. Surprisingly, the related sequence in the present H6N1 HA sequence was GIFG, the same as A/chicken/7-5/99 (accession number: AF310983 in the GenBank). In the critical HA receptor-binding site, the 228-related position (226 in H6) of the HA from the present isolates was S, like the human-adapted H3 or H2 strains (225-203 of H3 numbering, G-Q-R-S-RI).

The NP full-length gene segments of 2829/00, 2838N/00, 2838V/00, 2896/01, 2937/01, 3072/03, 3115/03, 3127/03 and 3153/03 were determined. This RNA segment 5 contained 1487 nucleotides and was coded for protein of 495 a.a., begin from SQGTK to AEEYD. The nucleotide percent identity among Taiwan H6N1 strains were from 95.8 to 99.3 %. The anchor residue of the cytotoxic T lymphocyte epitope SRYWAIRTR (383 to 391) was found, which could be recognized by specific cytotoxic T lymphocytes [13].

The NA full-length gene segments of 2829/00, 2838N/00, 2838V/00, 2896/01, 2937/01, 3072/03, 3115/03, 3127/03 and 3153/03 were determined. This RNA segment 6 contained 1375 nucleotides and was coded for protein of 456 a.a., begin from MDVNP to LGRQK. The nucleotide percent identity among Taiwan H6N1 strains were from 93.7 to 99.7 %. The glycosylation of N at 144 a.a. conserved from the functional requirement was also found[11]. Some strains showed 12 a.a. deletion in the NA stalk and some not.

The M full-length gene segments of 2829/00, 2838N/00, 2838V/00, 2896/01, 2937/01, 3072/03, 3115/03, 3127/03 and 3153/03 were determined. This RNA segment 7 contained 903 nucleotides. The encoded polypeptide divided into M1 (250 a.a.), begin from MDVNP to LGRQK and M2 (97 a.a.). The nucleotide percent identity among Taiwan H6N1 strains were from 95.6 to 99.8 %. RNA segment 7

coded for 252 a.a. of M1 and 97 a.a. of M2 proteins, respectively. The a.a. positions 27 to 30 of M2 protein associated with resistance was VIAS. The a.a. change from I to T or S at position 27 and from A to T or S at position 30 of M2 are associated with amantadine resistance [1]. The position 30 were S, indicating those strains might resistant to amantadine.

The NS full-length gene segments of 2829/00, 2838N/00, 2838V/00, 2896/01, 2937/01, 3072/03, 3115/03, 3127/03 and 3153/03 were determined. This RNA segment 8 contained 890 nucleotides and was coded for protein of 230 a.a. (NS), begin from MDSNT to IESEV. The nucleotide percent identity among Taiwan H6N1 strains were from 94 to 99.9 %. RNA segment 8 coded for 230 a.a. of NS1 and 121 a.a. of NS2 proteins, respectively. The position 92 of NS1 protein was D, which was sensitive to cytokine and less virulent than highly pathogenic H5N1 [12].

Phylogenetic data.

All of the H6N1 influenza viruses isolated from Taiwan were clustered together and different from viruses of other countries (Fig. 1,2,3,4,5,6,7,8). They could be subgrouped into two different clades from all the eight gene fragments (Fig. 1,2,3,4,5,6,7,8), with 3115/03 and 2838V/00 in a clade and the others in another clade.

DISCUSSION

Consistent with accepted dogma, genes from influenza viruses isolated from birds are in evolutionary stasis and the majority of the genes clustered together are separated from viruses from other areas. Interestingly, Taiwan AIVs are quite unique because they are clustered together and separate from AIVs of other areas. All of the isolates are clustered in spite of their origin. From the phylogenetic trees of PB1, HA, NP, NA, M, and NS, 2838N/00 and 2838V/00 are clustered into the same clade as 3115/03 in spite of their origin was quite different. The percentage identity between 2838N/00 and 3115/03 were PB1: 99.7%, HA:99.1%, NP:99.3%, NA:97.2%, M:99.7%, and NS:99.9%. Isolate 2838/00 was from a broiler breeder in Yilan and 3115/03 was from a broiler farm in Taoyen. The broilers were not from that breeder farm. The similar sequences indicated that AIVs in Taiwan are circulated over the all island but not restricted to a specific area.

Another unique finding in this study is the presence of AIVs spread by wind from clinical epidemiologic investigation. Those viruses infected in a flock and then transmitted to adjacent flocks in spite of restricted biosafety management even the movement of workers and equipments between different premises were restricted.

The host range of influenza A viruses is associated with differences in the specificity of the HA to attach sialic acid-containing receptors on susceptible cells [10]. HAs of avian influenza viruses preferentially recognize sialic acid linked to galactose by α 2,3 linkage, whereas HAs of human influenza viruses preferentially recognize sialic acid α 2,6Gal linkages [9]. Residues 226 and 228 in the HAs of the H2 and H3 subtypes of human influenza viruses may be important in determining receptor specificity [5]. In all H6N1, the HA posses a Q residue at the position 226 and an S residue at position 228. This combination of amino acids binds preferentially to sialic acid α 2,3 if there is an additional substitution of Q226L in; it may enable this virus to recognize the mammalian receptor and significantly increase the potential of the virus to infect mammals [5]. The potential of human transmission of the present H6N1 viruses should be further studies.

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Table 1. Histories of the flocks having H6N1 viruses by isolation.

