Molecular epidemiology and antigenic analyses of influenza A viruses H3N2 in Taiwan

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Abstract

The severity of an influenza epidemic season may be influenced not only by variability in the surface glycoproteins, but also by differences in the internal proteins of circulating influenza viruses. To better understand viral antigenic evolution, all eight gene segments from 44 human H3N2 epidemic strains isolated during 2004–2008 in Taiwan were analyzed to provide a profile of protein variability. Comparison of the evolutionary profiles of the HA, NA and PB2 genes of influenza A (H3N2) viruses indicated that they were derived from a group of H3N2 isolates first seen in 2004. However, the PA, M and PB1 genes were derived from a different group of H3N2 isolates from 2004. Tree topology revealed the NP and NS genes could each be segregated into two groups similar to those for the polymerase genes. In addition, new genetic variants occurred during the non-epidemic period and become the dominant strain after one or two seasons. Comparison of evolutionary patterns in consecutive years is necessary to correlate viral genetic changes with antigenic changes as multiple lineages co-circulate.

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Introduction

Influenza viruses cause yearly epidemics worldwide in humans, and are some of the most active pathogens in Taiwan leading to significant morbidity and mortality [1,2]. Conventionally, analyses of influenza evolution have focused on individual viral genes, most often HA, without exploring the interactions among them. However, the evolutionary behaviour of the virus often involves cooperative changes within and between genes. Thus, there is important information about influenza evolutionary behaviour that may be deduced from correlated changes between nucleotide positions both within and between genes.

With the cocirculation of different influenza A subtypes, genetic reassortment can play an important role in antigenic variation [3,4]. Reassortment of the eight segments of influenza A viruses can lead to complicated phylogenetic patterns on the genomic scale [5–7]. Previous studies have shown that the virulence and growth characteristics of influenza viruses are influenced by changes in the internal proteins [8,9]. The M1 protein is a multifunctional protein that contributes to the control of virulence, growth [10–13] and host specificity [14,15]. Changes in the NP, PB2 and M1 proteins were shown to play a role in host restriction in monkeys [14]. Considering these observations, the severity of an influenza epidemic season may be influenced not only by variability in the surface glycoproteins, but also by differences in the internal proteins of circulating influenza viruses. In the present study, we examined the evolutionary dynamics of influenza A (H3N2) virus at genomic and epidemiological scales, as isolated during 2004–2008 in Taiwan, to provide a complete profile of protein variability as well as the viral antigenic evolutionary patterns of all gene segments of these viruses.

Materials and Methods

Isolates

Influenza strains circulating in 2004–2008 seasons were isolated from combined nose and/or throat swabs from selected patients with influenza-like illness presenting to physicians in sentinel practices (http://www.cdc.gov.tw). The specimen were inoculated into Madin-Darby canine kidney cells and were confirmed by the indirect immunofluorescence assay (IFA, Chemicon, Inc., Temecula, CA, USA) and RT-PCR. RT-PCR reactions were performed as described previously [16]. Supernatants from positive cultures were harvested and stored at −80°C for subsequent antigenic and genetic analyses.

Genetic and antigenic analysis

Phylogeny construction and evaluation were performed with the maximum likelihood and Neighbour-joining (NJ) methods in the PHYLIP software [17]. Empirical transition/transversion ratio was estimated by the TREE-PUZZEL software [18] to calculate the evolutionary distances. To determine the overall selection pressures of each gene, we estimated the mean numbers of nonsynonymous substitutions (d_N) and synonymous substitutions (d_S) per site (ratio d_N/d_S) using the SLAC method within the HYPHY package [19] through the Datamonkey web-based interface (http://www.datamonkey.org). The d_N/d_S estimates were based on NJ trees under the GTR substitution model. The full-genome sequences of 44 influenza A (H3N2) viruses obtained in the present study have been submitted to GenBank and assigned accession numbers: FJ805464–FJ805743.

Viruses were characterized by haemagglutination inhibition (HI) assays, as described previously [20,21], using post-infection ferret antisera obtained from CDC, Atlanta, Georgia.

Results

Epidemic activities and antigenic analysis

Although the relative prevalence of influenza virus varies from season to season, influenza A (H3N2) viruses were the major epidemic strains isolated during the study period with the exception of the 2005–2006 season. H3N2 viruses frequently cocirculated with influenza B viruses as seen during the 2004–2005 and 2006–2007 seasons in Taiwan (Fig. 1).

