

# Genetic diversity of swine influenza viruses isolated from pigs during 2000 to 2005 in Thailand

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**Background** Recent studies have revealed the existence of genetic diversity in swine influenza viruses (SIVs) in the world. In Thailand, there has been a little information on the molecular characteristics of the SIVs since the first isolation of viruses of H1N1 and H3N2 subtypes in the late 1970s. Our previous study demonstrated that Thai H1N1 SIVs possessed the classical swine H1 and avian-like swine N1 genes (Takemae *et al.*, Proceedings of the Options for the Control of Influenza VI.2007;350–353).

**Objectives** In the present study, we genetically characterized 12 SIVs including those of H1N1, H1N2 and H3N2 subtypes isolated between 2000 and 2005.

**Methods** We determined the entire nucleotide sequences of the eight gene segments of those isolates.

**Results** Phylogenetic analysis revealed the existence of nine distinct genotypes amongst the Thai SIVs. These genotypes arose

from multiple introductions of classical swine, avian-like swine and human viruses. The existence of two distinct sublineages within classical swine H1 and NS, avian-like swine PA and M and human H3 and N2 genes of the Thai SIVs suggested that introduction of viruses of classical swine, avian-like swine and human origins occurred twice respectively into the Thai pig population. The predominance of avian-like swine genes amongst the Thai SIVs was evident. In particular, three polymerase (PB1, PB2 and PA) and matrix genes of avian-like swine origin were retained in all the Thai SIVs examined.

**Conclusions** These observations may suggest that genes of avian-like swine lineages have some advantages to be maintained in pigs as seen in the SIVs established through multiple introductions in other regions.

**Keywords** Genetic diversity, influenza virus, pig, Thailand.

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## Introduction

Swine influenza was first described in the United States in 1918 when the Spanish Flu pandemic occurred in the human population.<sup>1,2</sup> Typical clinical signs in pigs are respiratory diseases with fever, coughing and nasal discharge, as in humans. Anorexia and diarrhoea that are directly associated with reducing body weight are also occasionally involved.<sup>3</sup> These signs may result in serious economic losses when pigs are raised and fattened on farms. Thus, although swine influenza results in low mortality, it has been recognized as an important pathogen in the swine industry.<sup>2</sup>

Recent genetic and antigenetical analyses have revealed complicated features of the evolutionary diversity of swine influenza viruses (SIVs). In Europe, H3N2 viruses possessing genes encoding internal proteins related to avian-like swine H1N1 and surface antigens related to human H3N2

viruses were isolated from Italian pigs in the 1980s and continued to circulate in Italian pigs since then.<sup>4,5</sup> In 1998, at least two different genotypes of SIV of the H3N2 subtype emerged amongst the pig population in the USA by the introduction of human, classical swine and avian viruses<sup>6</sup> and one of them has established new lineage in North America.<sup>7</sup> In addition to those, SIVs of the H4N6 subtype were isolated from pigs in Canada,<sup>8</sup> and SIVs of the H9N2 subtype have been reported to circulate in China.<sup>9,10</sup> The segmented nature of the influenza genome and receptor molecules in the swine respiratory tract contributes to such evolutionary diversity of SIVs.<sup>1</sup> This segmented nature enables influenza viruses to exchange their genes when viruses of two or more different origins infect a host at the same time. The existence of 2-3 and 2-6 linked sialic acid in the swine respiratory tract allows type A influenza viruses of human and avian origin to infect pigs.<sup>11</sup>

The pig industry in Thailand has developed as one of the major livestock industries since 1970s. Production of pig meat in Thailand had tripled in the 37 years from 1971 to 2006.<sup>12</sup> The number of pig farms including breeding and fattening farm also has increased about four times in the last decade.<sup>13</sup> About 34 000 pig farms were scattered throughout the country in 2006, in which over 70% was dominated by small farms having 10–50 pigs; large producers having more than 500 pigs were only 6%.<sup>13</sup>

Although SIVs of H1N1 and H3N2 subtypes were first isolated in the 1970s in Thailand,<sup>14,15</sup> little information on SIVs had been obtained in Thailand until recently. Continuous surveillance by the National Institute of Animal Health in Thailand since 2000 demonstrated the co-circulation of three subtypes, H1N1, H1N2 and H3N2, amongst the swine population in Thailand,<sup>16</sup> raising the possibility that evolutionary diversity of SIVs may exist in Thailand as seen in other region of the world. Our previous phylogenetic study demonstrated that H1N1 Thai isolates possessed an evolutionarily distinct set of surface antigens, that was the H1 gene derived from the classical swine lineage and the N1 gene from the avian-like swine lineage,<sup>17</sup> this finding has been also supported by Chutinimitku *et al.*<sup>18</sup> In order to explore the extent of the genetic diversity, we determined the whole sequences of all eight gene segments of the Thai strains isolated between 2000 and 2005, consisting of one H1N1 strain and five strains of the H3N2 subtype as well as all gene segments encoding internal proteins of five H1N1 and one H1N2 strains whose surface antigen genes were sequenced in our previous study.<sup>17</sup> Phylogenetic and regression analyses were applied to those sequences to identify the genetic origin and the genetic relationship between the Thai isolates and other SIVs circulating in the world and to elucidate the evolutionary pathways of the Thai isolates.

