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S1 and N Gene Analysis of Avian Infectious Bronchitis Viruses in Taiwan

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SUMMARY. The disease caused by infectious bronchitis virus (IBV) produces great economic loss for the poultry industry. The purpose of this study is to investigate the molecular epidemiology of IBV in Taiwan. An old IBV strain isolated in 1964 and another 31 strains isolated from 1991 to 2003 were selected for N-terminal S1 gene analysis. Based on their phylogenetic tree, 13 strains were selected for sequencing the entire S1 and partial nucleocapsid (N) genes. The results indicated that Taiwanese IBV strains could be divided into two distinct lineages, Taiwan Group I and Taiwan Group II, with one Massachusetts strain and one Chinese strain. No recombination was found between H120 and the Taiwanese strains in the S1 gene. However, the S1 gene showed a noticeably higher divergence than the N gene. The phylogenetic trees constructed from the S1 and N genes indicate that intergenic recombination has occurred. Since most local strains are in Taiwanese clusters, developing vaccines from local strains is necessary for IBV control in Taiwan.

RESUMEN. Análisis de los genes S1 y N de cepas del virus de bronquitis infecciosa en Taiwan.

La enfermedad ocasionada por el virus de la bronquitis infecciosa ocasiona grandes pérdidas económicas a la industria avícola. El objetivo de este estudio es investigar la epidemiología molecular del virus de la bronquitis infecciosa en Taiwan. Se seleccionó una cepa del virus aislada en 1964 y 31 cepas aisladas entre los años 1991 y 2003, para analizar la zona terminal amino de sus genes S1. Tomando como base su árbol filogenético, se seleccionaron 13 cepas del virus con el fin de secuenciar la totalidad de sus genes S1 y parcialmente sus genes N. Los resultados indican que las cepas del virus de la bronquitis infecciosa en Taiwan pueden ser divididas en dos grupos diferentes, Taiwan I y Taiwan II, este último con una cepa tipo Massachussets y una cepa China. No se observaron recombinaciones en el gen S1 entre la cepa H120 y las cepas Taiwanesas. Sin embargo, se observó una divergencia mucho mayor en el gen S1 que en el gen N. Los árboles filogenéticos construidos con los genes S1 y N mostraron la presencia de recombinaciones a nivel de las secuencias intergénicas. Debido a que la mayoría de las cepas locales se encuentran en los grupos Taiwaneses, es necesario desarrollar vacunas con cepas locales para controlar el virus de la bronquitis infecciosa en Taiwan.

Key words: avian infectious bronchitis, coronavirus, S1 gene

Abbreviations: IBV = infectious bronchitis virus; Mass = Massachusetts; N = nucleocapsid; RT-PCR = reverse transcription–polymerase chain reaction; TW I = Taiwan Group I; TW II = Taiwan Group II

Infectious bronchitis virus (IBV), with a large positive-sense RNA genome of 27.6 kb, is a prototype of the *Coronaviridae* virus family (3,9). IBV is an important pathogen in poultry. Despite routine vaccine use, variant strains continue to cause outbreaks in field situations (4). The continuing emergence of new strains may be explained in part by the apparent common occurrence in nature of recombination between IBV strains (7,8,10,18).

Like most RNA viruses, the coronavirus has a high mutation frequency that involves discontinuous transcription and polymerase jumping (10). Several point mutations can be accumulated during each round of RNA replication. Thus, even a plaque-purified coronavirus stock would include a population

