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Identification of Taiwan and China-like recombinant avian infectious bronchitis viruses in Taiwan

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ABSTRACT

Infectious bronchitis virus (IBV) infections in poultry cause great economic losses to the poultry industry worldwide. The emergence of viral variants complicates disease control. The IBV strains in Taiwan were clustered into two groups, Taiwan group I and Taiwan group II, based on the S1 gene. A variant was previously identified and showed a distinct S1 gene homology with other local strains. This study investigated the 3' 7.3 kb genome of eight Taiwan strains isolated from 1992 to 2007. The genes of interest were directly sequenced. Sequence analyses were performed to detect any recombination event among IBVs. The results demonstrated that all of the examined viruses maintained the typical IBV genome organization as 5'-S-3a-3b-E-M-5a-5b-N-UTR-3'. In the phylogenetic analyses, various genes from one strain were clustered into separate groups. Moreover, frequent recombination events were identified in the Simplot analyses among the Taiwan and China CK/CH/LDL/971-type strains. Putative crossover sites were located in the S1, S2, 3b, M genes and the intergenic region between the M and 5a genes. All of the recombinants showed chimeric IBV genome arrangements originated from Taiwan and China-like parental strains. Field IBVs in Taiwan undergo genetic recombination and evolution.

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1. Introduction

Avian infectious bronchitis virus (IBV), with a large infectious RNA genome (27.6 kb), belongs to the family *Coronaviridae*, the group 3 *Coronavirus*. The RNA molecule of the IBV is linear, single-stranded, positive sense and possesses a 5' cap and a 3' poly-A tail. IBVs can replicate in respiratory, alimentary and urogenital tracts in chickens of all ages, resulting in great economic losses to the poultry industry worldwide (Cavanagh and Naqi, 2003). Clinical signs associated with respiratory and enteric tracts and renal damage might be found in infected chickens (Cavanagh, 2007). During infection, IBV possesses a unique discontinuous transcription system, i.e. a nested set of 3' co-terminal subgenomic mRNAs sharing a common leader sequence in the 5' end are transcribed in the presence of negative-stranded RNA intermediates. The viral polymerase "jumping" possibly contributes to the high RNA recombination frequency in coronaviruses (Lai, 1992; Lai and Holmes, 2001).

The IBV genome encodes four structural proteins: spike glycoprotein (S), envelope protein (E; also known as sM), membrane glycoprotein (M), and nucleocapsid protein (N) (Cavanagh and Naqi, 2003). The spike glycoprotein is post-translationally cleaved into S1

and S2 subunits. The S1 subunit anchors onto the viral outer membrane using the S2 subunit to form a club-shaped projection on the mature virion (Cavanagh, 1983). The S1 glycoprotein is involved in cell attachment and carries epitopes for serotype-specific hemagglutination-inhibition and virus-neutralization antibodies (Cavanagh and Davis, 1986; Hodgson et al., 2004; Ignjatovic and Sapats, 2005; Koch et al., 1990). The S2 glycoprotein, in which two antigenic determinants were identified (Koch et al., 1990), may possess different secondary structures that affect the S1 specific antibody binding (Callison et al., 1999). The membrane glycoprotein is associated with virus assembling and budding (Lai and Holmes, 2001). The nucleocapsid protein interacts with genomic RNA to form the viral nucleocapsid, playing a role in viral RNA synthesis and cell immunity (Lai and Holmes, 2001). In addition, four non-structural proteins of unknown function are expressed by the polycistronic genes, gene 3 and 5 (Britton et al., 2006). Virus mutants carrying truncated 3b genes demonstrate increased virulence and growth advantages *in vitro* and *in ovo* (Shen et al., 2003). The open reading frames (ORFs) of 3a, 3b, 5a, and 5b encode accessory proteins not essential for IBV replication (Casais et al., 2005; Hodgson et al., 2006; Youn et al., 2005).

As a signature of avian coronaviruses, IBV strains are continuously evolving through point mutations and recombination of their genomes. Those variants have better adaptation or increased virulence advantageous to IB outbreaks. To date, a large number of IBV sero- or genotypes have been identified worldwide (Cavanagh, 2007). Most molecular epidemiologic studies have focused on the

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Table 1
Eight IBVs isolated in Taiwan from 1992 to 2007.

Strain	Year of isolation	Chicken type	Age (weeks) ^a	Location	Pathogenicity	Genotype ^b
1171/92	1992	Broiler	3	Taoyuan	Nephropathogenic	TW-I
2296/95	1995	Broiler	2	Taoyuan	Nephropathogenic	TW-II
2575/98	1998	Broiler	4	Changhua	Nephropathogenic	TW-I
2992/02	2002	Broiler	4	Yilan	Nephropathogenic	Variant
3071/03	2003	Broiler	5.5	Yilan	Nephropathogenic	TW-I
3263/04	2004	Broiler	5.2	Yilan	Nephropathogenic	TW-II
3374/05	2005	Broiler	NA ^c	Changhua	NA	Variant
3468/07	2007	Taiwan country chicken	12	NA ^c	NA	TW-I

^a Age of chickens at the time of virus isolation.

^b Genotype is determined based on the S1 gene. TW-I, Taiwan group I; TW-II, Taiwan group II; Variant, neither TW-I nor TW-II.