## Materials

### Viruses

Nasal swabs of pigs have been periodically collected from the farms mainly in the central regions of Thailand by the National Institute of Animal Health, Thailand since 2000. All the 12 viruses available at the National Institute of Animal Health, Thailand, which were isolated from six provinces of Thailand between 2000 and 2005 were subjected to the study. They included six H1N1 isolates designated as A/Sw/Ratchaburi/NIAH1481/00(Rat1481), A/Sw/Ratchaburi/NIAH550/03(Rat550), A/Sw/Chonburi/NIAH977/04(Chon977), A/Sw/Chonburi/NIAH9469/04(Chon9469), A/Sw/Chachonegsao/NIAH587/05(Chacho587) and A/Sw/Chonburi/NIAH589/05(Chon589), one H1N2 isolate designated as A/Sw/Saraburi/13021/05(Sara13021) and five H3N2 isolates designated as A/Sw/Chachoengsao/

03(Chacho03), A/Sw/Udon Thani/NIAH464/04(UT464), A/Sw/Ratchaburi/NIAH59/04(Rat59), A/Sw/Ratchaburi/NIAH874/05(Rat874) and A/Sw/Nakhon Pathom/NIAH586-1/05(NP586-1). Subtypes of these viruses were identified by a standard polymerase chain reaction (PCR) method with subtype-specific primers designed in this study. Rat1481, Chon977, Chon9469, UT464 and Rat59 were isolated from pigs showing respiratory diseases with coughing and/or sneezing. Low appetite and/or diarrhoea were recorded in the pigs from which Rat1481 and Chon9469 were isolated. Viruses were propagated in allantoic cavities of embryonated eggs.

### DNA sequencing and analyses

The entire nucleotide sequences of the coding region were determined for all eight genes of each virus. Viral RNA was extracted from infectious allantoic fluid by using MagNA Pure LC (Roche Diagnostics Corp., Basel, Switzerland) as specified by the manufacturer. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out under standard conditions with specific primers designed for this study. PCR products were purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified products were subjected to sequence reactions with gene-specific primers using Big Dye terminator version 3-1 (Applied Biosystems, Foster City, CA, USA). Reactions were analysed by the ABI model 3130 DNA sequencer (Applied Biosystems, USA).

The sequences obtained in this study along with other sequences from humans, pigs, birds and equines available from GenBank were aligned, and the gaps were removed using BioEdit software version 7.0.5.3.<sup>19</sup> Phylogenetic trees were constructed by the neighbour-joining method with 1000-times bootstrapping replicates.<sup>20</sup> A total of 157 H1 genes including the Thai SIVs examined in this study, 281 H3, 186 N1, 294 N2, 128 PB2, 148 PB1, 145 M, 136 PA, 423 NS and 167 NP were analysed. The genes from Genbank were collected from a variety of viruses isolated in different years and from different subtypes. The lengths of the nucleotide bases used were 1695bp for H1, 1611bp for H3, 1197bp for N1, 1242bp for N2, 2235bp for PB2, 2154bp for PB1, 945bp for M, 2079bp for PA, 770bp for NS and 1459bp for NP respectively. Regression analysis by plotting the number of nucleotide substitutions against the year of isolation were applied to the clusters Thai SIVs belonging in all phylogenetic trees obtained with all eight segments to estimate the year (with a 95% confidence limit) when the strains within a particular cluster derived from a common ancestor. The coefficient of determination ( $R^2$ ) was used as a measure of the reliability of the estimation years. All of the sequences belonging to one particular lineage for each segment were used for regression analysis.

### Nucleotide sequence accession numbers

The sequences determined in this study are available from GenBank under accession numbers AB434285–AB434380, AB434389–AB434420.

## Results

Phylogenetic analysis of the H3 HA1 genes (Figure 1) and the N2 NA genes (Data not shown) revealed the existence of at least two distinct evolutionary pathways in the human virus lineage, designated as clusters Ha and Hb respectively, amongst the Thai SIV isolates examined. Rat59 and Rat874 belonged to the cluster Ha whereas Chacho03, UT464 and NP586-1 belonged to the cluster Hb (Table 1). The H3 Ha Thai isolates were closely related to a human virus isolated in Thailand in 1997 (Figure 1), suggesting that an ancestral Ha strain may have acquired an H3 HA gene from a human H3N2 strain circulating in the late 1990s. The H3 Hb Thai isolates were estimated to have derived from a hypothetical common ancestor in 1976, which had acquired an H3 HA gene from a human-like H3N2 swine strain circulating in the early 1970s. Similar evolutionary pattern was observed in the N2 tree (data not shown). N2 gene of the H1N2 isolate, Sara13021, which HA gene was of classical swine origin, also belonged to the cluster Hb. The branch points of common ancestors for those clusters of N2 genes were estimated to be 1998 for Ha and 1976 for Hb respectively.