A total of 2080 samples that tested positive for influenza viruses in the 2004–2005 season were confirmed by RT-PCR. Of these, 726 (34.9%) were influenza A (H3N2) viruses, 305 of which were selected for characterization in HI tests. Of those HI analyzed, 172 (56.4%) were A/Wyoming/3/03-like, with the remaining 133 (43.6%) isolates showing reduced titres with antisera produced against A/Wyoming/3/03 (Fig. 2).

In the 2005–2006 season, a total of 858 samples tested positive for influenza and 228 (26.6%) of these were H3N2. Ninety-five isolates of the H3N2 positive samples were selected for further characterization by HI testing. Of those HI analyzed, 68 (71.6%) of them were A/California/7/04-like, with the other 27 (28.4%) showing reduced titres with antisera produced against A/California/7/04. In the subsequent 2006–2007 season, a total of 2175 samples tested positive for influenza. Of these, 770 (35.4%) were H3N2, 213 of which were selected for characterization in HI tests. Ninety-

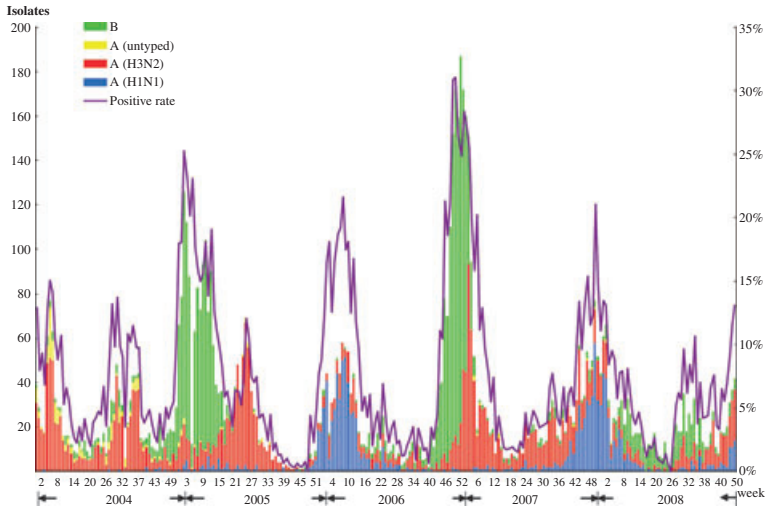

FIG. 1. Weekly distribution of Taiwanese influenza A (H3N2) isolates based on cell culture from 2004 to 2008, together with the positive rate of culture confirmed cases.