Isolate	Location	Host	Week age	Egg[b] production drop	Weekly mortality	NA stalk deletion[C]
2829/00	Yilan	Broiler breeder	46	0%	0.3%	No
2838/00[a]	Yilan	Broiler breeder	45	33%	3.8%	Yes
2896/01	Miaoli	Broiler breeder	45	10%	2.5%	No
2937/01	Tainan	Layer	4	NR	0.5%	Yes
3072/03	Taichung	Layer breeder	43	10%	2.8%	Yes
3115/03	Taoyen	Broiler	5	NR	6%	Yes
3127/03	Taichung	Broiler breeder	14	NR	0.5%	Yes
3153/03	Yilan	Broiler	5	NR	3%	Yes

a. From which, 2838N/00 (nonvirulent) and 2838V/00 (virulent) were obtained.
b. Usually, the egg production drop lasted for 5 days and recovered. ND: no record because those chickens are immature.
c. NA stalk deletion site: 120-159 (except 137-139)

Table 2. Primers used for PB2 gene cloning and sequencing

Gene	Strains	Primer[a]	Sequence	Position
PB2	2838N/00	F: Ba-PB2-1	Ref. Hoffmann, 2000	-41 － -14
		R: Ba-PB2-2341r	Ref. Hoffmann, 2000	2329－2296
		Internal primer:		
		F: D PB2 F/F1	CAGACAGAGTGATGGTGT	257 － 274
		R: D PB2 F/R2	GCACTGAGATCTGCATGG	467 － 450
		F: D PB2 F/F2	GGTAAGGATGGTGGACAT	873 － 890
		R: D PB2 F/R1	TCCTCAGGATAGCTGTTG	1126－1109
		F: PB2-1286f	TCGTAAACAGAGCAAACCAAAG	1259－1280
		R: PB2-1458r	TCCCATCTTACTAACTCTCACC	1452－1431

a. F: forward primer, R: reverse primer
b. The same primers were used for strain 2838V/00 and 2937/01.

Table 3. Primers used for PB1 gene cloning and sequencing

Gene	Strains	Primer	Sequence	Position
PB1	2838N/00	F: Ba-PB1-1	Ref. Hoffmann, 2000	-38 － -11
		R:Ba-PB1-2341r	Ref. Hoffmann, 2000	2332－2300
		Internal primer:		
		F: PB1-639f	AGGGAAGAAGAAGCAGAGG	615－634
		R: K PB1 F/R1	TTCTCACAGATGCTCCTC	794－777
		F: PB1-990f	AACATACATCACAAGAAGCCAG	966－987
		R:PB1-1259r	ATCATCATTCCAGGACTCAA	1226－1207
		F: PB1r-630f	ACAATATGATAAACAACGACCTTGG	1595－1619
		R: PB1r-777r	CCCACAGCCTCTTCAGCTCG	1741－1722

a. F: forward primer, R: reverse primer
b. The same primers were used for strain 2838V/00, 2896/01, 2937/01, 3072/03, 3115/03, 3127/03,and 3153/03.

Table 4. Primers used for PA gene cloning and sequencing

Gene	Strains	Primer	Sequence	Position
PA	2838N/00	F: Bm-PA-1	Ref. Hoffmann, 2000	-38 － -10
		R: Bm-PA-2233r	Ref. Hoffmann, 2000	2224－2192
		Internal primer:		
		F: PA-525f	CAGGGCAAGAATCAAAACCAG	501－521
		R: K PA F/R1	GCATCATACAGTGGTATAC	920－902
		F: PA-1009f	GGCATAAACCCCAATTACCTC	985－1005
		R: PA-1155r	CCATATTCTCACCAAGTGCCC	1123－1103

a. F: forward primer, R: reverse primer
b. The same primers were used for strain 2838V/00 and 2937/01.

Table 5. Primers used for HA gene cloning and sequencing

Gene	Strains	Primer	Sequence	Position
HA	2838N/00	F: P1(+)[b]	5'-GGAAAATGATTGCAATCATT	-5 — 15
		R: P4(-)[b]	5'-TTATATACATATCCTGCATTGCAT	1704—1681
		Internal primer:		
		F: P2(+)	CCTTGGTATGCATACAAATT	802 — 821
		R: P3(-)	GGTAAATTTGACTTGAAGAC	866 — 847
	2829/00	F: Bm-HA-1	Ref. Hoffmann, 2000	-31 — -4
		R: Bm-NS-890r	Ref. Hoffmann, 2000	1745—1711
		Internal primer:		
		F: HA-400f	AAATGGGATTTGCTACCCAGG	306 — 326
		R: HA-600r	GATTGGCTGGTTTCCAGTGTTG	570 — 549
		F: HA-1206f	CAAAATGAACACACAATTCGAAGC	1206—1229
		R: HA-1398r	CCCTTAGCTGCGATTTGACC	1417—1398
	3115/03	F:Bm-HA-1	Ref. Hoffmann, 2000	-31 — -4
		R: P4(-)	Dr. Chang	1704—1681
		Internal primer:		
		F: P2(+)	CCTTGGTATGCATACAAATT	802 — 821
		R: P3(-)	GGTAAATTTGACTTGAAGAC	866 — 847

a. F: forward primer, R: reverse primer
b. From Dr. Chang PC of National ChungHsing Unviersity
c. The same primers used for 2838N/00 were used for strain 2838V/00.
d. The same primers used for 2829/00 were used for 2896/01, 2937/01, 3072/03, 3115/03, 3127/03, and 3153/03.