^c NA: Not available.

spike glycoprotein gene. (Bochkov et al., 2006; Dolz et al., 2006; Dolz et al., 2008; Jackwood et al., 2007). It was reported that slight sequence differences in the S1 gene probably lead to poor cross-protection (Cavanagh et al., 1997). Viruses of different types can co-circulate within a region (Capua et al., 1999; Liu et al., 2006), raising the inter-strain RNA recombination frequency (Bochkov et al., 2007; Jia et al., 1995; Lee and Jackwood, 2000). The wide use of live virus vaccine may also critically contribute to the genetic evolution of IBVs by acting as a heterologous RNA donor template (Kusters et al., 1990; Wang et al., 1993). The emergence of viral variants has complicated disease control requiring persistent IBV molecular surveys.

IBV strains in Taiwan were previously clustered into two groups, Taiwan group I (TW-I) and Taiwan group II (TW-II), on the basis of the S1 gene (Wang and Tsai, 1996). However, a variant isolated in 2002 showed an unusually high S1 gene homology with China strains, but not in the N gene, suggesting an inter-strain recombination event (Huang et al., 2004). In this study, the 3' 7.3 kb genomes from Taiwan IBV strains were investigated to elucidate the genetic diversity of viruses.

2. Materials and methods

2.1. Viruses

Eight IBVs isolated in Taiwan from 1992 to 2007 (Huang et al., 2004) and the vaccine strain H120 (ABIC Biological Laboratories Teva Ltd., Israel) were recovered for this study. The case histories of local strains are listed in Table 1. Viruses were propagated in the allantoic cavity of 9–11-day-old specific pathogen free embryonated eggs (Animal Health Research Institute, Council of Agriculture, Tamsui, Taiwan). Each egg received 0.1–0.2 ml inoculation. After 48–72 h incubation, allantoic fluid was collected and frozen at –80 °C until use.

2.2. Viral RNA extraction, RT-PCR and DNA sequencing

Viral RNA was extracted from 200 µL of virus-infected allantoic fluid using a viral nucleic acid extraction kit (Geneaid Biotech Ltd., Taipei, Taiwan) following the manufacturer's protocol. Previously published primers (Huang and Wang, 2007) were employed in this study to amplify the gene fragments. For sequencing the 3' untranslated region (UTR) of H120, one additional forward primer was designed as 5'-GGAACAATGCACAGCTGGAA-3' from strain H120-GD (GenBank accession no. AY028296).

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with one step in a reaction volume of 50 µL containing 0.5 µL of 5 U/µL RealTaq DNA polymerase (Real Biotech, Taipei, Taiwan), 5 µL of 10× buffer (Real Biotech), 12 µL of 2.5 mM dNTPs (GeneTeks BioScience, Taipei, Taiwan), 0.5 µL of 50 pmol/µL upstream primer (Mission Biotech, Taipei, Taiwan),

0.5 µL of 50 pmol/µL downstream primer (Mission Biotech), 0.4 µL of 40 U/µL Recombinant RNasin ribonuclease inhibitor (Promega, Madison, WI), 0.1 µL of 200 U/µL M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA), 10 µL of viral RNA and 21 µL of diethyl pyrocarbonate-treated water. Reverse transcription was carried out at 40 °C for 30 min, and followed by initial DNA polymerase activation at 94 °C (3 min). PCR was then performed for 35 cycles of denaturation at 94 °C (30 s), annealing at 53 °C (30 s), and polymerization at 72 °C for (1 min 40 s). The cycling reaction was completed with the final polymerization step at 72 °C for 10 min. The amplified products were analyzed in 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

DNA sequencing in both strands from separate RT-PCR products was conducted by a commercial service (Mission Biotech). Each nucleotide was determined from at least four identical results.

2.3. Sequence analysis

The obtained nucleotide sequences were compiled and the amino acid sequences were deduced using DNASTar software (DNASTar, Madison, WI). The IBV reference strains were retrieved from the GenBank database with the accession number listed in Table 2. The AlignX program of the Vector NTI Suite 8 software

Table 2
IBV reference strains included in this study.

Strain	Country	Accession numbers
Armidale	Australia	DQ490205 (S1-N) ^a
Vic	Australia	DQ490221 (S1-N)
BJ	China	AY319651 (S1-N)
CK/CH/LDL/971	China	EF030996 (S1), EF602445 (S2-N)
CK/CH/LDL/981	China	DQ167132 (S1), EF602446 (S2-N)
J2	China	AF286303 (S1)
LX4	China	AY338732 (S1-N)
SAIBK	China	DQ288927 (S1-N)
T3	China	AF227438 (S1)
Q1	China	AF286302 (S1)
QXIBV	China	AF193423 (S1), AF288146 (S2), AF221667 (M-ORF 5), AF199412 (N)
1171/92	Taiwan	DQ646406 (S1-N)
2296/95	Taiwan	DQ646404 (S1-N)
2575/98	Taiwan	DQ646405 (S1-N)
Beaudette	USA	NC_001451 (S1-N)
Cal99	USA	AY514485 (S1-N)
CU-T2	USA	U49858 (S1-N)
DE072	USA	AF274435 (S1), AY024337 (S2), AF202998 (ORF 3), AF202999 (M), AF203000 (ORF 5), AF203001 (N)
Gray	USA	L18989 (S1), AF394180 (S2), AF318282 (E), AF286180 (M), AF469011 (ORF 5), M85245 (N)
M41	USA	AY851295 (S1-N)

^a Sequences of the gene fragments used within the parenthesis.