Two distinct clusters of H1 HA genes of the Thai SIVs that were demonstrated in the classical swine lineage<sup>17</sup> were designated as Cla and Clb (Table 1). The Cla viruses (Rat550 and Rat1480) and the rest Clb H1 viruses were estimated to have derived from a hypothetical common ancestor in 1981 with 95% confidence limits at 1979 and 1983 for Cla and in 1990 (95% confidence limit; 1988–1991) for Clb.

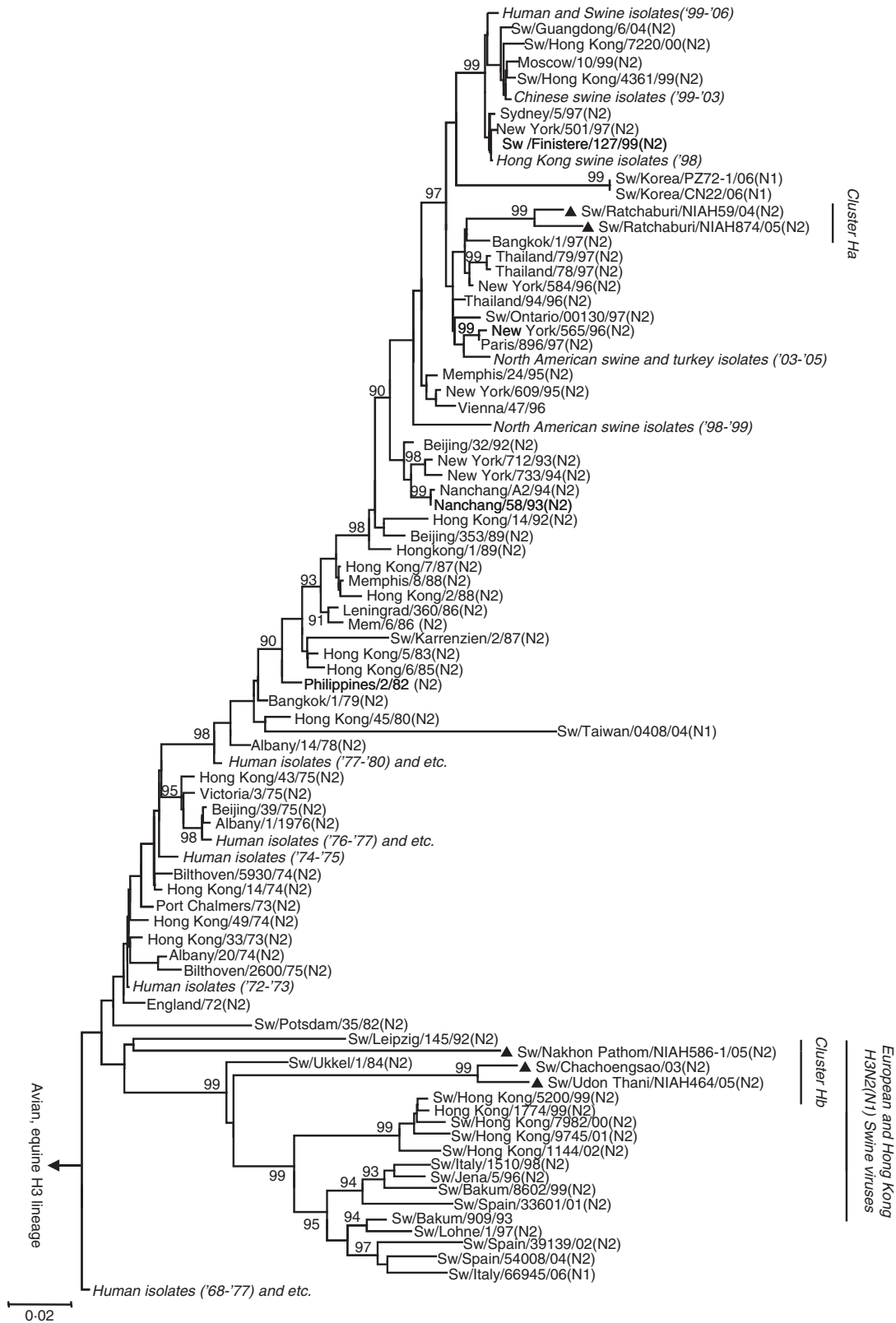
PB2 (Figure 2) and N1 NA<sup>17</sup> genes of all the Thai SIVs formed a single cluster, designated as ALa within the avian-like swine lineage (Table 1). Regression analysis estimated that they were derived from a hypothetical common ancestor in 1983 (95% confidence limit; 1981–1986) for N1, and 1990 (95% confidence limit; 1988–1992) for PB2. The topologies of the Thai SIVs in those phylogenetic trees coincide with each other, providing supporting evidence for the single introduction of those genes. The PB1 gene of all the strains, except Rat59, showed a similar evolutionary pathway to those seen in N1 and PB2 genes. They were derived from a hypothetical common ancestor in 1983 (95% confidence limit; 1980–1987). The PB1 gene of Rat59 was located in another cluster within the avian-like swine lineage, sharing a common ancestor with European swine viruses isolated between 1995 and 2004. Because of the low bootstrap value (52%) at the node where Rat59 and the

other Thai isolates diverged, it was not clear whether or not the PB1 gene of Rat59 had been introduced in another occasion.