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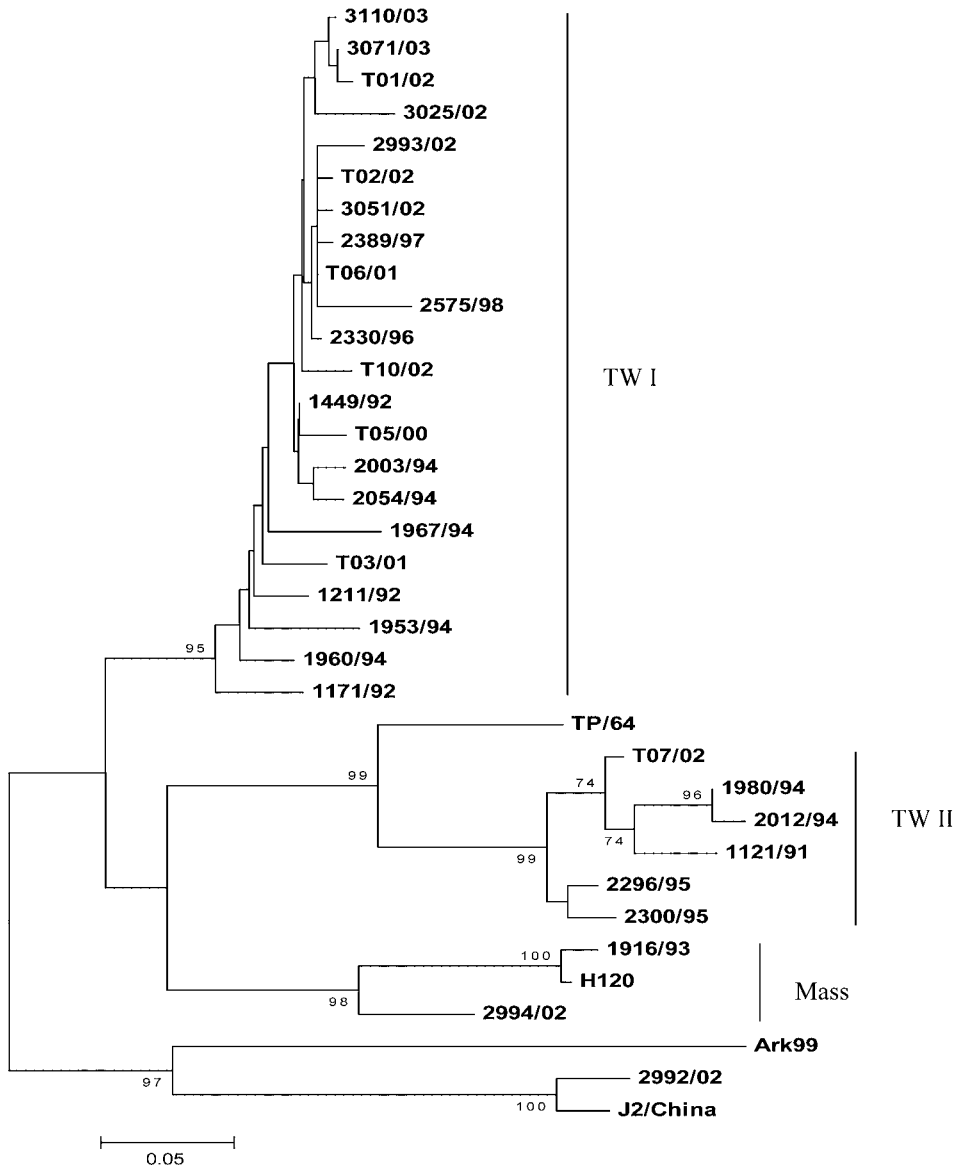

Fig. 1. Phylogenetic tree of Taiwanese IBV and reference strains (H120, J2/China, and Ark99) based on the N terminus of the S1 gene (rC2U-rC3L region). Most Taiwanese strains are grouped into Taiwan Group I (TWI); some are in Taiwan Group II (TW II). Only one strain is in Mass group, except one vaccine strain, 1916/93.

of related quasi-species rather than homogeneous virus. For example, the recombination frequency of the entire mouse hepatitis virus reaches 25% (1).

Vaccination with Massachusetts (Mass) group strains for IBV control in chickens is very common in Taiwan. These vaccine strains are suggested to provide a large donor pool for IBV recombination. The importance of this pool has not been determined, although recombination and mutation have been confirmed by several reports (7,8,9,10,18). IB has occurred frequently in Taiwan in spite of vaccination (12,14,15,16,17) since it first appeared in 1965 (12). The reason for vaccination breaks has been due to different serotypes among imported

vaccine strains and Taiwan-filed IBVs. However, recombination evidence in Taiwanese IBVs is lacking. The aim of this project is to determine the recombination of IBV in Taiwan during the past 10 yr.