Table 6. Primers used for NP gene cloning and sequencing

Gene	Strains	Primer	Sequence	Position
NP	2838N/00	F: Bm-NP-1	Ref. Hoffmann, 2000	-59 — -31
		R: Bm-NP-1565r	Ref. Hoffmann, 2000	1593—1559
		Internal primer:		
		F: K- NP/F1	5'-TGATGCCACATACCAGAG	432—449
		R: Bm-NP-756r	5'-TGTTTGGAATTTCCCTTTGAGG	755—734
		R: K-NP/R1	5'-CTCTTGGKACCACTCTTG	1125—1108
		F: NP2	5'-YAGATATTGGGCTATAAGAAC	1208—1228
	2829/00	F: NPf	5'-TCTCAAGGCACCAAACG	7 — 23
		R: NPr	5'-TTGTCATACTCCTCTGC	1493—1477
		Internal primer:		
		F: K- NP/F1	5'-TGATGCCACATACCAGAG	432—449
		R: Bm-NP-756r	5'-TGTTTGGAATTTCCCTTTGAGG	755—734
		R: K-NP/R1	5'-CTCTTGGKACCACTCTTG	1125—1108
		F: NP2	5'-YAGATATTGGGCTATAAGAAC	1208—1228

a. F: forward primer, R: reverse primer
b. The same primers used for 2838N/00 were used for strain 2838V/00 and 2896/01.
c. The same primers used for 2829/00 were used for 2937/01, 3072/03, 3115/03, 3127/03, 3152, and 3153/03.
d. K=G/T, Y=C/T

Table 7. Primers used for NA gene cloning and sequencing

Gene	Strains	Primer	Sequence	Position
NA	2838N/00	F: Ba-NA-1	Ref. Hoffmann, 2000	-34 — -6
		R: Ba-NA-1413R	Ref. Hoffmann, 2000	1411—1376
		Internal primer:		
		F: Naf	5'-ACGGACCAAGTAATGGACAG	689 — 708
		R: Nar	5'-TTCAATACAGCAACAGCCCC	578 — 559
	2829/00	F: NC1	5'-ATTGGRTCAATCTGTATGG	28 — 46
		R: NC2	5'-CAAYGGTGAATGGCAACT	1360—1343
		Internal primer:		
		F: Naf	5'-ACGGACCAAGTAATGGACAG	689 — 708
		R: Nar	5'-TTCAATACAGCAACAGCCCC	578 — 559

a. F: forward primer, R: reverse primer
b. The same primers that used for 2838N/00 were used for strain 2838V/00 and 3153/03.
c. The same primers that used for 2829/00 were used for 2896/01, 2937/01, 3072/03, 3115/03, 3127/03, and 3152.
d. Y=C/T, R=A/G

Table 8. Primers used for M gene cloning and sequencing

Gene	Strains	Primer	Sequence	Position
M	2838N/00	F: M52F	5'-CTTCTAACCGAGGTCGAAACG	7 — 27
		R: Bm-M-1027r	Ref. Hoffmann, 2000	1017—982
		Internal primer:		

		R: M423R	5’-CCTATGCTGTGAATCAGC	480－463
		F: M440F	5’-TGACTATCACCAATCCAC	497－514
	2829/00	F: M52F	5’-CTTCTAACCGAGGTCGAAACG	7 － 27
		R: M912R	5’-TCCCTCATAGACTCAGGCACTCC	909－887
		Internal primer:		
		F: M440F	5’-TGACTATCACCAATCCAC	497－514
		R: M423R	5’-CCTATGCTGTGAATCAGC	480－463

a. F: forward primer, R: reverse primer
b. The same primers used for 2838N/00 were used for strain 2838V/00.
c. The same primers used for 2829/00 were used for 2896/01, 2937/01, 3072/03, 3115/03, 3127/03, 3152, and 3153/03.

Table 9. Primers used for NS gene cloning and sequencing

Gene	Strains	Primer	Sequence	Position
NS	2829/00	Bm-NS-1	Ref. Hoffmann, 2000	-40 － -12
		Bm-NS-890r	Ref. Hoffmann, 2000	879 － 845
		K-NS/F1	5’-GGGACTGGTTAATGCTCA	299 － 316

a. F: forward primer, R: reverse primer
b. The same primers used for 2829/00 were used for strain 2838N/00, 2838V/00, 2896/01, 2937/01, 3072/03, 3115/03, 3127/03, 3152, and 3153/03.