M (Figure 3) and PA (data not shown) genes of the Thai isolates also belonged to the avian-like swine lineage. Furthermore, at least two distinct evolutionary pathways clearly existed amongst the Thai isolates in both these genes. The one that diverged earlier was considered to be an equivalent of the ALa in the previous segments, and the other was designated as ALb (Table 1). The years of divergence of the ALa were estimated as 1990 (95% confidence limit; 1988–1992) for the M gene and 1987 (95% confidence limit; 1985–1989) for the PA gene. Those for the ALb cluster were estimated as 2000 (95% confidence limit; 1999–2001) for the M gene and 1999 (95% confidence limit; 1998–1999) for the PA gene. The cluster ALa of the PA gene was divided into two groups with a low bootstrap value (21%) at the node of the hypothetical common ancestor of that cluster. In addition, NP586-1 possessed M gene of ALa and PA gene of ALb.

Nonstructural (NS) and nucleoprotein (NP) genes of the Thai SIVs were contained in both the classical swine lineage and the avian-like swine lineage (Figure 4 and Table 1). Furthermore, two distinct clusters were found within the classical swine NS lineage (Figure 4). One cluster, consisting of exclusively H3 isolates (Rat874, Rat59 and Chacho03), diverged from 1987 (95% confidence limit; 1986–1988), was suggested to be the cluster Cla. The other cluster including three H1 viruses (Sara13021, Chon977 and Chon9496), which diverged from 1986 (95% confidence limit; 1986–1987), appeared to be similar to that of the Clb H1 HA gene. NP586-1, instead of Rat59, clustered with Rat874 and Chacho03 within the classical swine lineage in the NP tree (data not shown). The similarity of the topology with the NS tree suggested that those three isolates share a common genetic origin with the cluster Cla. However, the estimated year of origin of the NP Cla gene was older (1963; 95% confidence limit; 1955–1971) than that calculated for the NS and H1 Cla. This discrepancy was probably due to the low  $R^2$  value for the estimation of the NP gene ( $R^2 = 0.488$ ). All the NS genes of Thai isolates belonging to the avian-like swine lineage were considered to be the cluster ALa (Table 1). A common ancestor of the ALa strains was estimated to have occurred in 1985 (95% confidence limit; 1982–1988) for NS and 1989 (95% confidence limit; 1988–1990) for NP.

Taken together, the existence of nine genotypes of the SIVs was evident in the Thai pig populations within the three subtypes examined (Table 1). All of the avian-like swine segments possessed by the Thai SIVs were closely related to the European avian-like swine lineage represented by A/Swine/Arnsberg/6554/79(H1N1),<sup>21</sup> but not to those found in the North American swine viruses