MATERIALS AND METHODS

Virus strains. One IBV strain, TP/64, isolated from layers with respiratory signs and egg drop in Taipei in 1964, and from 31 strains isolated from chickens with nephropathogenic IBV infection in Taiwan during 1991 to 2003 were used in this study (Fig. 1). IBV 1121/91 was isolated in 1991, and IBV 3110/03 was isolated in 2003. Thirteen of these strains were selected for the entire S1 gene and partial N gene sequencing (Table 1). Viruses were propagated in specific-pathogen-free chicken embryos (Animal Health Research Institute, Council of Agriculture, Tamsui). The allantoic fluid was harvested 48–72 hr after inoculation, frozen, and stored at −70 C.

N terminus sequences of the S1 IBV gene. For each strain from the field, the hypervariable region 1 in the N terminus of the S1 gene was amplified with a primer set, rC2U/rC3L, modified by Tseng (13) from C2U/C3L by Wang *et al.* (16,17). The sequences for these primers were rC2U (forward): 5′-TGGTT GGCA(T/C) TTACA (A/C/T)GG(A/G/T)-3′ and rC3L (reverse): 5′-(A/G)CAAT GTGTA ACAAA (T/C) ACT-3′. The size of the expected reverse transcription–polymerase chain reaction (RT-PCR) product was 231 bp. The rC2U-rC3L fragment from nt 114 to nt 341 in the N terminus of the S1 gene was selected because the phylogenetic tree based on this fragment is similar to that based on the entire S1 gene (16).

Complete S1 IBV sequences. Thirteen strains selected from the phylogenetic tree based on the rC2U-rC3L fragment, the virus-isolated year, and farm locations were used for sequencing the entire S1 IBV gene (Table 2). The primer, Oligo5′/IBVc2 primer was used for amplifying the entire S1 segment. The RT-PCR products were double sequenced from both sites (all together, at least four times). The primer set sequences were oligo5′: 5′-AAACT GAACA AAAGA CAGAC TTAG-3′ (20305-20328) and IBVc2: 5′-GCCAT AACTA ACATA TGGAC AAC-3′ (22023-22001).

Partial N IBV sequences. The partial N gene of the 13 selected strains was sequenced using the following primers (12). The primer sequences for this gene were NP1: 5′-GGTA G(C/T)GG(C/T) GTTCC TGATA A-3′ (26029-26048) and NP2: 5′-TCATC TTGTC (A/G)TCAC CAAAA-3′ (26647-26628), which were conserved among 34 different IBVs (data not shown). The NP1-NP2 fragment was from nt 157 to nt 775 in the N gene. The RT-PCR products produced with this primer set were sequenced and compared as described previously. For the above RT-PCR products, each region was sequenced at least twice from at least two RT-PCR products to make sure that the sequences were identical.

Recombination among IBVs. The entire S1 gene sequences for the selected 13 IBV strains obtained by direct sequencing from RT-PCR products, except IBV 1171/92 and IBV 1211/92, were used for checking recombination between H120 and the Taiwanese strains. The sequence CTT(A/T)(A/T)G found in the S1 IBV gene was similar to the consensus leader sequence used as the fragment junction for identifying the cross-over sites. These fragments were nt 1–30, 31–136, 137–1057, and 1058–1620. The phylogenetic trees constructed from the sequences for the S and N genes were used to compare the individual strain clustering for interstrain recombination.

Phylogenetic analysis. DNA sequences were compiled and edited using the Vector NTI software package. Phylogenetic trees were based on the N terminus of the S gene; the entire S1 gene and partial N gene were constructed using Neighbor-Joining and bootstrap analysis ($n = 2000$) to determine the best-fitting tree for each gene.

