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Phylogenetic analysis of influenza B virus in Taiwan, 1997 to 2001

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Seventeen strains of influenza B virus were isolated and identified from 1997 to 2001. Throat swabs were collected in children who presented in medical centers in both central and northern parts of Taiwan. To clarify the molecular characteristics of these isolates, both partial hemagglutinin (HA) gene and nonstructural (NS) gene nucleotide sequences were cloned and subjected to nucleotide sequence analysis. The phylogenetic analysis of the HA gene revealed that 16 out of 17 strains were similar to B/Yamagata/16/88-like virus, but grouped together to form an independent cluster. Only one strain, B/Taiwan/21706/97, was similar to the B/Victoria/2/87-like lineage. In addition, all isolates, except for B/Taiwan/21706/97, were similar to B/Beijing/184/93 and B/Yamanashi/166/98, which were chosen as the recommended vaccine strains in 1999 and 2001. In contrast, the NS gene of these isolates was evolved from B/Guangdong/8/93. Based on the accumulation of antigenic drift in our isolates, we conclude that influenza B virus is still prevalent in Taiwan and the accumulation of nucleotide mutations indicated that our isolates form a new cluster that evolved from the YA88 lineage.

Key words: Antigenic variation, disease outbreaks, evolution, genetic recombination, influenza B virus

Influenza A and B viruses are included in the family *Orthomyxoviridae* and share antigenic, genetic and structural similarities [1-3]. Both viruses have the unique capacity to undergo a high degree of antigenic variation within a short period of time. This property has made it difficult to control the seasonal outbreaks of influenza in the human population [4,5]. However, there are significant differences between the epidemiology, host range, genetic coding strategies and evolutionary patterns for influenza A and B viruses [6-8]. In fact, the influenza A viruses are subdivided into 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes, all of which were isolated from waterfowl species, poultry and mammalian species, the latter including humans, horses, minks, whales, swine, and seals [9]. In contrast, influenza B viruses had not been found to naturally infect animals other than humans until an outbreak in harbor seals was documented in 1999 [10]. While both influenza A and B viruses have a high frequency of point mutation on their HA and NA genes, genetic reassortment is frequently observed only in the influenza A viruses. Although genetic reassortment among the influenza B viruses has also been suggested by phylogenetic analysis, no direct evidence of this has been obtained [8,11,12]. Instead, the insertion-deletion of nucleotides in the HA gene was presumed to be another unique strategy of the influenza B viruses in the evolutionary pathway [11,13].

Since 1987, the influenza B viruses have been categorized as belonging to 2 distinct evolutionary lineages, the B/Victoria/2/87-like viruses (VI/87) and the B/Yamagata/16/88-like viruses (YA/88), according to the genetic variation in the HA gene [14-16]. These 2 lineages have been redefined into lineage II (YA/88) and lineage III (VI/87), whereas influenza B viruses identified earlier (isolates from 1940 to 1976) were redefined as lineage I [7]. Except for the early influenza B viruses, antigenic drift variants were identified from each lineage of influenza B virus every year. The accumulation of nucleotide point mutations in

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these variants prompted the classification of a sub-lineage for each major influenza B virus branch [12]. Furthermore, phylogenetic analysis also provided evidence of multiple lineages for the internal genes. For example, there were 3 lineages each in the matrix protein (M1) and nucleoprotein (NP) gene, and up to 4 lineages in the non-structural protein (NS) gene [7,12].

Influenza B viruses were isolated during 4 of the last 5 influenza seasons in Taiwan. Two of the major influenza B viruses, YA/88 and VI/87, were isolated during 1997. However, the YA/88-like viruses became dominant in the following seasons. Interestingly, viruses derived from B/Taiwan/3143/97 became dominant over the VI/87 lineage in the following seasons [17]. In this study, the HA and NS genes from influenza B viruses isolated in 1997 and from 1999 to 2001 were further characterized by using molecular techniques. The accumulation of these variants in the HA gene indicated that influenza B viruses continue to be prevalent in Taiwan and the accumulation of nucleotide mutations indicated that our isolates form a new cluster that evolved from the YA88 lineage.

Materials and Methods

Specimen collection and virus growth

Throat swabs were collected at the Department of Pediatrics of Taipei Veterans General Hospital and Chung Shan University Hospital, respectively. These specimens were processed using a previously described method [18]. The viruses were grown in the Madin-Darby canine kidney cell line.

RNA extraction

Virion RNA was extracted using the commercialized RNA isolation reagent, TRI REAGENT™LS (Molecular Research Center, Inc., Cincinnati, OH, USA) as described previously [19]. Briefly, 1 volume (0.25 mL) of virus-infected cells was mixed with 3 volumes (0.75 mL) of TRI REAGENT™LS. After the sample was homogenized, 0.2 mL of chloroform was added and the sample was shaken vigorously for 15 seconds and then stored at room temperature for 2 to 3 minutes. The resulting mixture was centrifuged at 12,000 *g* for 15 minutes. The aqueous phase was collected and viral RNA precipitated using isopropanol and then washed with 70% ethanol. Finally, the purified RNA was dissolved with 9 μL sterilized deionized water.