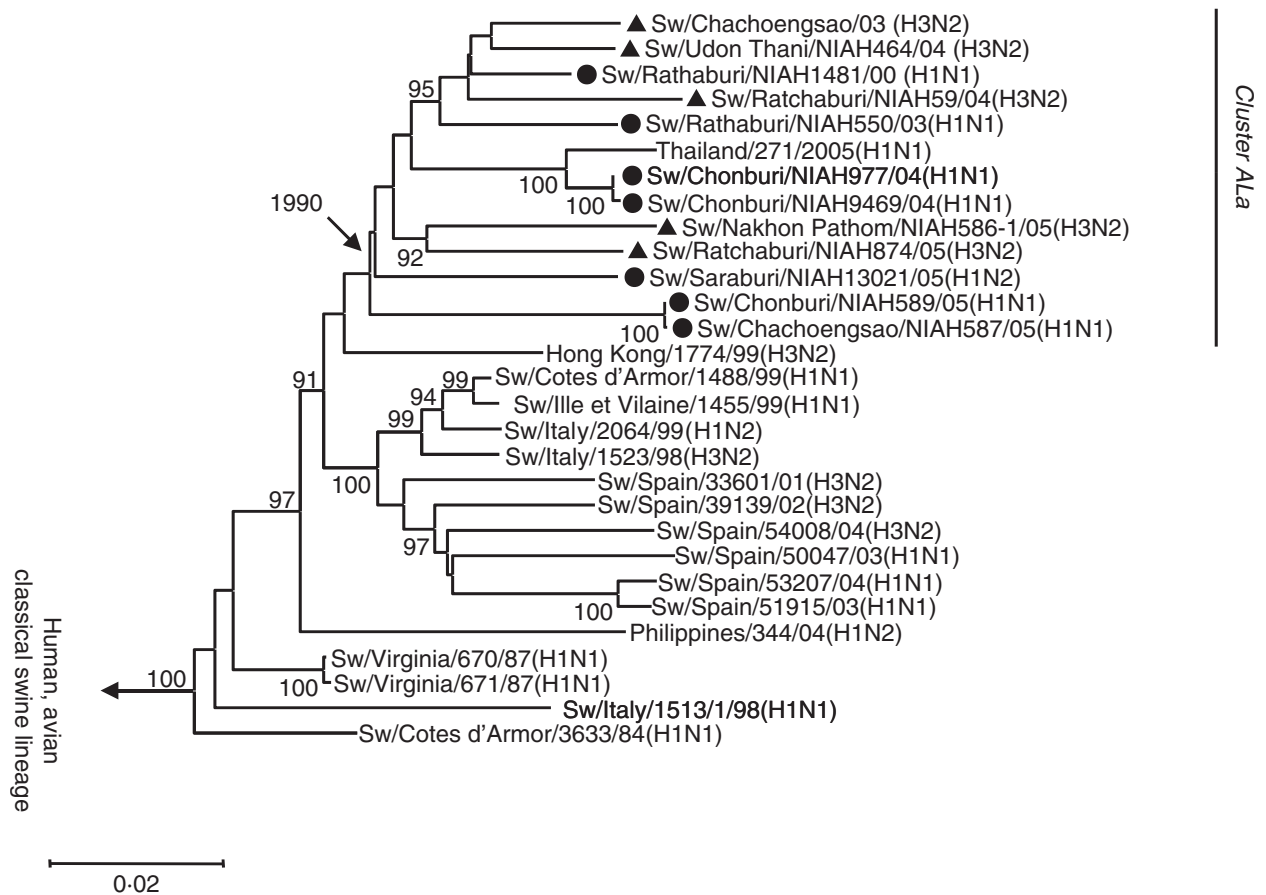
**Figure 1.** Phylogenetic tree of H3 HA genes of human lineage using HA1 region. Bootstrap values of more than 90% are shown at the nodes. Thai isolates are indicated by ▲. Sw represents swine.