GenBank accession numbers. GenBank accession numbers for the genes submitted through this study included 2575/98 S1, AY606314; T03/01 S1, AY606315; 2993/02 S1, AY606316; 3025/02 S1, AY606317; 3051/02 S1, AY606318; 3071/03 S1, AY606319; TP/64 S1, AY606320; 2296/95 S1, AY606321; T07/02 S1, AY606322; 2992/02 S1, AY606323; 2994/02 S1, AY606324; 1171/92 N, AY606325; 1211/92 N, AY606326; 2575/98 N, AY606327; T03/01 N, AY606328; 2993/02 N, AY606329; 3025/02 N, AY606330; 3051/02 N, AY606331; 3071/03 N, AY606332; TP/64 N, AY606333; 2296/95 N, AY606334; T07/02 N, AY606335; 2992/02 N, AY606336; and 2994/02 N, AY606337. GenBank accession numbers for the reference IBVs included H120 S1, M21970; J2/China S1, AF286303; Ark99 S1, L10384; 1171/92 S1, AF250005; 1211/92 S1, AF250006; H120 N, AY028296; and Ark99 N, M85244.

RESULTS

Comparison of N terminus sequences of the S1 IBV gene. The phylogenetic tree based on the N terminus sequences of the S gene showed that Taiwanese IBVs were divided into two lineages, Taiwan Group I (TW I) and Taiwan Group II (TW II), except 1916/93, 2992/02, and 2994/02 (Fig. 1). Most Taiwanese strains belonged to TW I and only a few belonged to TW II. IBV 1916/93 was considered a vaccine strain (17). IBV 2994/02 was a Mass group field strain. A new strain, IBV 2992/02 strain, whose S sequence was quite similar to that

Table 1. Amino acid sequences in the N terminal region of the S1 gene in Taiwanese IBV strains and H120, J2/China, and Ark99 reference strains.