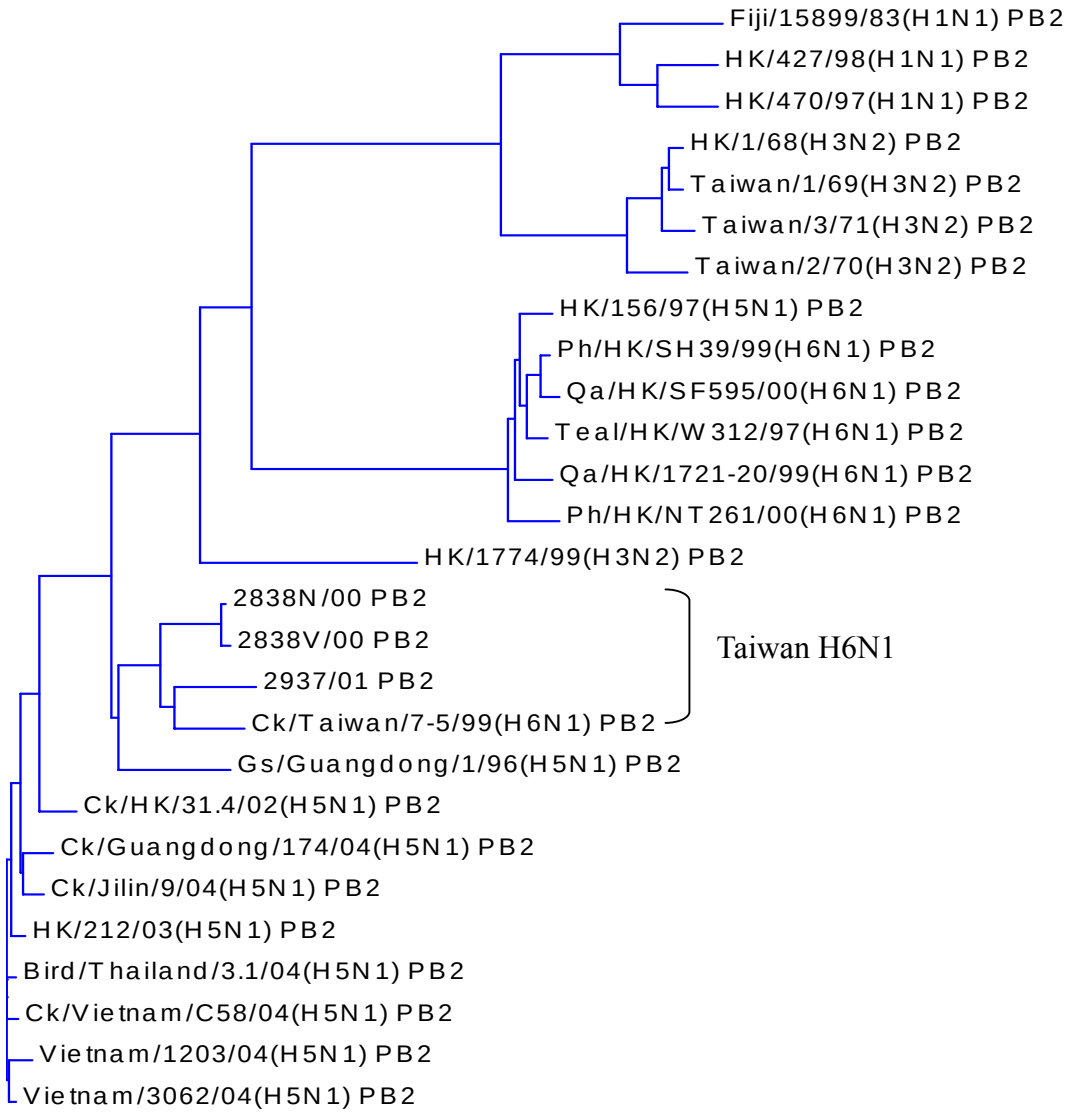

Fig. 1. Phylogenetic tree constructed from PB2 gene nucleotide sequences (1–2277). Note Taiwan H6N1 is in different cluster from other viruses including human H3N2, H1N1, and 2004 Asian H5N1.

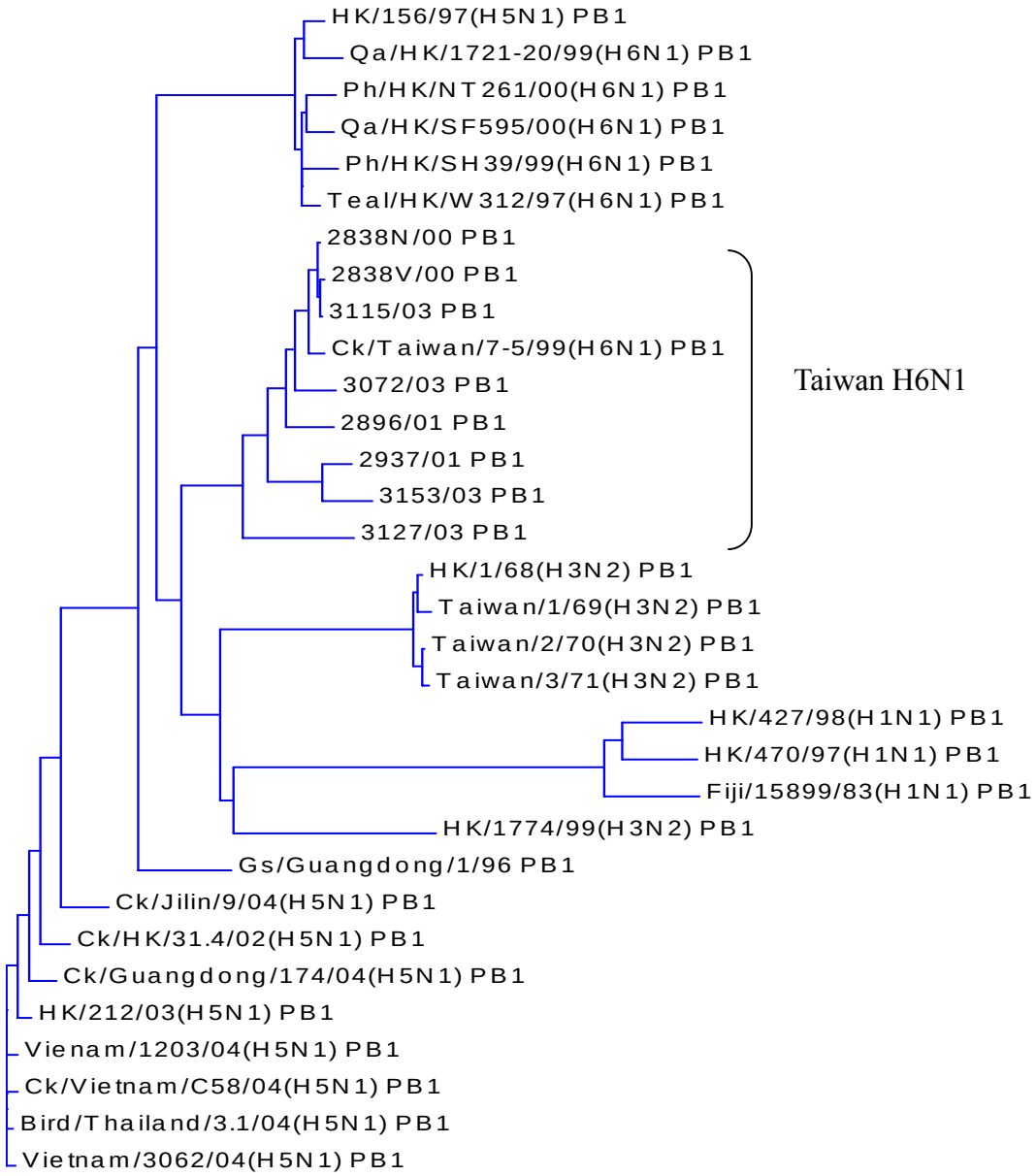

Fig. 2. Phylogenetic tree constructed from PB1 gene nucleotide sequences (1–2271). Except Fiji/15899/83(H1N1) (1-2089), Ck/HK/31.4/02(H5N1) (1-2163), HK/1774/99(H3N2) (1-2268), Bird/Thailand/3.1/2004(H5N1) 1-2269), and Vietnam/3062/04(H5N1) (1-2269).
Note Taiwan H6N1 is in different cluster from other viruses including human H3N2, H1N1, and 2004 Asian H5N1.