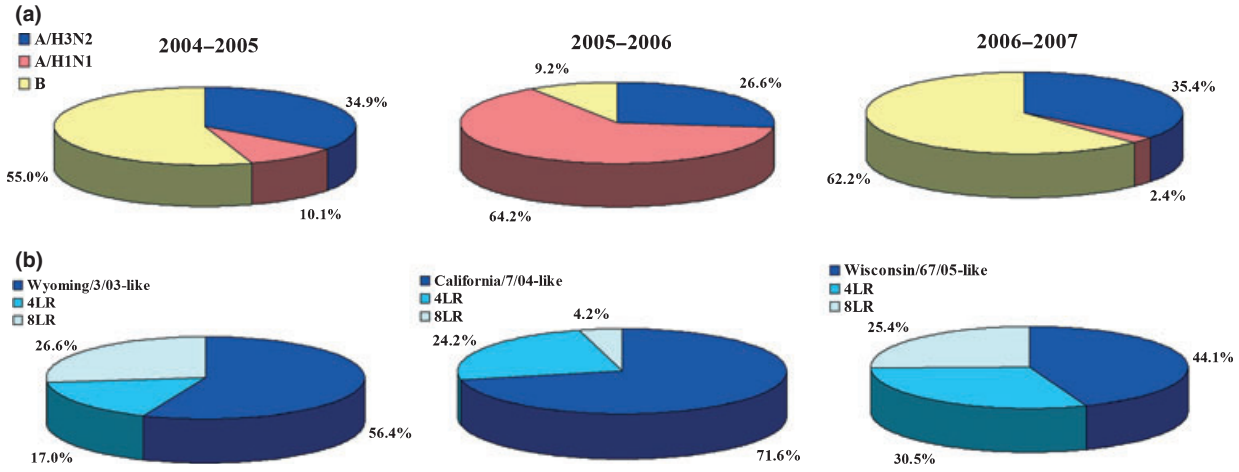

FIG. 2. (a) Antigenic characterization of influenza viruses isolated from 2004 to 2007 in Taiwan. (b) Haemagglutination inhibition reactions of influenza A (H3N2) virus using post-infection ferret antisera. Reference strains for 2004–2007 seasons, A/Wyoming/3/03, A/California/7/04, A/Wisconsin/67/05, respectively.

four (44.1%) of them were A/Wisconsin/67/05-like, with the remaining 119 (55.9%) having reduced titres with antisera produced against A/Wisconsin/67/05.

Analysis of individual gene segments

HA and NA genes. Comparison of the amino acid sequences encoded by the HA1 genes with that of the A/Wyoming/3/03 vaccine strain revealed six conserved amino acid changes (Table 1). The phylogenetic tree of these HA genes could be divided into three subgroups according to different influenza seasons: I, II and III (Fig. 3a). All isolates except those in subgroup Ia had an additional S227P change. Two additional amino acid changes at S193F and D225N were observed in both groups II and III. Isolates within subgroup IIIb had four additional changes at R142G, N144D, K173E and Y195H. Isolates in group IIIa lacked those four amino acids changes and could be further distinguished by an amino acid change at position G50E.

Similar to the results seen for HA, NA genes could be divided into three phylogenetic groups (Fig. 3b). This observation suggested that the NA genes of recent H3N2 viruses, similar to their HA counterparts, have evolved as two distinct phylogenetic branches. A total of 24 amino acid substitution were observed compared to the NA gene of A/Wyoming/3/03, and amino acid changes E199K, K221E and Q432E became fixed after 2004 (Table 1). The amino acids composition of group II was similar to group I with the exception of N93D, but differed from group III, which could be distinguished by the signature change H150R. N93D, V194I, Y310H L370S, S372L and N387K were signature changes for NA group IIIa, whereas group IIIb viruses all had changes at N86K, K296R, I307M and S335G. None of the established genetic markers for neuraminidase drug resistance at positions 119, 152, 274, 292 and 294 were found in our NA dataset [22].

Polymerase PB2, PB1 and PA genes. In the phylogenetic tree of the polymerase genes, the PB2 genes were divided into three groups, Using the classification of the HA genes, the PB2 genes of group Ia could be distinguished by amino acid

TABLE 1. Amino acid variation observed in HA1 and NA genes of human H3N2 viruses isolated from 2004 to 2008 in Taiwan

	HA1															NA													
Protein	50	128	142	144	145	159	173	186	189	193	195	219	225	227	86	93	150	194	199	221	296	307	310	335	370	372	387	432
Wyoming/03	G	A	R	N	K	Y	K	V	S	S	Y	Y	D	S	N	N	H	V	E	K	K	I	Y	S	L	S	N	Q
I																												
Ia		T			N	F		G	N			S				D			K	E								E
Ib		T			N	F		G	N			S		P		D			K	E								E
II		T			N	F		G	N	F		S	N	P					K	E								E
III																												
IIIa	E	T			N	F		G	N	F		S	N	P		D	R	I	K	E			H		S	L	K	E
IIIb		T	G	D	N	F	E	G	N	F	H	S	N	P	K		R		K	E	R	M		G				E