Reverse transcription and PCR

The cDNA synthesis and polymerase chain reaction (PCR) amplifications of the coding regions of the HA1 domains in the HA genes were carried out using primers corresponding to nucleotides 403-423 (5'-AATCTTCTCAGAGGATATGAA-3') and 961-937 (5'-GGCAATCTGCTTCACCAATTAAAGG-3'), yielding a 559-bp fragment, according to a previously described procedure [17]. The cDNA synthesis and PCR amplifications of the coding regions in the NS genes were carried out using primers corresponding to nucleotides 19-41 (5'-ATTTAGTCACTGGCAAA CGGAAAG-3') and 870-847 (5'-AAGAGATAAAGTT CTTCCGTGGCCAGT-3'), yielding an 897-bp fragment. Briefly, 9 μL of the RNA preparation was mixed with 16 μL of a buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$ and 0.02% gelatin, 1 mM each of the dNTP's, 2 units of RNase inhibitor, 50 pmol of oligonucleotide (primer 17) and 1 μL of avian myeloblastosis virus reverse transcriptase (AMV RT, RNase H minus; Promega, Madison, WI, USA) [5 to 10 units/μL]. The mixture was incubated at 42°C for 60 minutes to yield cDNA. For PCR, 100 μL of reaction mixture contained amplification buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.02% gelatin), 20 pmol each of the primers, 1 μL (4 units) of KlenTaq DNA polymerase (Clontech, Palo Alto, CA, USA) and 25 μL of cDNA solution. The amplification reaction was performed at 94°C for 0.5 of a minute, 55°C for 30 seconds, and 72°C for 1 minute for 30 cycles in a DNA thermal cycler (GeneAmp PCR System 2400; Perkin-Elmer, Foster City, CA, USA). One-tenth of the amplified product was applied to 1.5% agarose gel and stained with ethidium bromide. On agarose gel electrophoresis, the PCR products revealed a band of 562 base pairs visible under a UV illuminator.

Cloning and sequencing of HA genes

The PCR-derived dsDNA was ligated into the pGem-T vector (Promega) and transformed into *Escherichia coli* JM109. The positive clones were selected and cultured in L-broth containing 100 μg/mL ampicillin (Sigma, St. Louis, MO, USA) and incubated at 37°C overnight. The bacteria were centrifuged at 3000 rpm for 15 minutes. The pellet was treated with the Wizard™ Minipreps DNA Purification System (Promega) to extract the plasmid DNA used as a template for automated sequencing on an Applied Biosystem 373A automated DNA sequencer using cycle sequencing dye terminator chemistry (Perkin-Elmer). T7 and

Sp6 primers were used to sequence the HA1 domain of the HA gene and NS gene, respectively.

Sequence data and phylogenetic analyses

Nucleotide sequences, prediction of amino acid sequences, alignments, pairwise distances, number of amino acid differences, and phylogenetic tree construction were completed using MEGA@ software, version 2.01 [20]. Phylogenetic tree construction was based on the neighbor-joining method and bootstrap analysis (n = 1000) to determine the best-fitting tree for the HA1 and NS1 genes [21]. In addition to the sequence data determined in this study, previously reported nucleotide sequences for the HA gene [4,10,13,14,16, 22-30] and NS [7,14,31] gene were also used in phylogenetic tree construction. The abbreviations used for the influenza B virus strains are as follows: B/Lee/1/40 (Lee40), B/Great Lakes/54 (GL54), B/Maryland/59 (ML59), B/Singapore/64 (SG64), B/Ann Arbor/1/66 (AA66), B/Russia/69 (RU69), B/Hongkong/8/73 (HK73), B/Baylor/4/78 (BA78), B/Paris/1/79 (PS79), B/Singapore/222/79 (SG79), B/Oregon/5/80 (OR80), B/USSR/100/83 (USSR83), B/Houston/18513/84 (HT84), B/Norway/1/84 (NOR84), B/Ibaraki/2/85 (IBA85), B/Georgia/1/86 (GA86), B/Idaho/1/86 (ID86), B/Memphis/6/86 (MS86), B/Ann Arbor/1/86 (AA86), B/Victoria/2/87 (VI87), B/Beijing/1/87 (BJ87), B/Aichi/5/88 (AIC88), B/Ohio/10/88 (OH88), B/Taiwan/7/88 (TW88), B/Yamagata/16/88 (YA88), B/Guangdong/55/89 (GD89), B/Hongkong/22/89 (HK22/89), B/Hongkong/9/89 (HK9/89), B/Victoria/19/89 (VI89), B/Bangkok/163/90 (BK90), B/Panama/45/90 (PN90), B/Paris/329/90 (PS90), B/Texas/4/90 (TX90), B/Czechoslovakia/69/90 (CS90), B/Stockholm/10/90 (SH90), B/Switzerland/5241/90 (SW90), B/Finland/172/91 (FL91), B/Khazkov/224/91 (KK91), B/Leningrad/148/91 (LG91), B/Beijing/184/93 (BJ93), B/Finland/268/93 (FL93), B/Guangdong/8/93 (GD93), B/Mie/1/93 (MIE93), B/Guangdong/5/94 (GD94), B/Harbin/7/94 (HAR94), B/Argentina/218/97 (ARG97), B/Beijing/243/97 (BJ97) B/Henan/22/97 (HEN97), B/Chiba/447/98 (CHI98), B/Nagano/2038/98 (NAG98), B/Shiga/T30/98 (SHI30/98), B/Yamanashi/166/98 (YAM98), B/Sichuan/379/99 (SC99), B/Seal/Netherland/1/99 (SealNL99).