Strain	Residue 51	60	70	80	90
1171/92	N V S L E T N N A G A A S E C T I G I I S G G S G F N A S S I A M T A P V G P G M Q W S K S
1211/92	N V S S E T N N A G S A S E C T V G T I R G D R V V N A S S I A M T A P V G Q G M Q W S K S
1449/92	N V S S Q T N N A G T A Q E C T V G I I S G D R V V N A S S I A M T A P V G Q G M Q W S K S
1953/94	N V S S E T N N A D T R S E C T V G I I S G D R V V N A S S I A M T T P V G Q G M N W S K S
1960/94	N V S S E T N N A G S R S A C T V G I I S G G R I V N A S S I A M T V P V G Q G M Q W S K S
1967/94	N V S S E T N N A G H R S E C T V G I F R G D R V V N A S S I A M T A P V G H G M G W S K S
2003/94	N V S S Q T N N A G T A Q G C T V G I I S G D R V V N A S S I A M T A P V G Q G M Q W S K S
2054/94	N V S S E T N N A G T R Q E C T V G I I S G D R V V N A S S I A M T A P V G Q G M Q W S R S
2330/96	N V S S Q T N N A G T A Q E C T V G I I S G D R V V N A S S I A M T A P V G Q G M Q W S K L
2389/97	N V S S Q T N N A G T A S E C T I G I I S G D T V V N A S S I A M T A P V G Q G M Q W S K L
2575/98	N V S S Q P Y N A G T A S E C T V G I I S G D T V V N A S S I A I K A P V G Q G M Q W S K L
T05/00	N V S S Q T N N A G T A Q E C T A G I I R G D R V V N A S S I A M T A P V G Q G M Q W S K S
T03/01	N V S S E T N N A G Y A S E C T V G I I S G G K V V N A S S I A M T A P A G S G M Q W S K L
T06/01	N V S S Q T N N A G T A S E C T V G I I S G D T V V N A S S I A M T A P V G Q G M Q W S K L
2993/02	K A S S Q T N N A G T A S E C T V G I I S G D T V V N A S S I A M T A P V G Q G M Q W S K L
T01/02	N V S S Q T N N A G A A S V C T V G I I S G D T V F N A S S I A M T A P V G Q G M Q W S K S
T02/02	N V S S Q T N N A G T A S Q C T V G I I S G D T V V N A S S I A M T A P V G Q G M Q W S K L
T10/02	N V S S Q T N N A G S A S E C T V G I I S G D T V V N A S S I A M T A P V G Q G M R W S K S
3025/02	N V S S Q T N N A G T A S E C T V G I I S G D N V F N A S S I A M T A P V G Q G M N W S K S
3051/02	N V S S Q T N N A G T V S E C T V G I I S G D T V V N A S S I A M T A P V G Q G M Q W S K L
3071/03	N V S S Q T N N A G T A S E C T V G I I S G D T V F N A S S I A M T A P V G Q G M Q W S K S
3110/03	N V S S Q T N N A G T A S E C T V G I I S G D T V F N A S S I A M T A P V G Q G M Q W S K L
TP/64	N V S K E T N N A G T A P L C I G G S L Q G G R V V N A S S I A M T A P — P V G I S W S T S
1121/91	N V T N Q Y A N A G T A P V C I G G S I Q S G Y V F N A S S V A M T A P — N N G M T W S T A
1980/94	N V T T R Y A N A G Q R S V C I G G S I Q G G Y A I N A S S V A M T A P — N N G M T W S T T
2012/94	N V T T R Y A N A G N R S V C I G G S I Q G G Y A I N A S S V A M T A P — N N G M T W S T T
2296/95	N V T T Q F N N A G N A S V C I G G S I Q G G Y V F N A S S V A I T A P — N N G M T W S T P
2300/95	N V T T Q L N N A G N A S V C I G G S I Q G G Y V F N A S S V A M T A P — N N G M T W S T A
T07/02	N V T T Q Y A N A G N A P V C I G G S I Q G G Y V F N A S S V A M T A P — N N G M T W S T A
H120	N I S S E S N N A G S S S G C T V G I I H G G R V V N A S S I A M T A P — S S G M A W S S S
1916/93	N I S S E S N N A G S S S G C T V G I I H G G R V V N A S S I A M T A P — S S G M A W S S S
2994/02	N T S I E S N N L — — — R E C I V G I I G G D R V V N A S S I A M T A P — Q P G M D W S S R
Ark99	N V S S E N N N A G T A P S C T A G A I G Y S K N F S A A S V A M T A P — L S G M S W S A S
2992/02	N V S L E I N N A G T A S Q C T A G A I F W S K N F S A S A V A M T A P — E L G M K W S T S

Table 2. History of the selected infectious bronchitis virus strains isolated in Taiwan in 1964 and during the period from 1992 to 2003.

Strain[A]	Year isolated	Chicken type	Age (wk)	Location	Group[B]
1171/92	92	Broiler	3	Taoyuan	TW I
1211/92	92	Broiler	5	Pingtung	TW I
2575/98	98	Broiler	4	Changhua	TW I
T03/01	01	Broiler	4	Taoyuan	TW I
2993/02	02	Broiler	2	Yilan	TW I
3025/02	02	Fighting chicken	8	Taitung	TW I
3051/02	02	Broiler	4	Taoyuan	TW I
3071/03	03	Broiler	5.5	Yilan	TW I
TP/64	64	Layer	40	Taipei	TW II
2296/95	95	Broiler	2	Taoyuan	TW II
T07/02	02	Fighting chicken	6	Hsinchu	TW II
2992/02	02	Broiler	4	Yilan	China
2994/02	02	Broiler	2	Yilan	Mass

[A]All strains are nephropathogenic, except TP/64, which showed respiratory signs and egg drop but no renal lesion.
[B]TW I = Taiwan Group I; TW II = Taiwan Group II.

of the J2/China strain, was unusual, since the importation of animal products from China is prohibited.

Comparison of the S1 sequences of IBVs. Complete S1 sequences were determined for each of the 13 IBVs indicated in Table 2, except for those that were already available from the GenBank. The 13 IBVs could be divided into two groups: TW I and TW II. Most of the IBVs belonged to TW I, while IBV TP/64, 2296/95, and T07/02 belonged to TW II (Fig. 2). The S1 gene of those IBVs contained approximately 44.4% G+C residues. Among all strains in TW I, the S1 sequences were 1620 bp long and differed from one another by 5% to 15%, with IBV 2992/02 the most distant from the others.