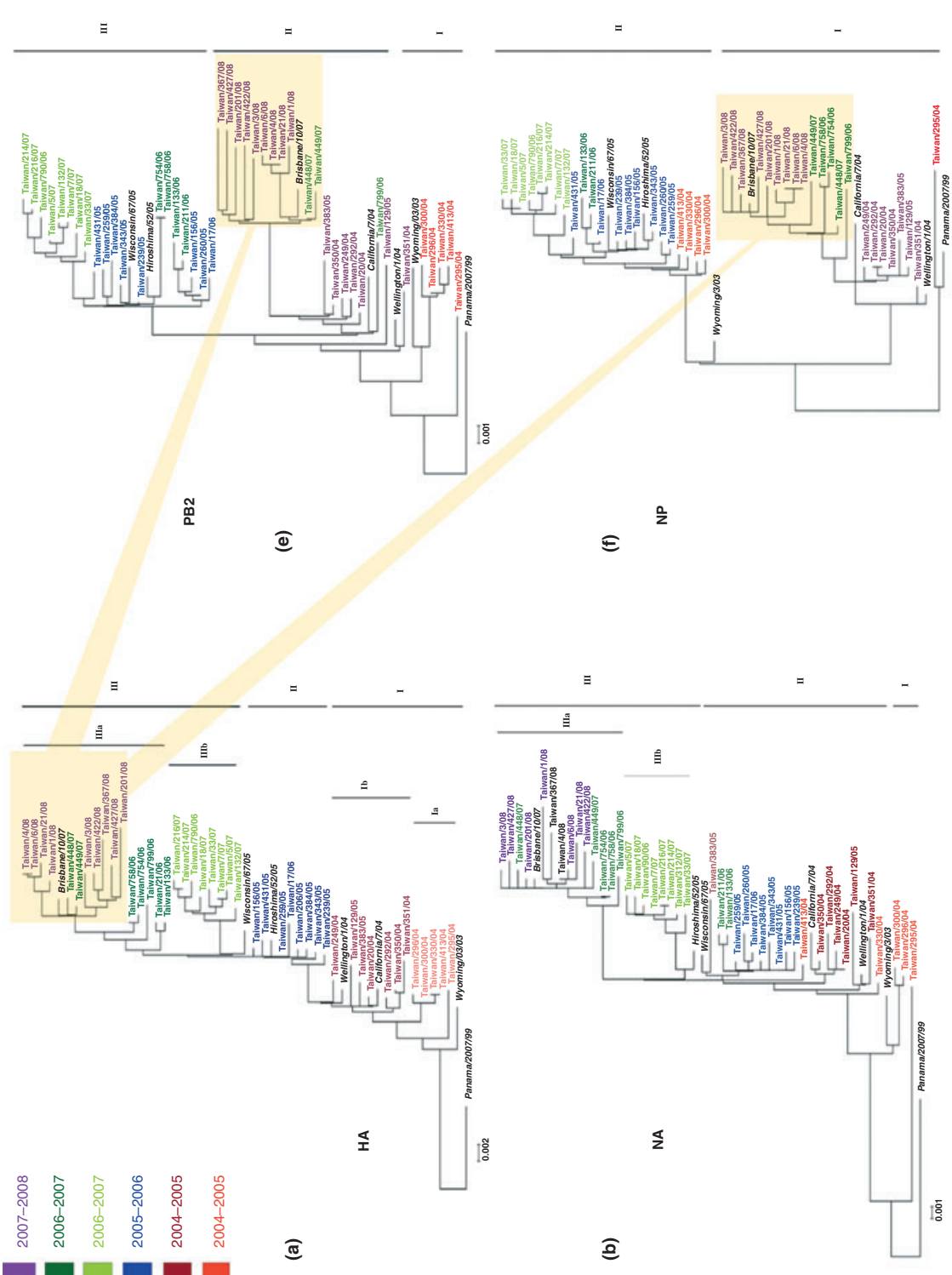

FIG. 3. Phylogenetic analysis of genetic evolution of full length, complete genome sequences of 44 human influenza A (H3N2) viruses isolated from 2004 to 2008 in Taiwan. The trees were inferred from HA (a), NA (b), PB2 (c), PB1 (d), PA (e), NP (f), MP (g) and NS (h) gene data by the Neighbour-joining method and using bootstrap analysis (n = 1000) to determine the best-fitting tree. The colored connectors indicate the major isolates that result from reassortment. The reference strains are represented in italic boldface. Vaccine strains for 2004–2008 seasons, A/California/7/04, A/Wisconsin/67/05, A/Brisbane/10/07, respectively.