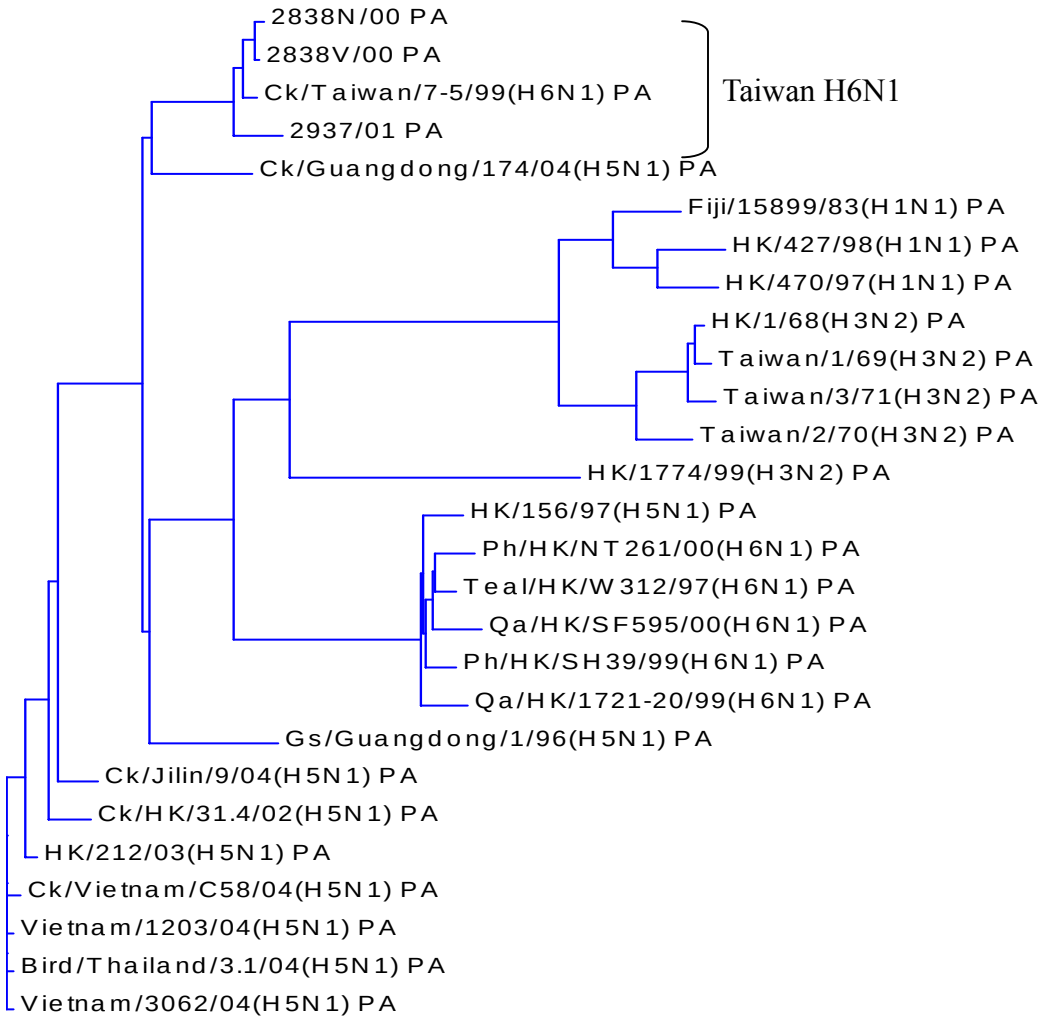

Fig. 3. Phylogenetic tree constructed from PA gene nucleotide sequences (1-2148) except Bird/Thailand/3.1/2004 (H5N1) (10-2148), HK/212/03 (H5N1) (13-2148), Vietnam/3062/04 (H5N1) (16-2148).