The deduced amino acid sequences of those strains showed several substitutions (Table 1). Among TW I group, the E at the residue 55 was replaced by Q, and the R at the residue 74 was replaced by T gradually. The S at the residue 96 was replaced by L. An amino acid deletion at position 87 was found in the Mass group strains, and strain 2994/02 showed deletion of three amino acids at position 60.

After checking the identity of the different fragments in Taiwanese IBVs and the H120 strain, no recombination was found between them (Tables 3, 4), because all of them were less than 90%, except the IBV 2994/02. All IBV 2994/02 fragments had similar sequences with H120 (Mass group); thus, IBV 2994/02 belonged to a Mass group.

The cleavage recognition sites between the S1 and S2 subunits of those 13 strains were compared. The oldest IBV strain, TP/64, had RLSRR, three strains (IBV 2992/02, 2994/02, 3025/02) had RRSRR, and the remaining nine strains had the RRFRR sequence. The most common cleavage recognition site was neither identical to most strains reported (6) nor correlated to genotypic grouping. TW I strains contained the same cleavage site as TW II strains.

Comparison of the N sequences of IBVs. The nt 177–755 fragment of the N gene had 86% to 90% nucleotide identity between H120 and the Taiwanese strains (Table 4). This value was much higher than that for the S1 gene ($P < 0.05$). The S1 and N genes' nucleotide sequences were compared for possible recombination. IBV 2992/02 was found to be a recombinant because its S1 gene was nearly identical to the S1 of the Chinese strain, J2/China; however, its N gene belonged to Taiwan cluster. IBV 2994/02 is another recombinant, because its S1 gene belonged to H120 while its N gene was similar to Ark99, a representative strain of the American group.

DISCUSSION

We have demonstrated that genomic recombination events can take place in the intergenic sequences between S and N genes. Evidence of recombination was found only in these strains, in which the 5′ segment of the recombinant sequence presented a similarity with one strain and the 3′