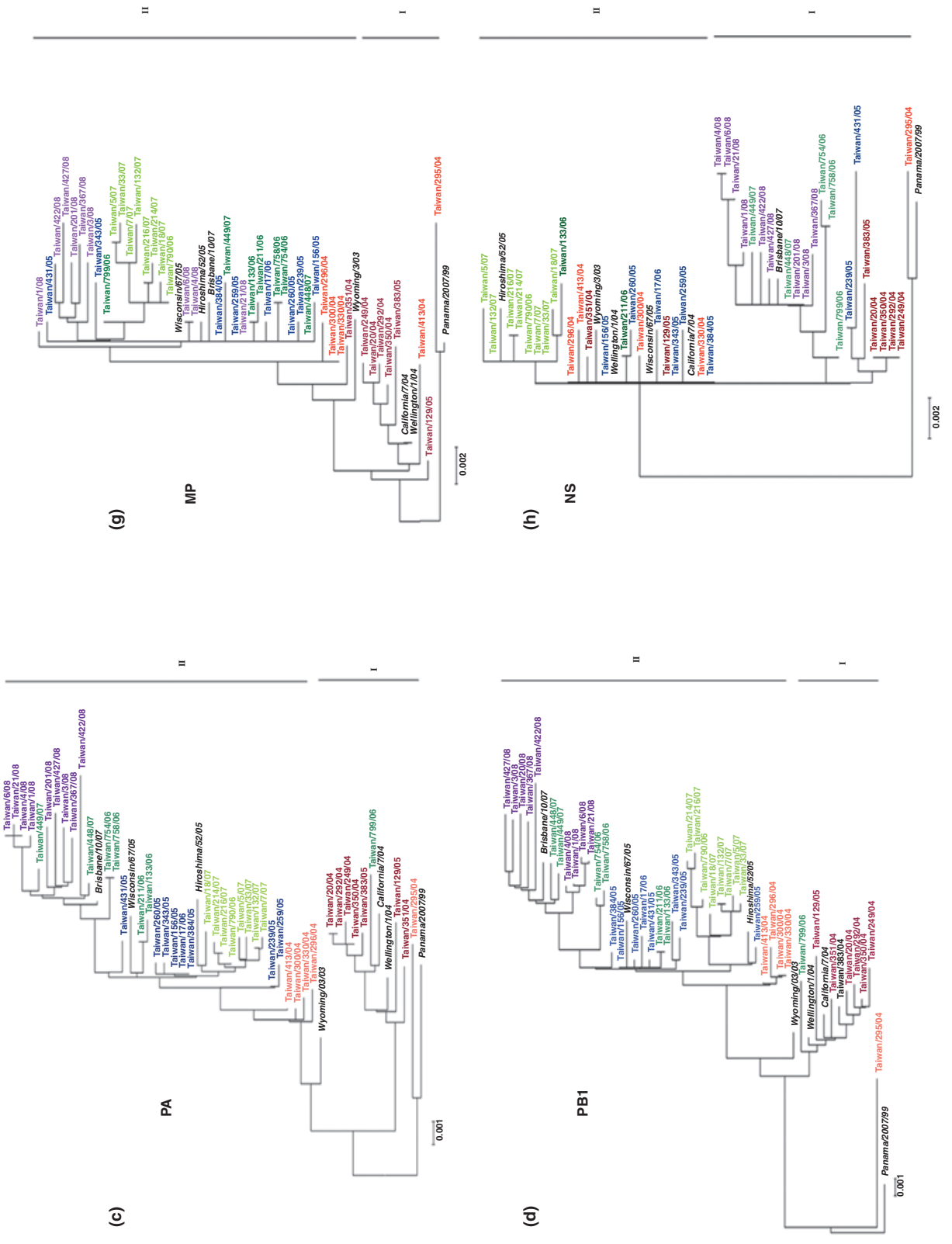

FIG. 3. (Continued)

TABLE 2. Amino acid variation observed in the internal genes of human H3N2 viruses isolated from 2004 to 2008 in Taiwan

	PB2				PB1					PA								NP						M1			M2			NS1	
Protein	**64**	**249**	**461**	**683**	**113**	**375**	**586**	**621**	**642**	**62**	**101**	**208**	**256**	**382**	**421**	**437**	**602**	**31**	**52**	**280**	**312**	**377**	**495**	**174**	**219**	**239**	**10**	**31**	**58**	**124**	**221**
Wyoming/03	T	E	V	T	V	N	R	R	N	V	E	S	Q	E	I	Y	V	K	Y	V	V	S	E	R	I	T	P	S	G	V	K
I																															
Ia	I			A	A	S			S		G				V	H						G						N		M	
Ib			I			S		Q		M			K	D			I		H	A	I			K	V					M	
II					A	S					G	T			V	H						G						N		M	
III																															
IIIa					A	S	K				G	T			V	H			H	A	I	G						N		M	E
IIIb		G			A	S					G	T			V	H		R				G	K			N	T	N	S	M	