Nucleotide sequence accession numbers

The nucleotide sequence data determined in this study will appear in DDBJ, EMBL, ISD and NCBI sequence databases under the accession numbers listed in Table 1.

Results

Comparative analysis of nucleotides of the HA1 domain

Influenza B viruses isolated in 1997, and from 1999 to 2001 are listed in Table 1. Despite B/Taiwan/3143/97 and B/Taiwan/21706/97 having been previously described,

Table 1. Influenza B virus isolates and genes sequenced in this study

Strain	Abbreviation	Accession number	
		HA gene	NS1 gene
B/Taiwan/3143/97	TW3143/97	AF026161	AF492478
B/Taiwan/21706/97	TW21706/97	AF026162	AF492479
B/Taiwan/1243/99	TW1243/99	AF363979	AF380504
B/Taiwan/2026/99	TW2026/99	AF148886	AF492481
B/Taiwan/2027/99	TW2027/99	AF148887	AF492480
B/Taiwan/2195/99	TW2195/99	AF148888	AF492482
B/Taiwan/1265/2000	TW1265/2000	AF363983	AF380508
B/Taiwan/1293/2000	TW1293/2000	AF492477	AF380509
B/Taiwan/4184/2000	TW4184/2000	AF363982	AF380507
B/Taiwan/12192/2000	TW12192/2000	AF363984	NA
B/Taiwan/31511/2000	TW31511/2000	AF363980	AF380505
B/Taiwan/41010/2000	TW41010/2000	AF363981	AF380506
B/Taiwan/114/2001	TW114/2001	AF492476	NA
B/Taiwan/202/2001	TW202/2001	AF366076	AF380512
B/Taiwan/1103/2001	TW1103/2001	AF363985	AF380510
B/Taiwan/2805/2001	TW2805/2001	AF400581	NA
B/Taiwan/11515/2001	TW11515/2001	AF366075	AF380511

Abbreviation: NA = not available

	110	120	130	140	150	160	170	180	190
VI87	NLLRGYEHIR	LSTHNVINAE	TAPGGPYKVG	TSGSCPNVTN	GNGFFATMAW	AVPKNDNNKT	ATNPLTVEVP	YICTEGEDQI	TVWGFHSDSE
YA88	.....N..	.....	R.....RL.	.....S	R.....	...RD--...	.....	....K.....	.....DK
FL93	.....	.....	R.....RL.	.....S	RS.....	...RD--...	.....	....K.....	.....NK
TW3143/97	.....R.T	...P...S..	K.....FNL.	.....A..	RS.....	...RD-....	.....	H..SKE....	.....NK
TW21706/97	.....R..	..N.....	K.....C.I.	.....	.....	.....E....	...S..I...	.....	.....N.
TW2026/99	.....K..	..**A**Q.....	K.....RL.	.....ANS	KS.....	...RD-....	.....	H...KE....	.....NK
TW2027/99	.....K..	..**A**Q.....	K.....RL.	.....ANS	KS.....	...RD-....	.....	H...KE....	.....NK
TW2195/99	.....K..	..**A**Q.....	K.....RL.	.....A.S	RS.....	...RD-....	.....	H...KE....	.....NK
TW1243/99	.....K..	...Q...II.	K.....RL.	.....A.S	RS.....	...RD-....	.....	H...KE....	.....NK
TW31511/2000	.....K..	...Q.....	K.....RL.	.....A.S	KS.....	...RD-....	.....I...	H...KE....	.....NK
TW41010/2000	.....K..	...Q.....	K.....RL.	.....A.S	KS.....	...RD-....	.....	H...KE....	.....DK
TW4184/2000	.....K..	...Q.....	K.....RL.	.....A.S	KS.....	...RD-....	.....G..	H...KE....	.....NK
TW1265/2000	.....K..	...Q...**D**..	K.....RL.	.....A.S	KS.....	...RE-....	.....	H...KE....	.....NK
TW1293/2000	.....K..	...Q...**D**..	K.....RL.	.....A.S	KS.....	...RE-....	.....	H...KE....	.....NK
TW12192/2000	.....K..	...Q...**D**..	K.....RL.	.....A.S	KS.....	...RE-....	.....	H...KE.V..	.....NK
TW114/2001	.....K..	...Q.....	K.....RL.	.....A.S	KS.....	...RD-....	.....	H...KE....	.....NK
TW202/2001	.....K..	...Q.....	K.....RL.	.....A.S	KS.....	...RD-....	.....	H...KE....	.....NK
TW11515/2001	.....K..	...Q...**D**..	K.....RL.	.....A.S	KS.....	...RE-....	.....	H...KE....	.....NK
TW1103/2001	.....K..	...Q.....	K.....RL.	.....A.S	KS.....	...RD-....	.....I...	H...KE....	.....NK
TW2805/2001	.....K..	...Q.....	K.....RL.	.....A.S	KS.....	...RD-....	.....	H...KE....	.....NK
BJ93	.....N..	...Q.....	K.....RL.	.....A.S	RS.....	...RD-....	.....	....K.....	.....NK
YAM98	.....K..	...Q.....	K.....RL.	.....A.S	RS.....	....-.....	.....	H...KE....	.....NK
SC99	.....K..	...Q.....	K.....RL.	.....A.S	KS.....	...R-.....	.....	H...KE....	.....NK
SealNL99	.....N..	...Q.....	K.....RL.	.....A.S	RS.....	...R-.....	.....	.V.....	.....NK