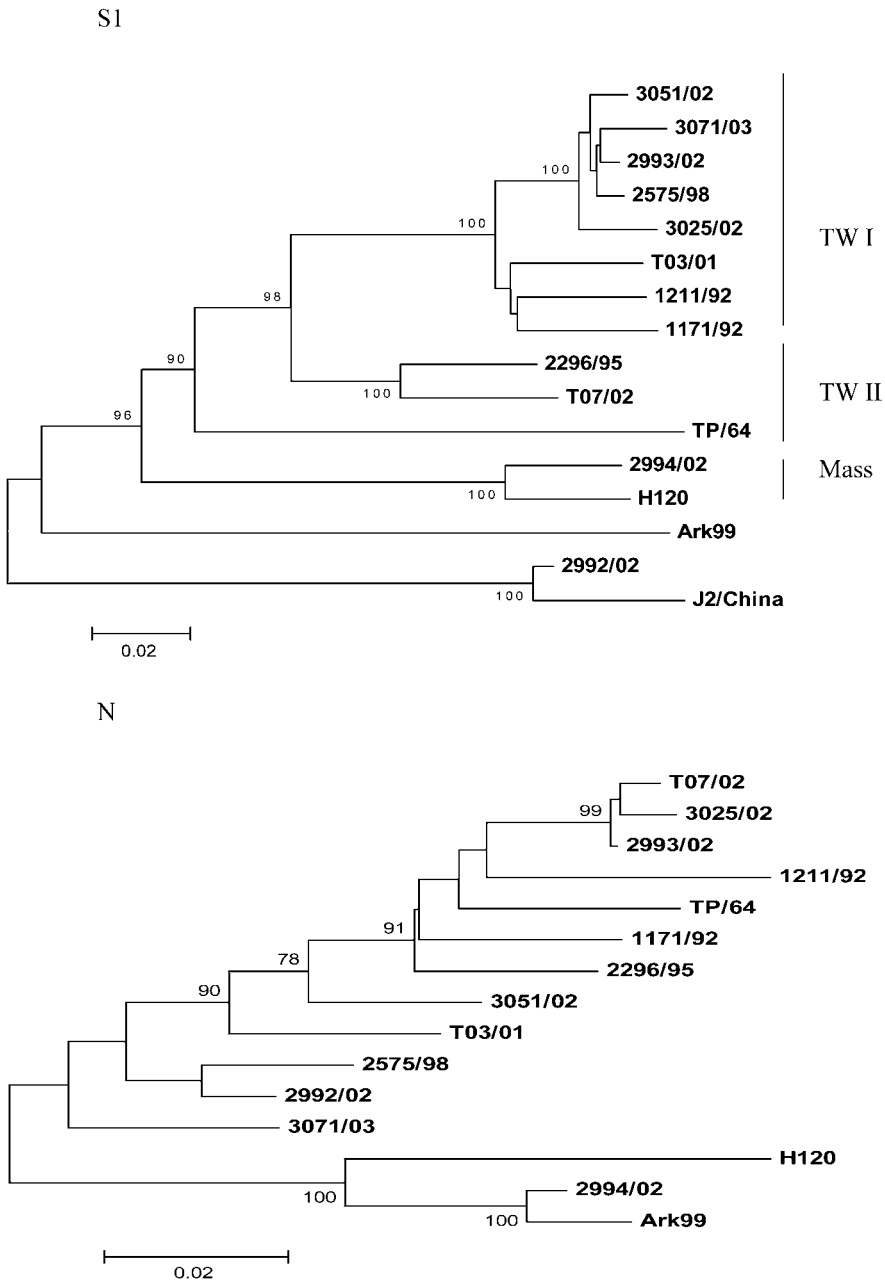

Fig. 2. Phylogenetic trees of the 13 IBVs based on the entire S1 gene and partial N gene. IBV 2994/02 belongs to a Mass group based on the S1 tree but is similar to Ark99 on the N tree. IBV 2992/02 is similar to J2/China strain on the entire S1 tree. No N gene sequence is available for J2/China strain.

segment had similarity with another strain. The same phenomenon was demonstrated in the case of *in ovo* inoculation with different strains (5). In a genome, if the strains that resemble each other in one gene are very different in another, it indicates that interstrain recombination may have occurred. This might be from the discontinuous transcription or replication during negative strain formation (2).

Table 3. The percent identity of the entire S1 gene and partial N gene between Taiwanese IBV strains.

	Percent identity—S1 gene											
Strain	1171/92	1211/92	2575/98	T03/01	2993/02	3025/02	3051/02	3071/03	TP/64	2296/95	T07/02	2992/02	2994/02
1171/92	***	94.4	93.6	93.9	93.5	92.9	93.1	93.4	81.0	87.1	85.7	77.3	80.0
1211/92	93.6	***	94.1	94.0	94.1	93.0	94.0	93.4	80.2	86.9	85.3	76.7	79.0
2575/98	92.2	91.0	***	94.4	98.9	97.3	98.5	98.0	80.1	86.9	86.6	77.1	80.0
T03/01	93.4	92.9	94.6	***	94.0	92.7	93.5	93.3	79.9	86.2	85.3	76.9	80.0
2993/02	96.2	95.7	92.4	93.8	***	97.3	98.7	98.1	80.5	86.5	86.4	77.0	79.5
3025/02	95.5	95.0	92.6	93.8	99.1	***	97.4	96.4	80.2	86.6	85.9	77.5	79.5
3051/02	95.2	92.6	94.6	94.6	95.5	95.5	***	97.4	80.3	86.6	86.1	77.0	80.0
3071/03	92.0	91.0	95.2	92.9	92.9	92.7	94.1	***	80.5	86.1	85.8	76.9	79.8
TP/64	95.2	94.6	91.2	93.3	95.8	95.0	93.8	91.3	***	84.6	84.1	74.1	79.6
2296/95	95.8	94.3	92.2	94.5	95.3	94.6	95.2	92.0	95.5	***	93.4	76.8	80.7
T07/02	95.7	95.2	92.2	93.6	99.5	99.0	95.3	92.7	95.3	94.8	***	76.4	80.7
2992/02	93.3	92.2	97.6	95.5	93.4	93.4	95.8	94.1	92.6	93.4	93.3	***	73.6
2994/02	87.9	86.7	92.7	91.5	87.5	87.7	89.8	92.2	87.7	87.5	87.4	92.0	***