(InforMax, North Bethesda, ML) was used to generate multiple sequence alignments and determine the nucleotide identity. Phylogenetic analyses were performed with the neighbor-joining method using MEGA version 4 (Tamura et al., 2007). The bootstrap values were determined from 1000 replicates of the original data.

2.4. Simplot analysis

The consecutive IBV nucleotide sequences from the S to N genes (6.8 kb) based on the multiple alignment results were introduced into similarity plots with SimPlot version 3.5.1 (Lole et al., 1999). The nucleotide identity was calculated using the Kimura 2-parameter

method with a transition-transversion ratio of 2 in each window of 500bp. The window was successively furthered along the alignment using a 20-bp increment. At least four sequences were required to initiate an analysis.

2.5. GenBank accession numbers

The IBV sequences resulting from this study were submitted to the GenBank database. The accession numbers are EU822336 (strain 3468/07), EU822337 (strain 3374/05), EU822338 (strain 3263/04), EU822339 (strain 3071/03), EU822340 (strain 2992/02), and EU822341 (strain H120).

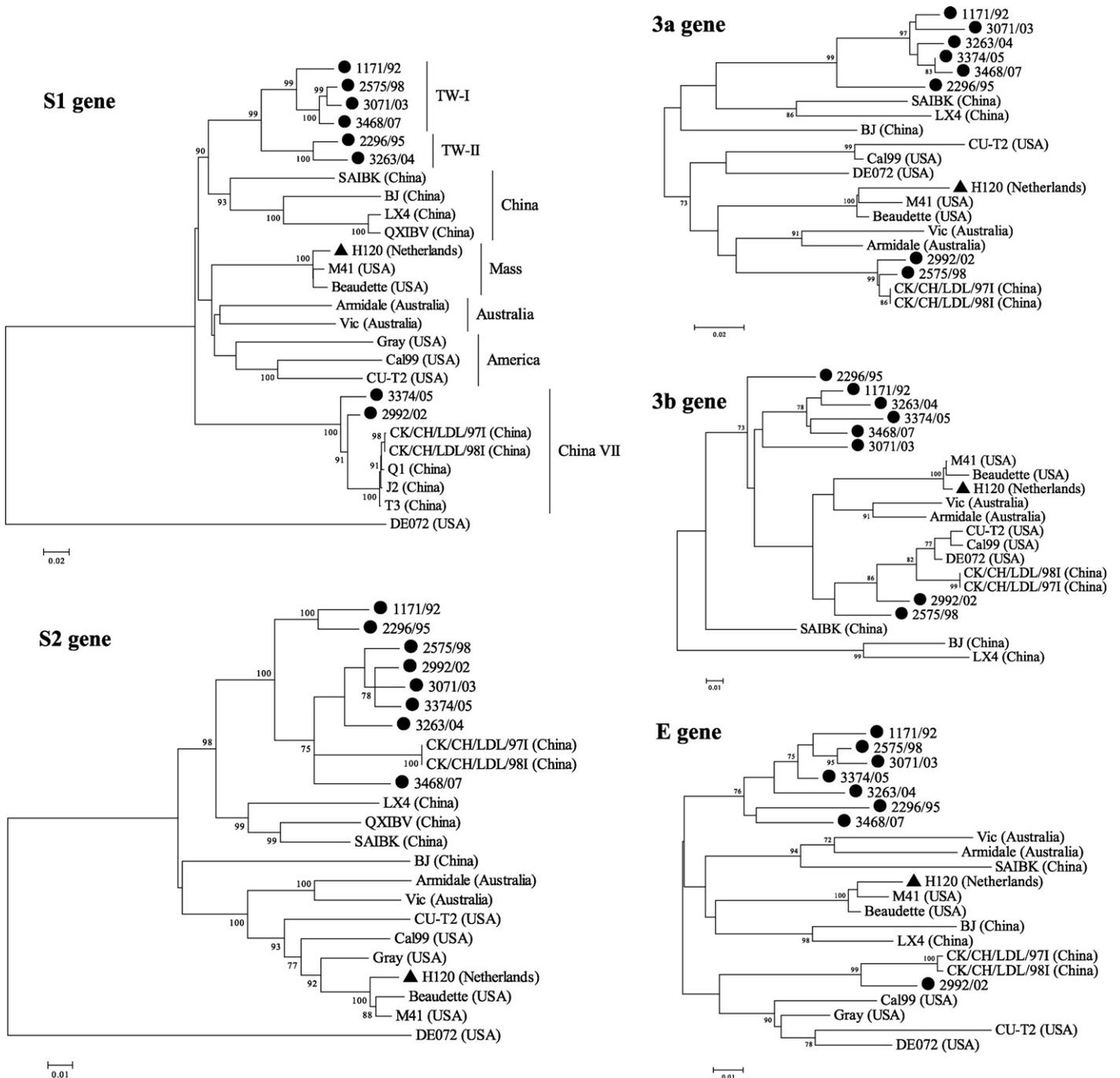


Fig. 1. Phylogenetic analyses of the Taiwan strains (●), H120 (▲) and reference strains for structural and non-structural protein genes of IBVs. The phylogenetic trees were constructed using the MEGA version 4 by the neighbor-joining method (bootstrapping for 1000 replicates with its value >70%).