Fig. 1. Comparison of the partial HA1 amino acid sequences from residues 109 to 295 of the influenza B viruses. Dots and dashes

Table 2. Pairwise nucleotide distances and difference in number of amino acids between HA1 genes among influenza B virus strains[a]

	VI87	YA88	FL93	3143/97	21706/97	2026/99	2027/99	2195/99	1243/99	31511/00	41010/00	4184/00
VI87	-	17	17	41	11	25	25	23	24	24	23	25
YA88	7.1	-	5	34	21	16	16	11	14	15	13	16
FL93	7.3	2.2	-	30	21	12	12	9	10	11	11	12
3143/97	15.1	10.3	10.3	-	41	28	28	27	25	27	27	28
21706/97	4.1	9.4	9.2	16.2	-	26	26	24	26	24	26	27
2026/99	10.4	5.1	5.1	7.6	12.7	-	0	5	6	3	3	4
2027/99	10.4	5.1	5.1	7.6	12.7	0	-	5	6	3	3	4
2195/99	9.4	3.7	4.1	7.0	11.6	1.3	1.3	-	5	6	6	7
1243/99	10.0	4.7	4.7	7.4	12.2	2.2	2.2	1.6	-	5	5	6
31511/00	10.7	5.3	5.3	8.2	12.4	1.3	1.3	2.2	2.4	-	2	3
41010/00	10.5	4.7	5.1	7.6	12.7	0.7	0.7	1.7	2.2	1.3	-	3
4184/00	10.8	5.5	5.5	8.2	13.1	1.3	1.3	2.2	2.8	1.8	0.9	-
1265/00	11.1	5.7	5.7	7.6	13.4	1.3	1.3	2.2	2.8	1.8	0.9	1.5
1293/00	11.1	5.7	5.7	8.0	13.4	1.3	1.3	2.2	2.8	1.8	0.9	1.5
12192/00	11.7	5.9	5.9	8.6	14.0	1.8	1.8	2.8	3.3	2.4	1.5	2.0
114/01	10.2	4.9	4.9	7.4	12.4	0.5	0.5	1.5	2.0	1.1	0.4	0.9
202/01	10.2	4.9	4.9	7.4	12.4	0.5	0.5	1.5	2.0	1.1	0.4	0.9
11515/01	11.1	5.9	5.5	8.2	13.4	1.5	1.5	2.4	3.0	2.0	1.1	1.6
1103/01	11.3	6.3	6.3	9.0	13.6	2.0	2.0	3.0	3.5	2.2	1.7	1.8
2805/01	10.2	4.9	4.9	7.4	12.4	0.5	0.5	1.5	2.0	1.1	0.4	0.9
BJ93	8.5	3.3	3.4	8.0	10.7	2.8	2.8	2.2	2.4	3.0	2.8	3.3
YAM98	9.0	4.1	4.1	6.6	11.1	1.3	1.3	0.7	1.3	1.8	1.3	1.8
SC99	10.4	5.1	5.1	7.6	12.7	0.7	0.7	1.6	2.2	1.3	0.4	0.9
SealNL99	8.8	3.5	3.2	8.4	10.5	3.0	3.0	2.4	2.6	3.2	3.0	3.5