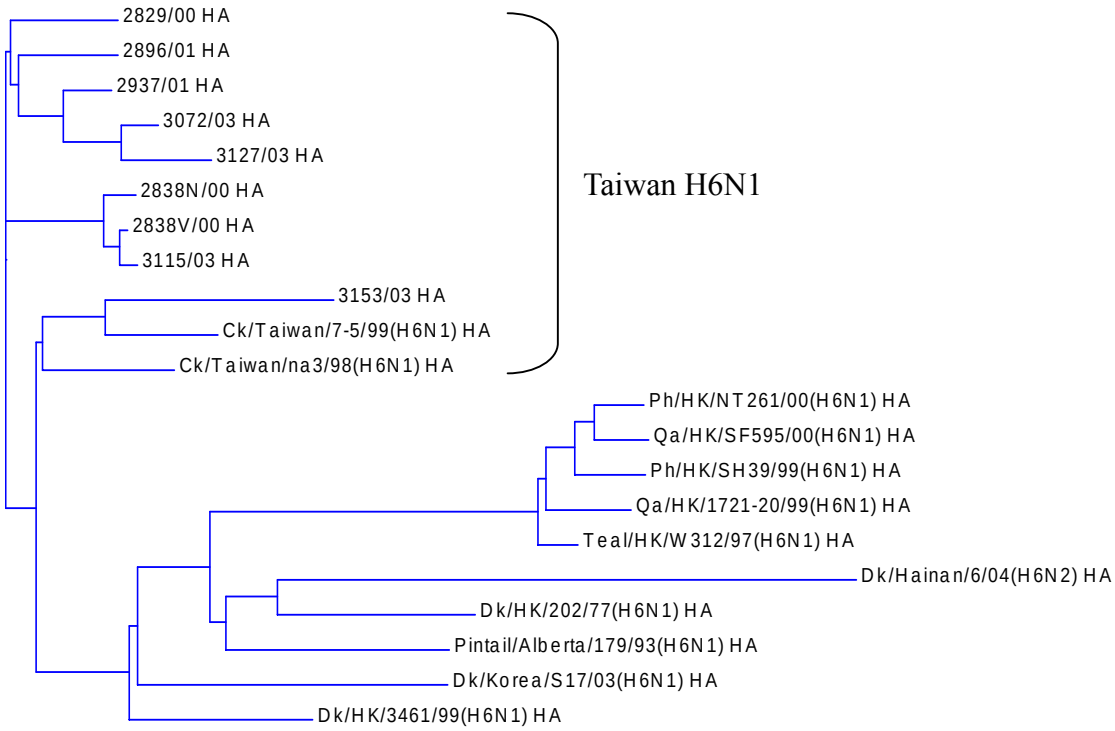

Fig. 4. Phylogenetic tree constructed from HA gene (H6) nucleotide sequences (1-1698) except Ck/Taiwan/7-5/99 (H6N1) (1-1110), Ck/Taiwan/na3/98 (H6N1) (1-1110)

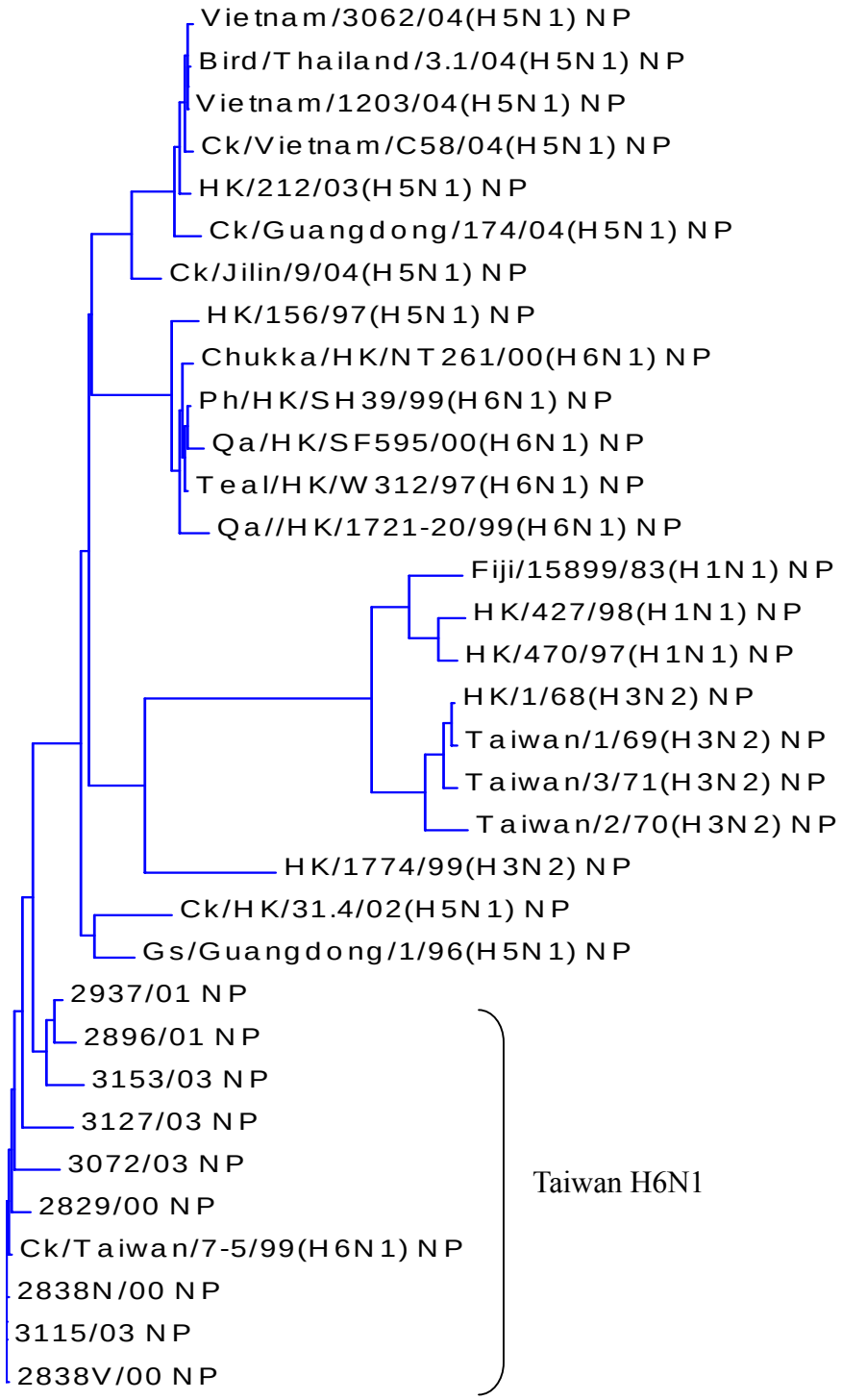

Fig. 5. Phylogenetic tree constructed from NP gene nucleotide sequences (7-1493) except Vietnam/3062/2004 (H5N1) (7-1479), Bird/Thailand/3.1/2004 (H5N1) (7-1484)

Note Taiwan H6N1 is in different cluster from other viruses including human H3N2, H1N1, and 2004 Asian H5N1.