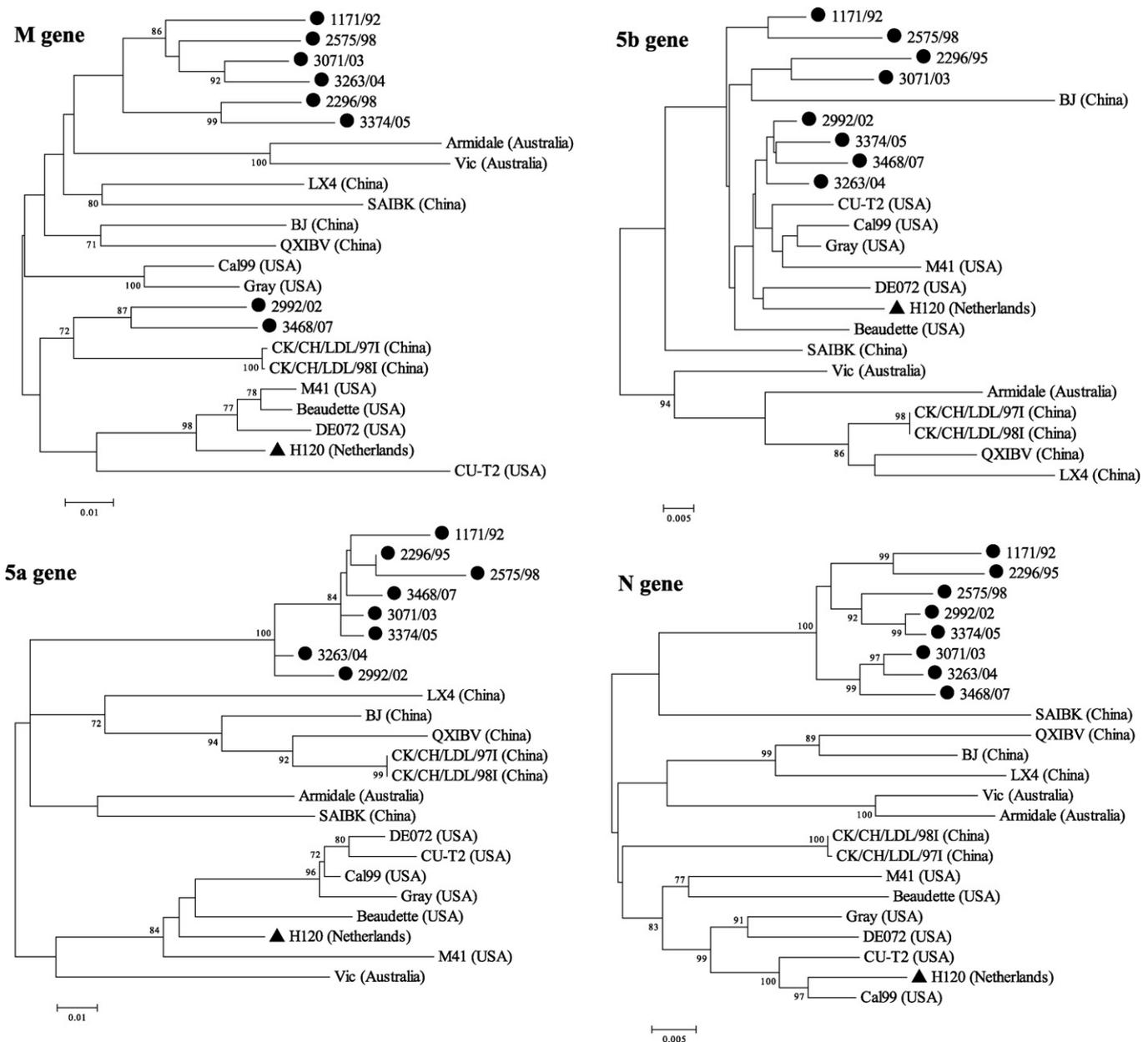


Fig. 1. (Continued).

3. Results

3.1. Sequence comparisons

Sequences that covered the 3' 7.3 kb genome were determined from the Taiwan IBVs and strain H120. All of the examined viruses were found to maintain the typical IBV genome organization as 5'-S-3a-3b-E-M-5a-5b-N-3' (data not shown). The S gene size ranged from a minimum of 3471 nucleotides (strain 3071/03) to a maximum of 3501 nucleotides (strains 2992/02 and 3374/05). Compared with other local strains, single base mutations in the 3' end of the S and 3b genes from the strain 3071/03 changed the genetic code from GAA to TAA (Glutamine → stop codon), resulting in 27- and 3-base truncated ORFs, respectively. Similarly, in the 3374/05, a single base T-insertion in the 3b gene created an early stop codon, leading to a 48-base truncation. However, the 3a, 5b, and N gene ORF sizes were conserved among IBVs. All

of the virus genomes carried an intergenic (IG) region located between the M and 5a genes with a size of 351–362 nucleotides. The IG region of the strain H120 was 55 bases longer than that of the strain Beaudette. The 3' UTR, downstream of the stop codon in the N gene, a region of 475 and 500 nucleotides was sequenced from local strains and H120, respectively. All of the local strains shared a high sequence identity (96–100%) in this region. However, only 52% and 73% identity were observed between H120 and its closely related M41 and Beaudette strains, respectively.