[a]Pairwise distances are presented as percentages in a triangular matrix. Nucleotide distances are presented in the lower half and amino acid

200	210	220	230	240	250	260	270	280	290
TQMVKLYGDS	KPQKFTSSAN	GVTTHYVSQI	GGFPNQAEDG	GLPQSGRIVV	DYMVQKSGKT	GTITYQRGIL	LPQKVWCASG	RSKVIKGSLP	LIGEADC
...K.....	N.....	.....	.D....T...	.....	......P...	...V....V.	.....	.....	.....
...KN.....	N.....	.....	......T...	.....	......P...	...V....V.	.....	.....	.....
.P.KN.....	N.....	.....H.	....DHT..R	...P....I.	..L...P...	R..VSL....	..H.....	Q.P.LE....	....P..
...A.....	N.....	.....	......T...	.....	.....	.....S..	.....	.....	.....
...KN.....	N.....	.I.....	....D.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.I.....	....D.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.....	.D....T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.....	....D.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.I.....	....D.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.I.....	....D.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.I.....	A...D.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.I.....	....D.T...	.....	......P...	...V.....	.....	.....	.....
...KN...G.	N.....	.I.....	....D.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.I.....	....D.T...	.....	......P...	...V....V.	.....	.....	.....
...KN.....	N.....	.I.....	....E.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.I.....	....E.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.I.....	....D.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.I.....	.S..D.T...	.....	.....RP...	...V.....	.....	.....F..	.....
...KN.....	N.....	.I.....	....E.T...	.....	......P...	...V.....	.....	.....	.....
I..KN.....	N.....	.....	....D.T...	.....	......P...	...V....V.	.....	.....	.....
...KN.....	N.....	.....	....D.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.I.....	....D.T...	.....	......P...	...V.....	.....	.....	.....
...KN.....	N.....	.....	....D.T...	.....	......P...	...V....V.	.....	.....	.....

indicate amino acids that are identical to those of B/Lee/1/40 and deletions, respectively.

1265/00	1293/00	12192/00	114/01	202/01	11515/01	1103/01	2805/01	BJ93	YAM98	SC99	SealNL99
24	25	27	23	23	24	27	23	22	21	23	21
15	16	16	14	14	15	17	14	9	13	14	10
11	12	12	10	10	11	14	10	6	9	10	7
26	27	29	27	27	26	30	27	28	25	27	29
26	27	29	25	25	26	27	25	24	23	25	23
3	4	6	3	3	3	6	3	9	5	3	10
3	4	6	3	3	3	6	3	9	5	3	10
6	7	9	5	5	6	8	5	8	4	6	9
4	5	7	5	5	4	8	5	7	3	5	8
2	3	5	2	2	2	3	2	8	4	2	9
2	3	5	2	2	2	5	2	8	4	2	9
3	4	6	3	3	3	6	3	9	5	3	10
-	1	3	2	2	0	5	2	8	4	2	9
0.4	-	4	3	3	1	6	3	9	5	3	10
0.9	0.9	-	5	5	3	8	5	9	7	5	10
0.9	0.9	1.5	-	0	2	5	0	8	4	2	9
0.9	0.9	1.5	0	-	2	5	0	8	4	2	9
0.5	0.5	1.1	1.1	1.1	-	5	2	8	4	2	9
2.2	2.2	2.8	1.6	1.6	2.4	-	5	11	7	5	12
0.9	0.9	1.5	0	0	1.1	1.6	-	8	4	2	9
3.4	3.4	3.5	2.6	2.6	3.5	4.1	2.6	-	6	8	3
1.8	1.8	2.4	1.1	1.1	2.0	2.6	1.1	1.8	-	4	7
0.9	0.9	1.5	0.4	0.4	1.1	1.6	0.4	2.8	1.3	-	9
3.5	3.5	3.7	2.8	2.8	3.7	4.3	2.8	1.3	2.0	3.0	-

differences in the upper half.

15 additional strains were identified in this study. The deduced amino acid sequences of partial HA1 domain of our isolates were aligned with the reference strains such as Lee40, VI87, YA88, TW88, and recommended vaccine strains such as BJ93, YAM98, and SC99. In addition, these sequences were also compared with that of SealNL99. Amino acid changes in the HA1 domain were found to distribute throughout the sequences (Fig. 1). Pairwise distances between the nucleotide and number of amino acid differences in the HA1 domain are shown in Table 2. While the distance among VI87, YA88, and FL93 (a YA88-like virus) ranged from 7.1 to 7.3%, the distance increased chronologically, ranging from 9.4 to 11.7% in 1999 to 2001 isolates. VI87 differed from YA88 in 17 amino acids. The difference increased to 24 amino acids when compared to our isolates. Interestingly, the difference between YA88 and our isolates ranged from only 11 to 17 amino acids.