**Table 1.** Phylogenetic origin and cluster of each RNA segment\*

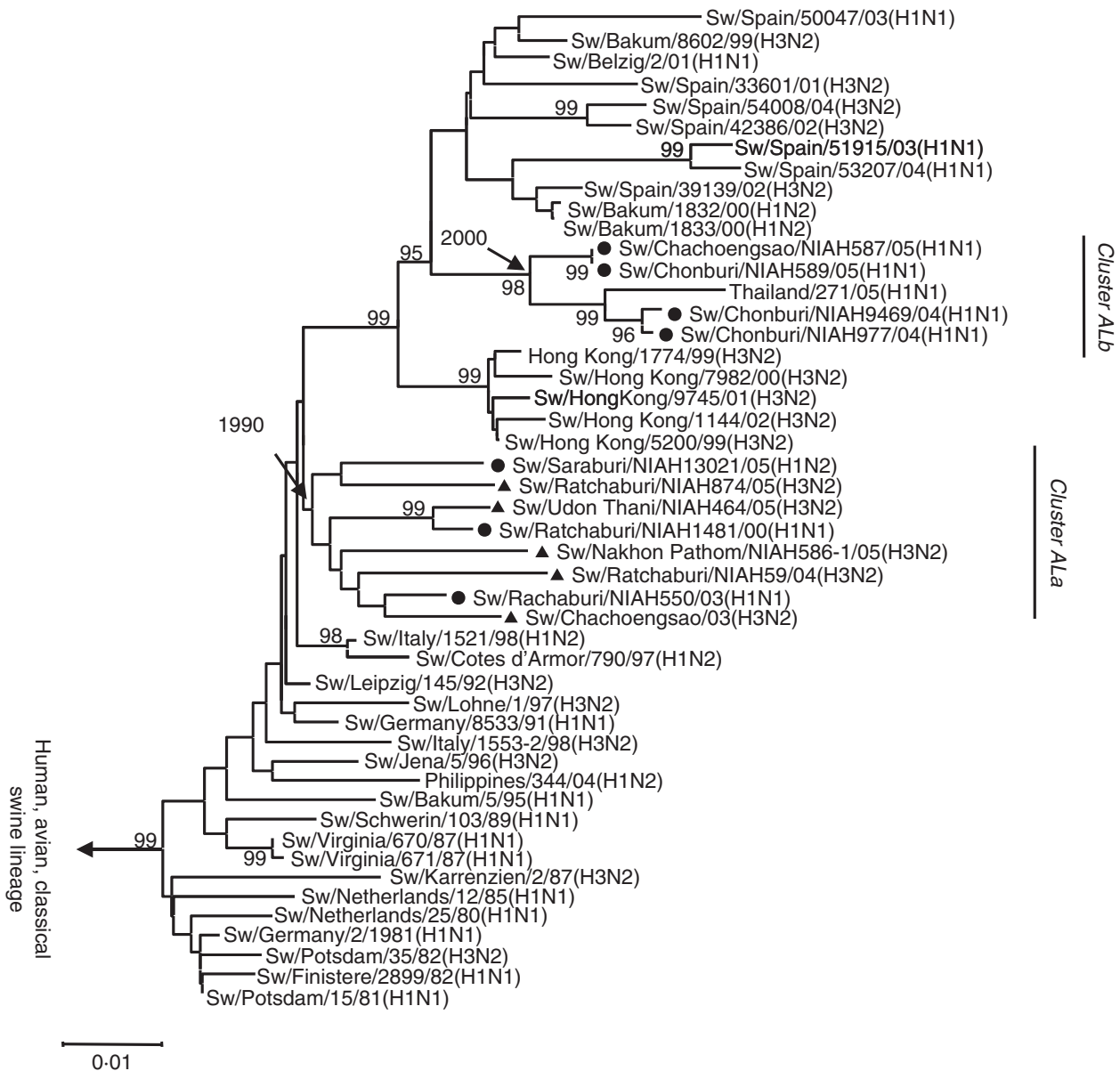
strain	subtype	HA	NA	PB2	PB1	PA	M	NP	NS
Sw/Ratchaburi/NIAH1481/00 Sw/Ratchaburi/NIAH550/03	H1N1	Cla	ALa	ALa	ALa	ALa	ALa	ALa	ALa
Sw/Chonburi/NIAH9469/04 Sw/Chonburi/NIAH977/04	H1N1	Clb	–	–	–	ALb	ALb	–	Clb
Sw/Chonburi/NIAH589/05 Sw/Chachoengsao/NIAH587/05	H1N1	Clb	–	–	–	ALb	ALb	–	–
Sw/Saraburi/NIAH13021/05 Sw/Chachoengsao/03	H1N2 H3N2	Clb Hb	Hb Hb	– –	– –	– –	– –	– Cla	Clb Cla
Sw/Udon Thani/NIAH464/04 Sw/Nakhon Pathom/NIAH586-1/05	H3N2 H3N2	Hb Hb	Hb Hb	– –	– –	– ALb	– –	– Cla	– –
Sw/Ratchaburi/NIAH59/04 Sw/Ratchaburi/NIAH874/05	H3N2 H3N2	Ha Ha	Ha Ha	– –	– –	– –	– –	– Cla	Cla Cla

\*Phylogenetic origins that differ from A/Sw/Ratchaburi/NIAH1481/00 are shown. The small characters a and b after the origins represent the clusters designated in this study. Cl, AL and H stand for classical swine, avian-like swine and human origins respectively.

\*\*It was not clear whether another introduction had occurred or not.



**Figure 2.** Phylogenetic tree of PB2 genes of avian-like swine lineage. Bootstrap values of more than 90% are shown at the nodes. Thai isolates are indicated by ● (H1N1/N2 strains) and ▲ (H3N2 strains) and the estimated years are indicated by arrows at the nodes of the hypothetical common ancestor. Sw also represents swine.