3.2. Phylogenetic analyses

Phylogenetic analyses were performed based on the nucleotide sequence alignment using each ORF from the S to N genes among eight Taiwan and reference strains (Fig. 1). In the S1 gene, the Taiwan strains showed the highest identity (84%) with the Beaudette

reference strain and the lowest (60%) with the DE072. The Taiwan strains (except for 2992/02 and 3374/05) could be clustered into two groups, TW-I and TW-II, based on the S1 gene. Strains 2992/02 and 3374/05 were closely related (>95% identity) to the China genotype VII strains (CK/CH/LDL/971, CK/CH/LDL/981, Q1, J2, and T3) (Liu et al., 2006). In the S2 gene, all of the local strains were grouped with CK/CH/LDL/971 and CK/CH/LDL/981 except for the two viruses isolated before 1995 (strains 1171/92 and 2296/95).

Strains 2575/98 and 2992/02 were classified with CK/CH/LDL/971 and CK/CH/LDL/981 in the analyses of the 3a and 3b genes. The E gene analysis segregated the 2992/02 into the China group. Strains 2992/02 and 3468/07 were distributed with the China strains in the M gene analysis. The 5a, 5b and N genes analyses revealed that all of the local strains were in the same group. Nucleotide sequences from the three ORFs were conserved among the Taiwan strains with >92% homology.