Phylogenetic analyses

The nucleotide sequences of the coding regions of the partial HA1 domains of HA genes obtained from viruses isolated in 1997, and from 1999 to 2001, were analyzed with the previously reported strains to show the genealogy of the HA1 genes. Based on the number of nucleotide substitutions, similarities among the HA1 genes from 54 viruses (15 new and 39 previously reported sequences) were calculated. The topology of the phylogenetic tree shown in Fig. 2 indicates that the 2 main lineages, YA/88-like (II) and VI/87-like (III) HA lineages, continue to exist. Particularly, 14 out of 15 isolates in Taiwan formed a cluster that originated from the YA/88 lineage. More importantly, the formerly recommended vaccine strain, BJ93, became the new ancestor of this cluster to which YAM98 and SC99 now belong.

To verify whether NS genes have a similar genealogy to that of HA1 genes, we also analyzed the nucleotide sequences of the coding regions for the NS1 protein of NS genes from 46 viruses isolated from 1940 to 2001 (14 new and 32 previously reported sequences). The topology of this tree indicated that there were 4 NS1 gene lineages in the influenza B virus. In contrast to the HA gene, it is interesting that the NS1 genes from all of our isolates obtained from 1997 to 2001 were related to lineage IV (Fig. 3).

Discussion

During 1997, 2 strains of influenza B virus, B/Taiwan/21706/97 and B/Taiwan/3143/97, were isolated and characterized in our laboratory. The phylogenetic data for the HA gene showed that B/Taiwan/21706/97 belonged to the VI87 lineage. In contrast, B/Taiwan/3143/97 belonged to the YA88 lineage, which was very different from the YA88-like viruses [17]. The influenza B virus strains continued to prevail during the successive years.

Some of our isolates were sent to the Centers for Diseases Control, Atlanta, USA for further verification of their antigenicity. The hemagglutination-inhibition test results indicated that B/Taiwan/2027/99 and B/Taiwan/2195/99 were BJ93-like; B/Taiwan/1265/2000, B/Taiwan/1103/2001, and B/Taiwan/11515/2001 were SC99-like, but were partially cross-reacted with BJ93 and YAM98 [data not shown]. These results revealed that the influenza B virus continued to drift in Taiwan. The predicted amino acid alignment data also showed that 3 of the 4 isolates in 1999 (B/Taiwan/2026/99, B/Taiwan/2027/99, and B/Taiwan/2195/99) changed an amino acid residue from alanine into threonine at position 121, while 3 out of the 6 isolates in 2000 (B/Taiwan/12192/2000, B/Taiwan/1265/2000, and B/Taiwan/1293/2000) and 1 out of the five 2001 isolates (B/Taiwan/11515/2001) changed an amino acid residue from asparagine into aspartic acid at position 126. The amino acid residue at position 121 was predicted to be a part of the virus receptor binding site [32].

In addition, both residues at position 121 and 126 have been shown to be located at the globular head structure of the HA protein in the influenza B virus. These variations may cause changes in the antigenicity and receptor-binding affinity among the 1999 to 2001 isolates [4,32]. Furthermore, the serologic data and amino acid sequence alignment results were generally in agreement with the findings of phylogenetic analysis.

Because of the rapid antigenic drift in the influenza B virus, the virus was examined every year for its potential use in producing an efficient vaccine. For example, BJ93-like and B/Shandong/7/97-like viruses have been used to produce vaccine globally since 1999 to 2000. The vaccine strains were changed into YAM98-like and SC99-like viruses in 2001 for vaccine preparation in the Northern and Southern Hemispheres, respectively. Comparing our isolates with the vaccine strains, our data showed that the strains isolated before 2000 were genetically close to the BJ93-like viruses. In contrast, we also showed that the strains isolated in 2001 were closer to SC99-like viruses, even though the government authority chose YAM98-like as the vaccine strain in Taiwan during that period.