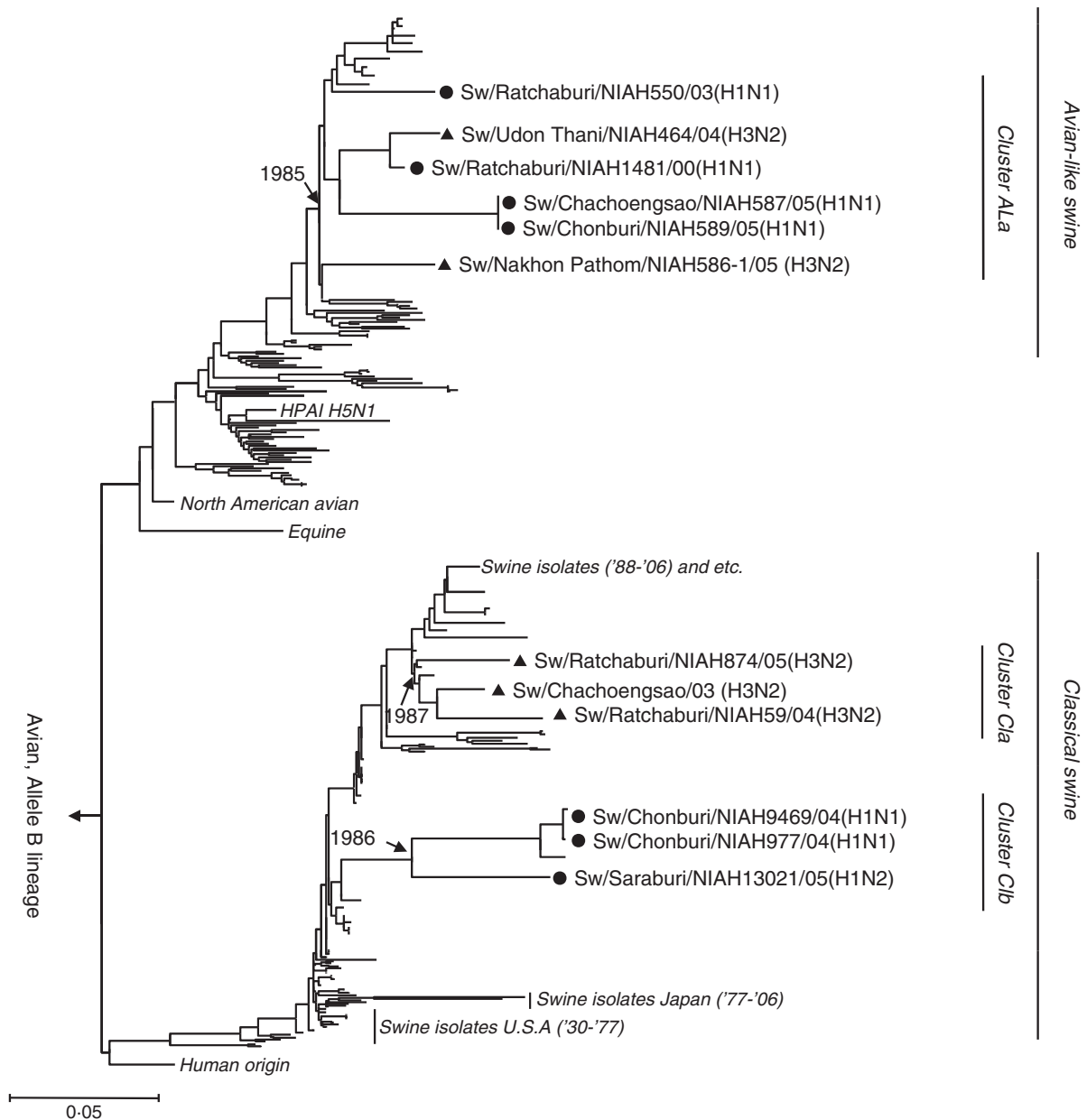
**Figure 3.** Phylogenetic tree of M genes of avian-like swine lineage. Bootstrap values of more than 90% are shown at the nodes. Thai isolates are indicated by ● (H1N1/N2 strains) and ▲ (H3N2 strains) and the estimated years are indicated by arrows at the nodes of the hypothetical common ancestor. Sw also represents swine.

represented by A/Swine/Texas/4199-2/98(H3N2),<sup>6</sup> indicating that the genetic resources introduced into the Thai SIVs were mainly from Europe. This is consistent with the fact that Thailand has imported breeding pigs from Europe rather than North America since late 1980s.<sup>13</sup>

## Discussion

The genetic diversity of SIVs as seen in other SIVs of the world was evident in Thai pig population even from a small number of viruses obtained between 2000 and 2005

(Table 1). The viruses containing genes from avian-like swine viruses appeared to have some advantages for being maintained in the pig population. All the Thai SIVs examined possessed PB2, PB1, PA and M genes of avian-like swine lineages. Avian H1N1 viruses entered the pig population in 1979 in Europe, and have gradually replaced classical swine H1N1 viruses, which circulated in Europe before 1979.<sup>21</sup> Although human-like swine H3N2 and H1N2 with human surface antigens emerged as a consequence of the introduction of human viruses, avian-like swine genes encoding internal proteins have been retained for nearly



**Figure 4.** Phylogenetic tree of (Nonstructural) NS genes. Thai isolates are indicated by ●(H1N1/N2 strains) and ▲(H3N2 strains) and the estimated years are indicated by arrows at the nodes of the hypothetical common ancestor. Bootstrap values and strains except for the Thai isolates examined are omitted. Sw represents swine.

30 years in the European pig population.<sup>4,22,23</sup> There was evidence that German swine H1N2 isolates in 2005 were reassortants of avian-like swine H3N2 and H1N2.<sup>24</sup> In North America, the reassortant H3N2 SIVs containing avian genes (PB2, PA) have spread more rapidly in the pig population since 1998 than other reassortant H3N2 SIVs without avian genes.<sup>25</sup> Interestingly, the introduction of classical swine H1N1 viruses into the former H3N2 SIVs

occurred and established the swine H1N2 lineage retaining the PB2 and PA genes of avian origin.<sup>26</sup>

The complicated genetic diversity of SIVs in southern China, where transmissions of avian viruses into pigs have been reported over the last 30 years, is evident.<sup>27</sup> In 1993, Eurasian avian H1N1 virus entered the Hong Kong pig population. It was an independent event from that observed in the European pig population in 1979.<sup>28</sup> Swine