Percent identity—partial N gene

The difference in the S1 gene among different IBVs varied from 1% to 40% (18). However, the difference in the N gene was 5%–15%, less than that in the S1 gene. The N gene is more conserved, making homologous recombination easier.

The result in Table 3 shows that recombination has not occurred in the S1 genes of the Taiwanese IBVs during the past 10 yr in spite of the extensive use of the H120 vaccine strain in the field for more than 30 yr. Although recombination may be a common mechanism for genetic variation for IBV, the factors that precipitate these events are not known. Recombination events could result from the polymerase jumping from one template to another in the synthesis of either the negative- or the positive-strain RNA (10).

Phylogenetic analysis of the IBV S1 gene permits the establishment of relationships between IBVs from different locations and different times. For example, the IBVs isolated 10 yr ago showed high similarity with the strains isolated recently, indicating that the same virus is still present after 10 yr (Fig. 1). The high level of sequence similarity indicates that a stable S1 gene lineage has been maintained in Taiwan, especially TW I.

An interesting finding was that the intruded strain, IBV 2992/02, is similar to the J2/China strain. The J2/China strain is very similar to the Q1 and T3 strains, despite being from geographically different areas in China (19,20). This strain was isolated from a broiler farm built near the migratory bird wetland in Yilan. The IBV vaccine strain Ma5 (Intervet, Holland) was used at 1 day of age. Some chicks showed respiratory signs and died at 2 wk of age. Six percent of these chicks died because of this infection through 4 wk of age. The dead chicks showed urate deposition in both kidneys (data not shown). However, no proventricular lesions were found, as was the case in China (19). Despite the similarity in the S1 gene, this pathogenesis is not the same, because the N gene of 2992/02 is similar to that of Taiwanese strains. Since no N gene sequences from these three Chinese strains were available, the similarity between them and 2992/02 is not known. Discovering the reason for this difference in lesions will require further study.

The IBVs in this study were isolated from chickens in infected flocks vaccinated with Mass group IBV strains. Most of the isolated strains belonged to Taiwanese clusters, except for one strain, 2994/02, which belonged to the Mass group. This might account for the difference in IBVs isolated from Liu's article (11), in which more Mass

Table 4. Nucleotide identity in different fragments of the S1 gene between H120 and Taiwanese strains.

H120	1171/92	1211/92	2575/98	T03/01	2993/02	3025/02	3051/02	3071/03	TP/64	2296/95	T07/02	2992/02	2994/02
1–30[A]	66[B]	73	70	67	70	73	73	69	73	73	73	70	97
−136	71	81	84	78	83	85	84	84	82	82	82	83	94
−1057	81	80	81	81	80	80	81	80	81	83	82	76	94
−1620	84	85	84	84	84	83	84	84	83	85	85	82	96
N[C]	88	87	90	88	86	87	87	89	87	87	86	89	93

[A]S1 fragment nucleotide number.
[B]Percent identity.
[C]N = nt 177 to nt 755 fragment in the N gene.

group IBVs were isolated. During the past 20 yr, nearly all Taiwanese IBVs were nephropathogenic (21), because renal urate lesions are considered to be caused by IBV, and those chickens that showed renal urate were taken to this laboratory for diagnosis. The presence of only two lineages makes the control of IB in Taiwan less difficult. These results indicate that most Taiwanese IBVs are still divided into two distinct lineages, TW I and TW II, as our previous reports suggest (16,17). This means that control of Taiwan IB might be possible using vaccines developed from Taiwanese strains.

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