changes at T64I and T683A. An amino acid substitution V461I was observed in group Ib, and the E249G amino acid substitution distinguished group IIIb. The grouping of the PA genes was similar to that seen for the PB1 gene (Fig. 3c,d,e). When examining the deduced amino acid sequences of the PB1 genes, all isolates had an N375S amino acid substitution, and all except group Ib contained a V113A amino acid change compared to A/Wyoming/3/03(Table 2). In addition, all of the PA genes had the amino acid changes E101G, I421V and Y437H, except for group Ib. An addition amino acid substitution at S208T was seen in both groups II and III. The subgroup Ib had unique changes at V62M, Q256K, E382D and V602I (Table 2).

NP, M and NS genes. Phylogenetic analysis of the NP, M and NS genes showed that these three genes of these viruses could each be segregated into two groups similar to those for the polymerase genes (Fig. 3f,g,h). Using the classification used for the HA genes (above), three amino acid substitutions (Y52H, V280A and V312I) were observed in the NP genes of both group Ib and group IIIa. The amino acid substitution S377G was present in all isolates except those in group Ib and additional amino acid changes at K31R and E495K were observed in group IIIb.

Signature change associated with adamantanes resistance S31N in M2 gene became fixed after 2004 in all isolates except those in group Ib. Group IIIb could be distinguished by the amino acid change T239N in the M1 gene and P10T and G58S changes in the M2 gene. The NS1 genes of Taiwanese isolates all have the V124M amino acid difference compared to A/Wyoming/3/03, with group IIIa having an additional amino acid change at K221E (Table 2). There were no changes in NS2 compared to the A/Wyoming/3/03 vaccine strain.

Measurement of selection pressures

The selection pressures acting on human H3N2 viruses were higher than for previous reports [23,24]. The highest d_N/d_S ratios were observed in the HA and NA (mean d_N/d_S 0.41 and 0.37, respectively), most likely reflecting immune selection pressure at a small number of amino acid sites [25], and also NP, NS and M (mean d_N/d_S 0.08, 0.32, and 0.08, respectively), which are essential for transcription and replication.

Discussion

To date, molecular and phylogenetic analyses of influenza A H3N2 viruses spanning successive seasons have not been reported in Taiwan. The changing patterns of genetic diversity in viral isolates provide insight into the seasonal dynamics of influenza A and reveal the evolutionary interaction between subtypes; for example, recent seasons dominated by H1N1 were observed following H3N2 epidemics (Fig. 1). The amino acids in the H3 HA1 from 2004 to 2008 had a total of 17 amino acid substitutions, all of which were located at well known antibody antigenic sites or receptor binding sites.

Changes in the 140–146 region of antigenic site A are characteristic for antigenically distinct viruses of epidemic significance [26]. The amino acid substitution K145N has been fixed subsequent to the 2004–2005 season and we found two additional amino acid changes, R142G and N144D, in the antigenic site A of viruses isolated in 2007. Moreover, one region in HA at amino acids 225–227, which affects antigenic site D, changed dramatically during the study period as a result of D225N and S227P mutations. The 225 and 227 positions are also located in the receptor binding pocket and thus could be expected to have effects beyond antigenic variation. Two amino acid substitutions at positions 128 (A to T; position 126–128 are N,W and T) and 144 (N to D; position 144–146 are D,N and S) were observed that could affect N-linked glycosylation, which could conceivably allow escape from human immune pressure.

The NA genes of the A/Hiroshima/52/05 reference strain apparently evolved along a separate evolutionary pathway

and cocirculated with A/California/7/04-like and A/Wisconsin/67/05-like viruses between 2004 and 2007, thus providing an opportunity for mixed infections that might lead to the emergence of reassortants. These reassortants would not be expected to have any increased epidemic potential compared to their parents and, at this stage, there is no definitive evidence that the influenza A H3N2 reassortant viruses were more virulent than the nonreassortant ones in terms of clinical outcomes. A previous study suggested that new antigenic variants may arise from changes that affect N2 antigenic site B [27]. H3N2 viruses isolated from Taiwan possessed E199K and K221E changes in the NA gene, both of which are found in antigenic site B. We also detected amino acid substitutions in the neuraminidase gene that were not associated with catalytic or framework sites, and none of the neuraminidase substitutions conferring resistance to neuraminidase inhibitors have been observed in our study. Similarly, we did not observe amino acid changes in the N2 protein that would be expected to lead to a decrease in virus replication in eggs [28]; the amino acids known to allow efficient replication in eggs (Q119, K136 and Y347) were all present in the viruses examined.