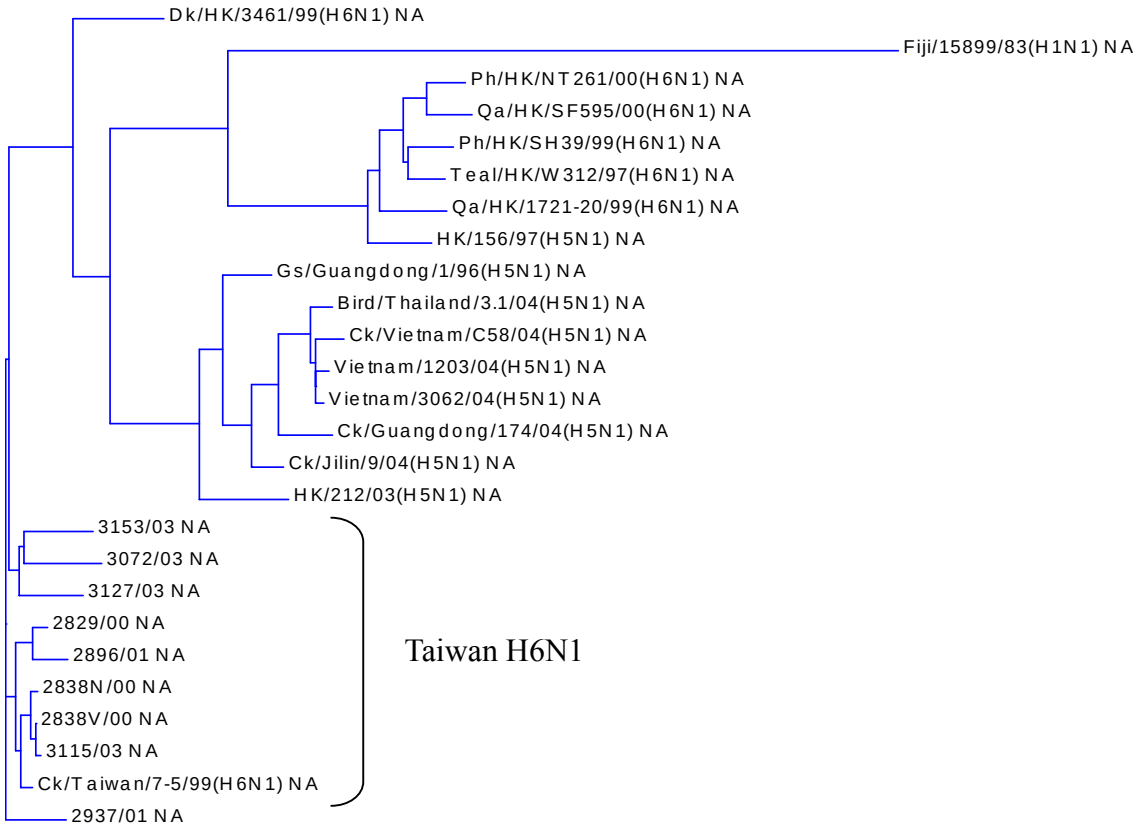

Fig. 6. Phylogenetic tree constructed from NA gene (N1) nucleotide sequences (28-1333).