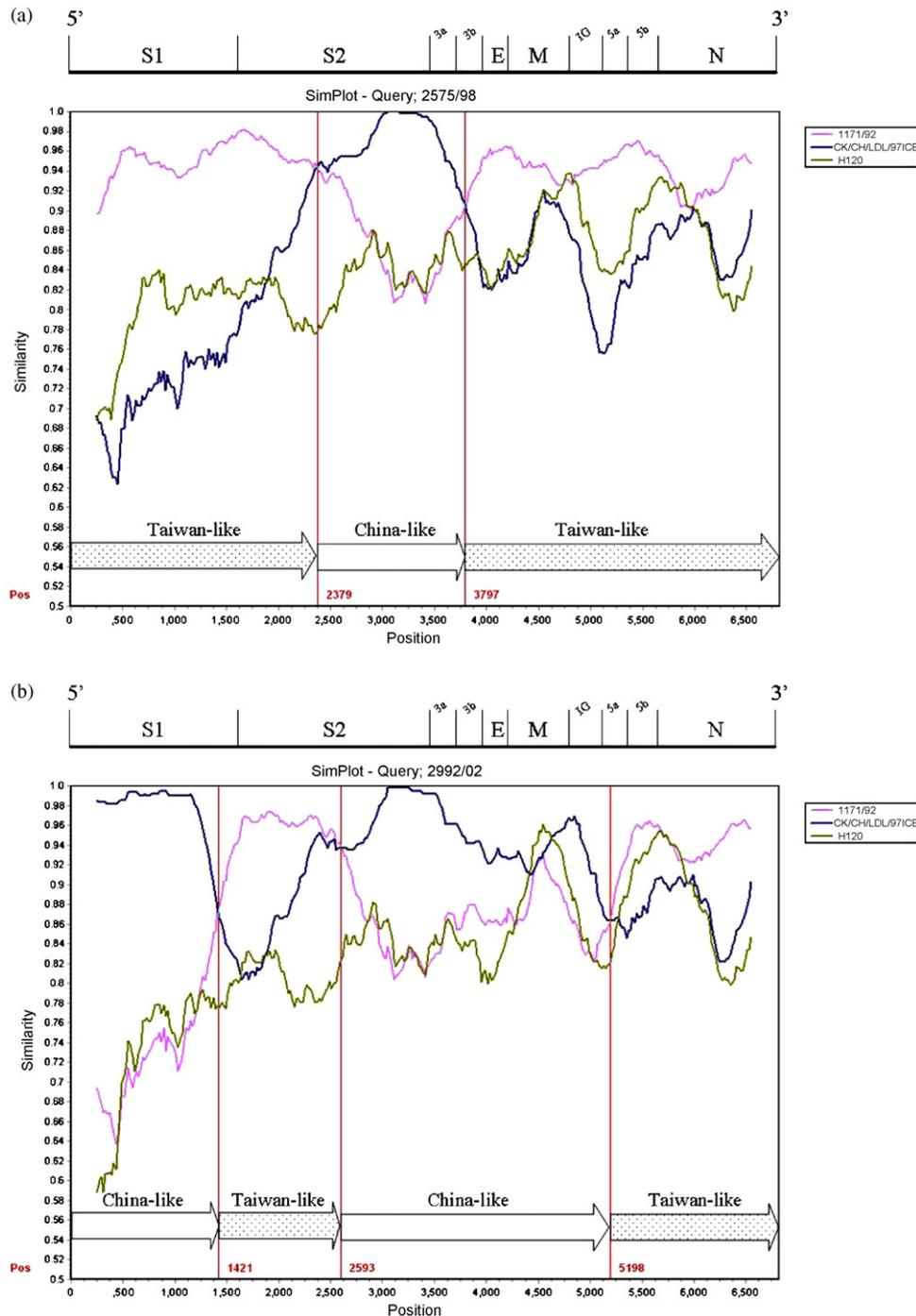


Fig. 2. Simplot analyses of the Taiwan IBVs. Strains 1171/92 (pink), H120 (green), and the China strain CK/CH/LDL/971 (deep blue) were used as putative parental strains when strains 2575/98 (a), 2992/02 (b), 3071/03 (c), 3374/05 (d), 3468/07 (e) were queried. The parental strain 1171/92 was replaced with 2296/95 (light blue) when the strain 3263/04 (f) was queried. Each graph displays the consecutive nucleotide identity (%) from the S to N genes among the queried strain and parental strains. The breakpoint where the parental strains have equal identity to the query strain is the predicted recombination site. Each putative recombinant was schematically assembled using Taiwan and China-like sequence regions. The genomic positions of the crossover sites were indicated by numbers in red. The genomic scale was given at the top of the plot. IG: Intergenic region. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

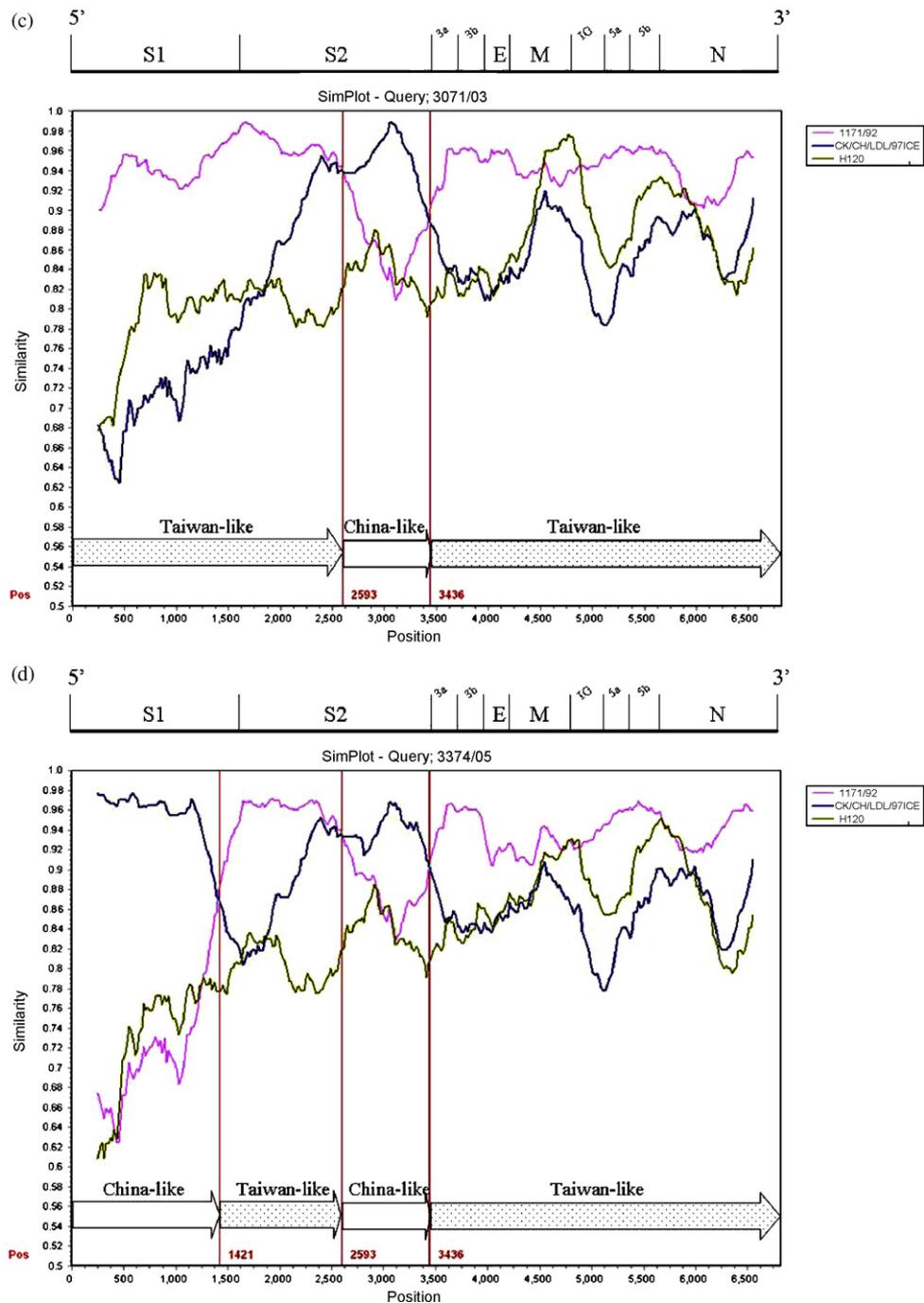


Fig. 2. (Continued)