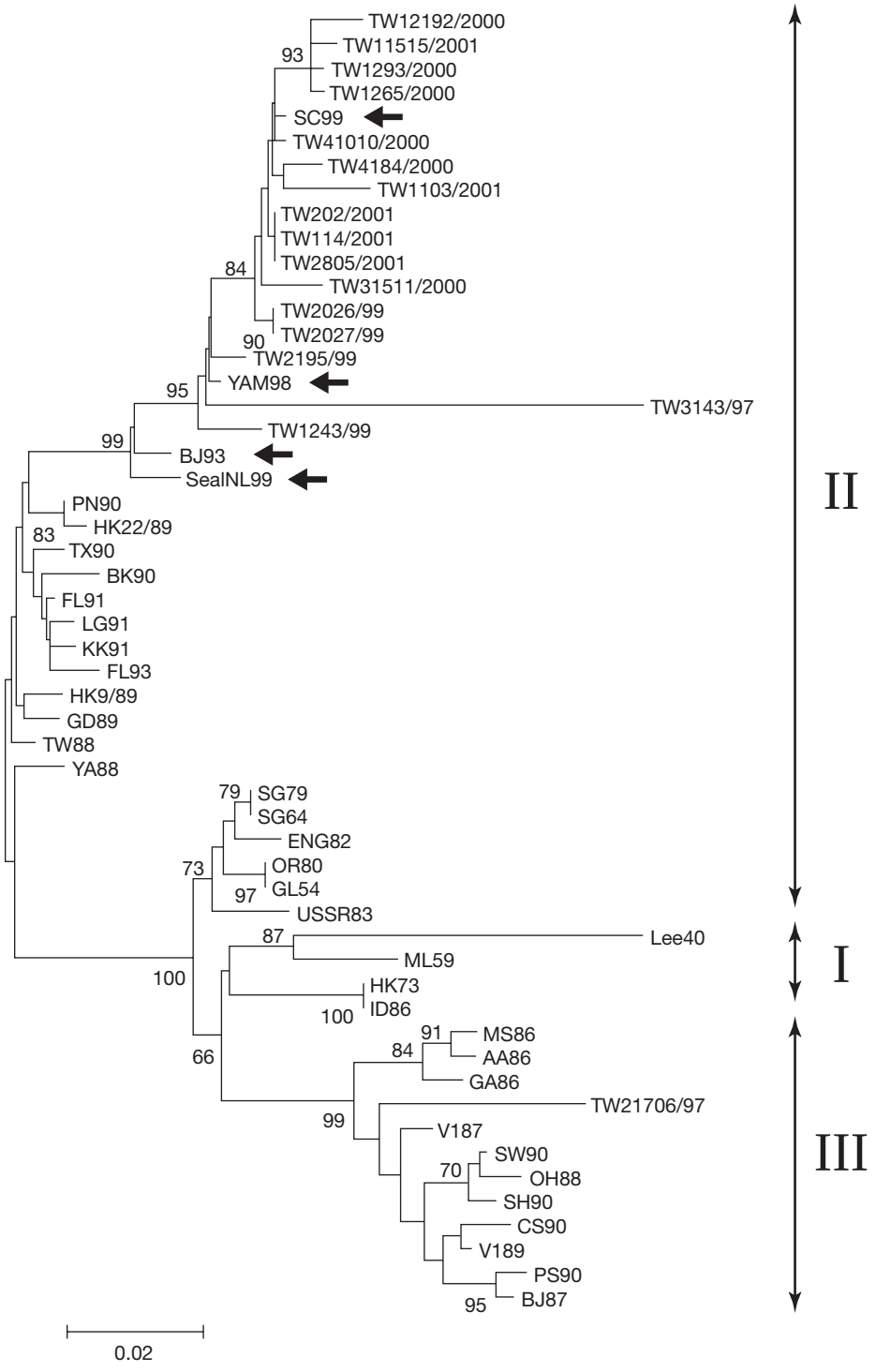

Fig. 2. Evolutionary tree for HA1 genes of the influenza B virus isolates from 1940 to 2001. The tree was constructed using the neighbor-joining method with bootstrap-resampling (n = 1000) as described in the text. The number at each branch point indicates the percentage probability that the resultant topology is correct. The lengths of the horizontal lines are proportional to the nucleotide changes between sequences. Vertical lines separate progeny virus lineages at the point where they branch from a theoretical common ancestor. Vaccine recommended strains and B/Seal/Netherland/1/99 are represented by arrows and an arrowhead, respectively.