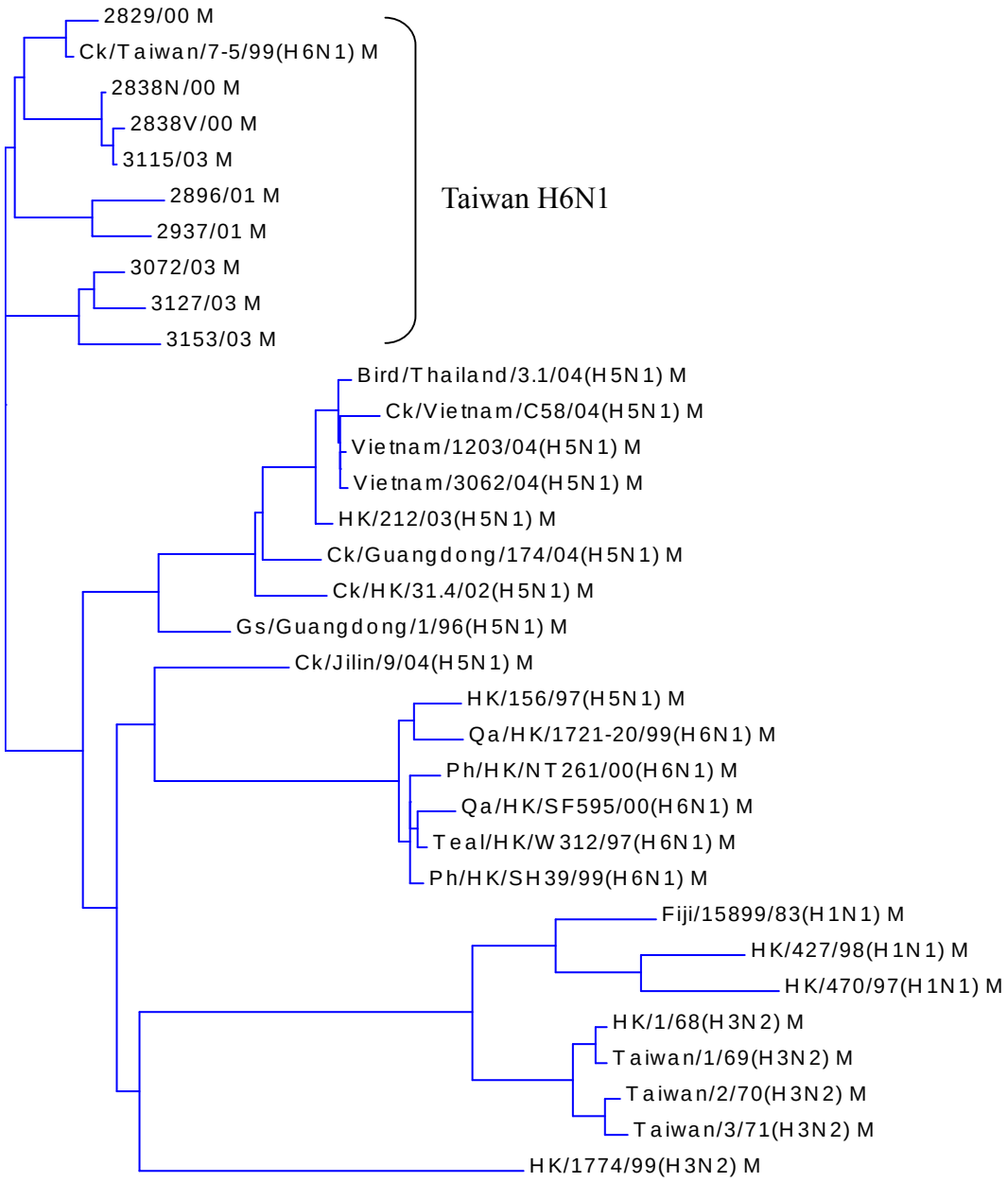

Fig. 7. Phylogenetic tree constructed from M gene nucleotide sequences (7-909). Note Taiwan H6N1 is in different cluster from other viruses including human H3N2, H1N1, and 2004 Asian H5N1.

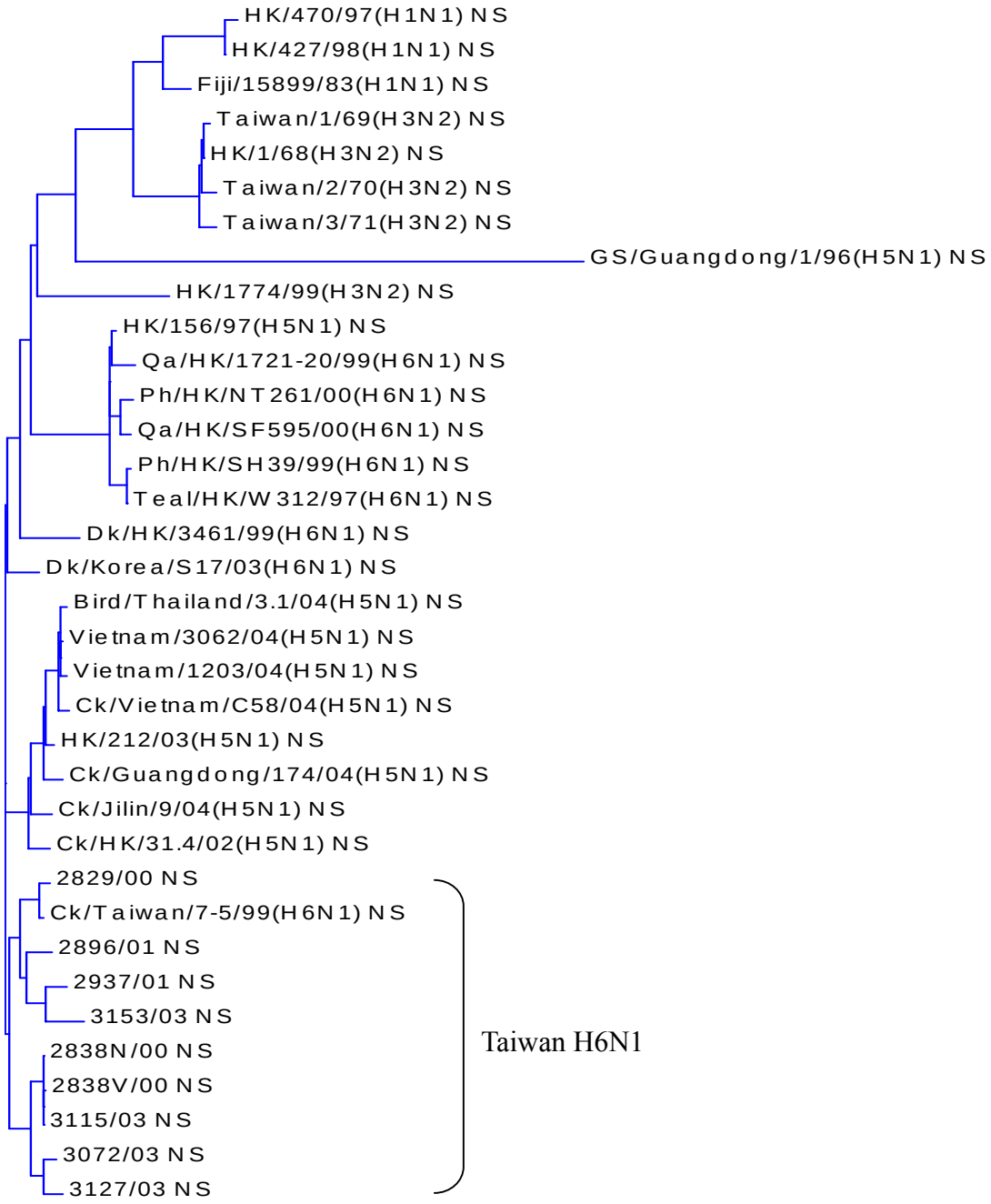

Fig. 8. Phylogenetic tree constructed from NS gene nucleotide sequences (1-829 to 864)

流感病毒預防及控制-禽流感病毒 H6N1 亞型基因定序及其與病原性的關係

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摘要　禽流感傳染人及提供基因給人類流感病毒為大家所關心的問題，雖然人流感基因資料逐漸累積，但有關流行於雞場的禽流感病毒基因則少有報告發表。本研究提供 2000 年至 2003 年九株由雞場分離的 H6N1 病毒於家禽畜舍流行情形，其中三株的完整序列及另外 6 株的六段基因，除進行定序外並將序列做分析比較，結果顯示這些禽流感病毒自成一個族群並未提供基因給人流感。

鍵詞：禽流感、H6N1、基因分析