3.3. Inter-strain recombinants identification

The 3' 6.8 kb genome sequence (S–N gene) of six Taiwan IBVs were queried in the Simplot analysis. Strains 1171/92, H120 and the China strain CK/CH/LDL/97/ICE were used as putative parental strains when strains 2575/98, 2992/02, 3071/03, 3374/05, 3468/07 (TW-I) were queried. The parental strain 1171/92 was replaced with 2296/95 when strain 3263/04 (TW-II) was queried. The similarity plot displays the consecutive nucleotide identity (%) among the queried strain and parental strains. Strains were considered as recombinants if any crossover event took place between two putative parental strains. The breakpoint in which the parental strains have equal identity to the query strain is the predicted recom-

bination site. As Fig. 2 demonstrates, crossover events between parental 1171/92 or 2296/95 and CK/CH/LDL/97/ICE were detected in each plot. The recombination sites were located in the S1, S2, 3b, M genes, and the 1G region between the M and 5a genes. Each putative recombinant was schematically assembled using Taiwan and China-like sequence fragments. The genomic positions of those crossover sites were indicated within the plot (numbers in red).

4. Discussion

In Taiwan, field outbreaks are frequently reported despite routine vaccine use. There were originally two IBV genotypes (TW-I and

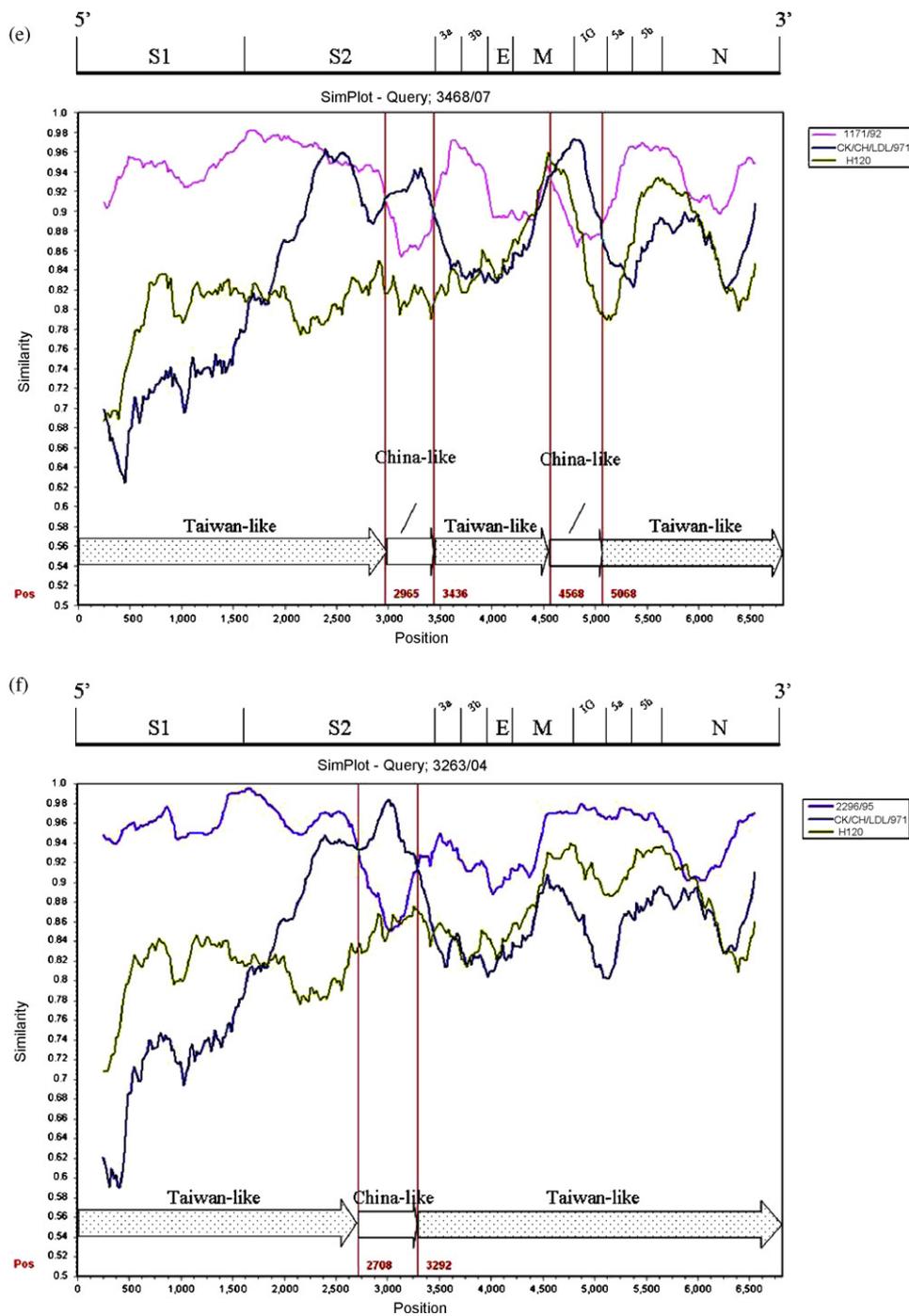


Fig. 2. (Continued).