Comparison of the evolutionary profiles of the HA, NA, and PB2 genes of influenza A (H3N2) viruses revealed that these genes of 2005–2008 isolates were derived from isolates having the group Ib HA gene, and PA, M and PB1 genes were derived from group Ia isolates of the 2004–2005 season. The phylogenetic trees of HA and NA showed seasonal clusters but also co-circulating lineages within each season. It should be noted that, in the 2006–2007 season, the HA, NA, PA, PB1 and M genes of three viruses (A/Taiwan/799/2006, A/Taiwan/448/2007 and A/Taiwan/449/2007) were in group IIIa but the PB2 and NP genes were in group Ib. These new genetic variants were detected during the 2006 and 2007 non-epidemic periods and the reassortants isolated in 2006 were antigenically similar to A/California/7/04; however, the reassortants isolated in 2007 demonstrated a four-fold HI difference compared to A/Hiroshima/52/2005, both of which were vaccine strains recommended for the 2005–06 and 2006–07 season, respectively. Indeed, these reassortant viruses continued to be isolated and become the dominant strains during year 2008 (Fig. 3).

Reassortment among co-circulating H3N2 viruses similar to that observed in present study has been described previously, including reassortment of the NA gene segment [29] and the internal segments [30–33]. However, a different phylogenetic pattern from previous report is observed during the 2007–08 season, and such a major difference in the phylogenetic signal strongly suggests that H3N2 viruses evolve rapidly, as a result not only of genetic drift, but also natural selection. The complete genome analysis of recently sampled Taiwan A (H3N2) influenza viruses in the present study has revealed the co-circulation of multiple distinct groups and frequent intra-subtype reassortment events among them, and such evolution plays an important role in antigenic drift.

In addition, the data presented here reveal that the M2 proteins in all groups except group Ib possessed the substitution S31N. It is interesting to note that all isolates with this S31N amino acid change also have amino acid substitutions in PA and PB1 genes, including E101G, S208T, K256Q, D382D, I421I and Y437H in PA, and the V113A amino acid change in PB1. The role of PA is not well established, although it is known to be involved in RNA replication [34,35]. Recent studies indicate that PA is likely to be involved in transcription as well as replication [36,37]. It has also been reported that the N-terminal region of PA plays a critical role in multiple functions, such as cap-binding, endonuclease activity, etc. [38].

Genetic analyses of influenza H3N2 viruses provide information that is important for understanding the mechanisms involved in the emergence of pandemic influenza viruses. The present study covered the phylogenetic relationships of the external as well as internal genes and clearly demonstrates the value of analyzing the complete genetic composition of influenza viruses in order to understand fully the evolutionary mechanisms and epidemiology of influenza. Furthermore, the present study explores in detail a reassortment event in internal genes leading to an epidemiologically significant outcome, namely the emergence of the '2008–2009 epidemic strain' in the 2006–2007 season. Importantly, the data presented here provide evidence suggestign that influenza A H3N2 viruses undergo internal gene reassortment and that important evolutionary processes may occur during non-epidemic periods at least one or two season ahead. Although it is not yet clear how variant clades manage to persist alongside dominant strains, the fact that they do suggests the influenza virus has multiple adaptive abilities. The overall selection pressure also reveals that the former have not yet reached a global fitness peak characterized by little amino acid fixation.

Analyses of the genetic and antigentic data, together with epidemiological data for the sequenced isolates, have provided insights into the pattern of virus spread, the genetic diversity during seasonal epidemics, and the dynamics of subtype evolution. Continuous monitoring of viral genetic changes throughout consecutive years is necessary to formulate hypotheses as to why antigenic evolution allows certain variants to become dominant when multiple lineages co-circulate.

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Transparency Declaration

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