H3N2 viruses that were closely related to those isolated from the European pig population were isolated from Hong Kong pigs.<sup>27</sup> Prior to that event, an H3N2 virus with similar gene set was isolated from a young child in 1999 in Hong Kong.<sup>29</sup> Additionally, H1N2 swine viruses containing classical swine H1 and human-like N2 were isolated in 2004 in China.<sup>30</sup> Our phylogenetic analysis indicated that none of those avian-like and classical swine genes identified in China were directly introduced into Thai pig population. This observation correlates well with the fact that <30 breeding pigs were imported from China from 1986 to 2006 to Thailand.<sup>13</sup>

Our phylogenetic analysis suggested two possibilities by which Thai H1N1(N2) SIVs, which possess the HA gene of classical swine origin and most of the other gene segments of avian-like swine origin, emerged. One possibility is that an avian-like swine H1N1 virus (ancestral ALa) was introduced into the Thai pig population around 1990, where two distinct classical swine viruses (Cla and Clb) had already been circulating. By means of this introduction, all of the gene segments except the HA of the Cla viruses were replaced by genes of ancestral ALa virus. For the viruses which retained the Clb HA gene, most of the segments were also replaced by genes of ALa origin. The fact that three strains (Chon9469, Chon977 and Sara13021) with the Clb HA had retained NS genes of Clb origin supports this possibility. Another possibility is that progenitor viruses with a combination of genes of Clb and ALa origin established elsewhere were introduced into the Thai pig population where only viruses with genes of Cla origin had been circulating. The coincidence of the divergent year of hypothetical ancestral genes for Clb and ALa genes supports this possibility. A weakness of the latter hypothesis is that there has been no report of such a progenitor virus in the world, although this does not completely exclude the possibility. Also, the existence of the Clb NS genes in the Thai SIVs discounts this possibility as well. In any case, the introduction of avian-like swine virus resulted in a variety of genetic constellations within the H1N1 Thai SIVs.

This study also revealed that a human-like swine H3N2, which may have been a provider of the Hb genes, was introduced into the Thai pig population. The Hb genes of the Thai SIVs shared a common ancestor with European and Hong Kong H3 (H1) N2 SIVs which possess human-like surface antigens and avian-like genes encoding internal proteins. SIVs with an avian internal protein gene constellation have been recognized in Europe for H3N2 and H1N2 subtypes since the mid-1980s and -1990s respectively.<sup>4,22</sup> Although it is not clear when the progeny Hb Thai viruses were introduced into the Thai pig population, our estimation revealed that the H3 and N2 genes of an ancestral Hb virus had diverged in 1976, indicating that the virus did not contain any avian-like swine genes at that

time. Then, genes encoding internal proteins of the H3 (H1) N2 SIVs possessing surface antigens of Hb origin were replaced with those of the H1N1 SIVs established in Thailand, although it is not clear that the ancestral H3N2 viruses containing Hb origin were circulating amongst the Thai pig population prior to the introduction of the ALa genes from the data analysed by us.

The introduction of another human H3N2 virus (ancestral Ha virus) into the Thai pig populations was estimated to have occurred around 1998. At that time, our analyses indicated that H1N1 and H3N2 viruses containing a variety of combination genes had already co-circulated in the Thai pig population. Interspecies transmissions from pigs to humans or vice versa have been described in many countries such as some European countries,<sup>31,32</sup> China<sup>29</sup> and Thailand.<sup>18,33</sup> Our analysis clearly demonstrated that the Ha viruses acquired the HA gene not from swine but from human virus (Figure 1). In contrast, Chutinimitku *et al.* concluded that Rat874, used in both studies, derived from an American or Asian SIV.<sup>18</sup> This discrepancy was referable to the data set used for the phylogenetic analysis. Chutinimitku *et al.*, included only seven human H3 viruses and 13 American and Asian H3 SIVs for the analysis; in contrast, 109 human H3 viruses and 37 American and Asian H3 SIVs were used in our study to improve reliability of the analysis. Their work might not have adequate number of human epidemic H3 viruses to lead to a correct conclusion.

The prevalence and pathogenicity of the Thai SIVs have not been well known, and neither has the economic impact caused by the SIVs in Thailand. The situation in Thailand where several genotypes of SIVs could be co-circulating in pig population certainly increases the chance of additional evolutionary events of the SIVs. Further surveillance would elucidate whether one genotype highly adapted to the pigs in each subtype gradually became dominant amongst various genotypes of the Thai SIVs. It is unclear whether different gene constellations observed in this study could affect the pathogenicity of the Thai SIVs. Van Reeth observed no differences of virulence in pigs amongst three subtypes (H1N1, H3N2 and H1N2) or strains.<sup>34</sup> In addition, dual infections with SIVs and other pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV)<sup>35</sup> and *Mycoplasma hyopneumoniae*<sup>36</sup> complicate the pathogenicity of SIVs. We need further analyses of the SIVs in Thailand to deepen our understanding regarding not only the ecology but also the pathogenesis of the SIVs.

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