TW-II) circulating in the field (Liu et al., 2003; Wang and Tsai, 1996) until genetic variants 2992/02 (Huang et al., 2004) and 3374/05 emerged. Both variants showed a high S1 gene homology with the proventriculus pathogenic strains J1, Q2, T3, CK/CH/LDL/971 and CK/CH/LDL/981, belonging to the China genotype VII (Liu et al., 2006; Yu et al., 2001). The 3' 6.8 kb gene of CK/CH/LDL/971 was used as a putative parental strain. Mass type viruses have been widely used as vaccine strains in Taiwan. Our primary concern was the possible viral recombination resulting from the co-circulation of heterologous vaccine strains in flocks. The consecutive sequence of 3' 7.3 kb genome in strain H120 was obtained first in this study and served as another putative parental strain. To our surprise, six local strains experienced crossover events with the strain

CK/CH/LDL/971 instead of H120. Thus, inter-strain recombination events had occurred between the IBVs from Taiwan and China. Taiwan and China are geographically separate. Neither live poultry nor processed poultry products from China have been allowed to import into Taiwan for years. How those recombinants arose remains unknown. A recently identified IBV isolate in Korea (Kr/D64/05) also revealed a close relationship to the China CK/CH/LDL/971-type strains (Lee et al., 2008). Furthermore, the appearance of the China-like strains in Taiwan is reminiscent of the spread of the China QXIBV strain in European countries (Beato et al., 2005; Domanska-Blicharz et al., 2006). In this case, it could be speculated that migrating birds provide the genetic sources of IBV variants in Taiwan. A chicken-nephropathogenic IBV strain was identified from

a non-diseased teal (*Anas* sp.) in China (Liu et al., 2005). Thus, the transport of IBVs over long distances by other avian species appears to be possible (Cavanagh, 2005). However, we still cannot overlook illegal trafficking or unapproved vaccine use in the field.

All six recombinants defined in this study were inter-genotypic recombination and the “China-like” sequence substitutions took place in multiple genes. In particular, partial S gene replacement was observed in every recombinant. Since coronaviruses possess different host range or cell tropism through the variance in the S gene (Casais et al., 2003; Kuo et al., 2000), alterations in the antigenic characteristic in those variants could be expected. In this study, however, nearly all of the strains showed nephropathogenicity (lesions of kidney) in chickens, rather than the proventricular lesions caused by the CK/CH/LDL/971-type strains. How a variant with a chimeric genome arrangement from heterologous strains demonstrates its tissue tropism or pathogenicity in a host is not clear. In addition, it was found that chicks challenged with virulent CK/CH/LDL/971 were incompletely protected by commercial vaccines and other heterologous strains (Liu et al., 2007). To effectively control the IB disease in Taiwan, the protective effect of vaccines against challenges from those recombinants awaits to be investigated.

To our knowledge, this is the first use of Simplot for genetic analyses of IBV strains. The similarity plot can depict the genetic distance among the aligned sequences in a graphical window. In this study IBV recombinants could be defined directly from the Simplot analyses, and the crossover events and corresponding genome positions were readily observed. In phylogenetic analyses, strains were deduced as recombinants if different genes from the genomes were clustered into separate phylogenetic groups. Parallel results were obtained from both analyses.

The emergence of IBV variants through RNA recombination was previously described (Brooks et al., 2004; Jia et al., 1995; Lee and Jackwood, 2000; Mondal and Cardona, 2007; Wang et al., 1993). Recombination events occurred in multiple genes. The consensus IG sequences CT(T/G)AACAA or the conserved regions around were assumed as the recombination “hot spots” in IBVs (Lee and Jackwood, 2000). In addition, the CTTTTG sequence was observed adjacent to the putative recombinant junction (Brooks et al., 2004; Wang et al., 1993). In this study, T-rich motifs (A/T/G)TTTTG, consensus among a recombinant and its parental strains, were located upstream of several crossover sites. For instance, the motifs were found in the S2 gene of the 3263/04, the 3b gene of the 2575/98, and the IG region of the 2992/02 and 3468/07. All of the recombinants experienced crossover events in the 3' end of the S gene, indicating a possible region for template switching in viral RNA synthesis. Similar results were obtained from the IBV strain CU-T2 and Cal99, whose recombination sites were deduced in the 3' 751 and 700 nucleotide of the S gene, respectively (Jia et al., 1995; Mondal and Cardona, 2007).

In addition to viral recombination, point mutation is a way of virus evolution. The single base nonsense mutations resulted in truncations of the S and 3b genes in the Taiwan strains 3071/03 and 3374/05. The naturally occurring IBV strains with mutations or complete deletions in non-structural ORFs 3a, 3b, 5a, and 5b revealed lower virus growth rate or titer *in ovo* (Liu et al., 2008) and *in vivo* (Mardani et al., 2008).

This study describes the dual viral genomes incorporation in the separated areas having no known interactions between poultry. The molecular investigation in the 3' 7.3 kb of the IBV genome demonstrates that inter-strain recombination events contribute to the genetic diversity of the Taiwan strains. Field IBVs in Taiwan undergo genetic recombination and evolution, which might lead to disease control difficulty. The biological and antigenic characteristics of the IBV variants await further studies.

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