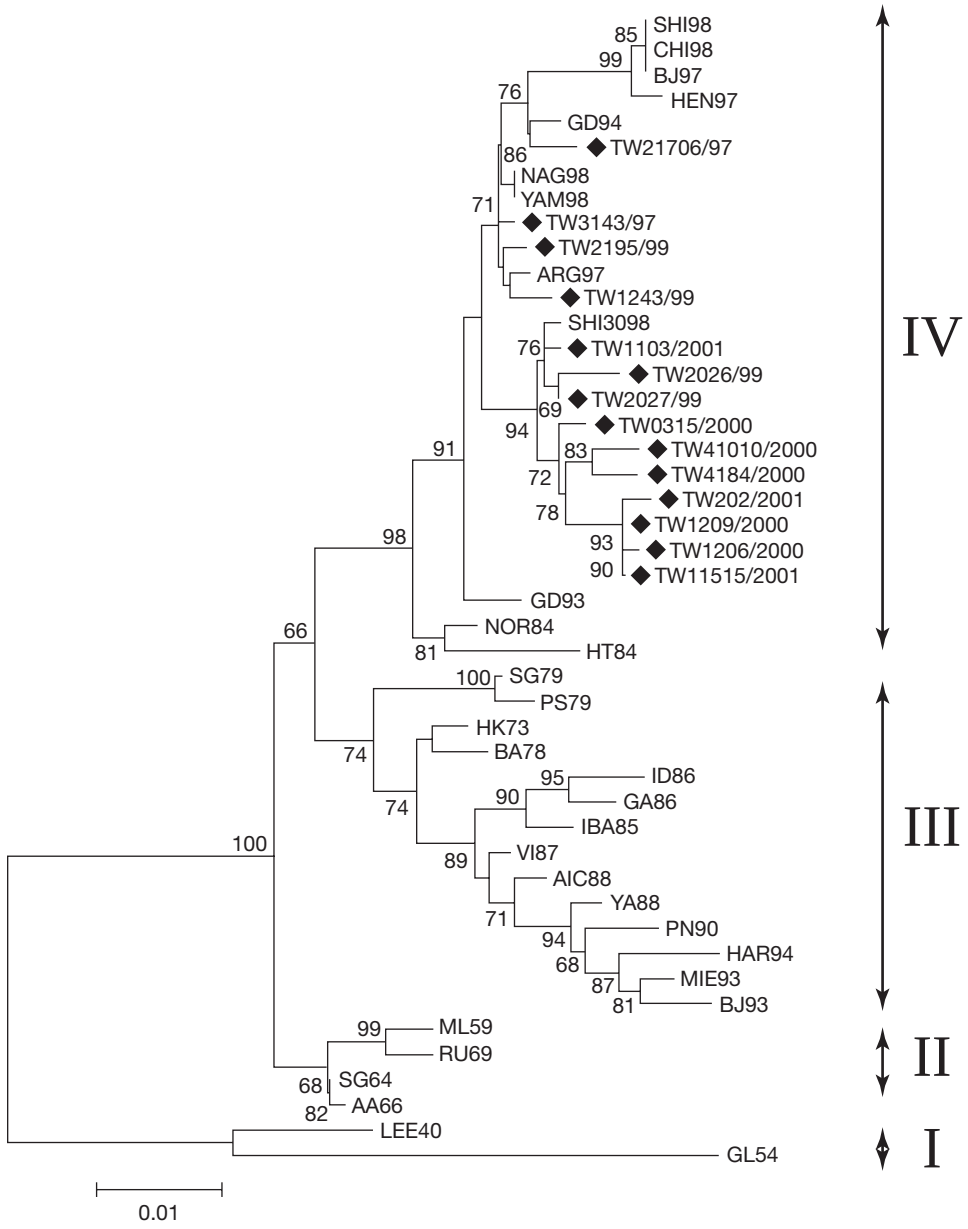

Fig. 3. Evolutionary tree for NS1 genes of the influenza B virus isolates from 1940 to 2001. The tree was constructed using the neighbor-joining method with bootstrap-resampling (n = 1000) as described in the text. The number at each branch point indicates the percentage probability that the resultant topology is correct. The lengths of the horizontal lines are proportional to the nucleotide changes between sequences. Vertical lines separate progeny virus lineages at the point where they branch from a theoretical common ancestor. Influenza B virus strains isolated during 1997 to 2002 are represented by diamonds.

The nucleotide distance between the YA88-like and VI87-like virus ranged from approximately 7 to 9% (Table 2). The distances between VI87-like viruses and our isolates increased from 9.4 to 11.7%. Comparing our isolates with the YA88-like viruses, the distances ranged from 3.7 to 6.3%. The high variation among our isolates suggests they should be categorized as a new cluster or sublineage in the phylogenetic tree.

The occurrence of frequent genetic reassortment of the influenza B virus internal genes has been hypothesized, although direct evidence has yet to be found [8,11,12]. Reassortment was suggested by phylogenetic analysis in BJ93 and B/Taiwan/21706/97 in this study. While the HA gene of BJ93 and other recommended vaccine strains (YAM98 and SC99) belonged to YA88 (lineage II), the NS1 gene of BJ93

belonged to lineage III. Similar reassortment patterns occurred in B/Taiwan/21706/97. The HA gene of B/Taiwan/21706/97 belonged to lineage III and the NS1 gene belonged to lineage IV. In contrast, no evidence of genetic reassortment was found in our 1999 to 2001 isolates.

The host range for the influenza B virus as well as its potential to infect other non-human species is still unclear. Although influenza B virus infection in harbor seals has been reported, the mechanism by which the virus was introduced from human beings into harbor seals or vice versa is still unknown [10]. Positive serological analyses in the seal population indicated that seals may constitute an animal reservoir of the virus. It is noted that the HA1 gene of BJ93 and SealNL99 were similar in the phylogenetic constellation. Further study is needed to determine the interspecies transmission mechanism of the influenza B virus.

In conclusion, the accumulation of nucleotide mutations indicated that our isolates form a new cluster that evolved from the YA88 lineage. Furthermore, BJ93 is proposed as the ancestor of this new cluster of viruses.

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