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Phylogenetic analysis of classical swine fever virus isolated from Taiwan

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Abstract

By analyzing the E2 sequences of classical swine fever virus from field outbreaks in Taiwan during 1993–2001, three virus populations with distinct genotypes were determined including one historical (subgroup 3.4) and two exotic (subgroup 2.1) strains. The first subgroup 2.1 virus was isolated in 1994 and further sporadic outbreaks occurred after 1996. Phylogenetic analysis using the E2 region has segregated the Taiwanese strains of 2.1virus into two different genotypes (termed 2.1a and 2.1b). The 2.1b viruses were only isolated in 2001 and shared approximately 94.8% nucleotide identities to the 2.1a viruses in the total genomic sequences. The results suggest that the 2.1a and 2.1b viruses may be introduced from different origins.

Keywords: Classical swine fever virus; *Pestivirus*; Epidemiology; Phylogenetics

1. Introduction

Classical swine fever (CSF) is the most insidious and devastating disease of swine and wild boars, causing significant economical losses in the pig industry over most regions of the world. CSF is caused by classical swine fever virus (CSFV), a member of the genus *Pestivirus* within the family Flaviviridae. The genome of CSFV comprises a single open reading frame (ORF), approximately 12.3 kb in length (Meyers et al., 1989; Meyers and Thiel, 1996). This ORF, flanked by a 5′-untranslated region (UTR) and a 3′-UTR, encodes a polyprotein composed of about 3898 amino acids, which is processed by viral and cellular enzymes into four structural (C, E0, E1 and E2) and eight nonstructural (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (Thiel et al., 1991; Meyers and Thiel, 1996).

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CSF viruses consist of one serotype, reflecting a narrow range of evolutionary divergence (Vanderhallen et al., 1999). Therefore, genetic typing of the virus has been used in understanding the evolution, spread of viruses and the origins of disease outbreaks. Three regions, 5′-UTR, E2 and NS5B, in the viral genome are most extensively used for genetic analysis and for studying virus diversity on the basis of sequence homology (Hofmann et al., 1994; Lowings et al., 1996; Stadejek et al., 1996; Greiser-Wilke et al., 1998; Paton et al., 2000). Analysis using a 96 nt region of the 5′-UTR, a 190 nt region of the E2 and a 409 nt region of the NS5B has resulted in similar resolution to classify CSFV into three major groups and their subgroups. Group 1 and its three subgroups (1.1, 1.2 and 1.3) comprise most of the historical strains (Lowings et al., 1996; Paton et al., 2000) distributed in most regions of the world. Group 2 containing most of the current viruses, which segregates into subgroup 2.1, 2.2 and 2.3, has increased activity and caused epidemic infection since the 1980s (Paton et al., 2000). The earliest 2.1 strain (VRI2277) was isolated from Malaysia in 1986 (Vilcek et al., 1996; Paton et al., 2000). In the 1990s, the 2.1 viruses have caused epidemic of outbreaks in Germany (Oleksiewicz et al., 2003), The Netherlands (Widjojoatmodjo et al., 1999; Stegeman et al., 2000), Switzerland, Austria, Italy, Belgium, Spain (Paton et al., 2000), China (Tu et al., 2001) and Taiwan. Nevertheless, Group 3 contains disparate viruses distributed in regions such as Taiwan, Korea, Japan, Thailand and the United Kingdom (Sakoda et al., 1999; Paton et al., 2000).

Although an attenuated lapinized live vaccine (LPC) has been used to protect pigs from CSF since the 1950s in Taiwan, outbreaks occur endemically. We have genetically analyzed the viruses obtained during 1993–2001. Our results not only identify that the 3.4 strains are the historical strains in Taiwan, which may become silent strains in fields after 1996, but also reveal that there has been a switch in the virus populations from the 3.4 to the 2.1 strains circulating wild types.

2. Materials and methods

2.1. Virus isolates

A total of 36 isolates of CSFV recovered from field outbreaks in domestic pig herds over a period of 9 years (1993–2001) in Taiwan (Table 1) were included in this study. These isolates were passaged twice in PK-15 cells and identified with fluorescence-labelled specific antiserum against CSFV. Virus information on their geographical origins (prefecture), years of isolation and genotypes was summarized in Table 1. Two of the 36 isolates, p97/FL/94 and 94.4/IL/94, have previously been studied by other investigators, and the relevant references are cited where applicable (Shiu et al., 1996; Paton et al., 2000). In addition, 59 strains of E2 nucleotide sequences retrieved from published data were listed in Fig. 1.

2.2. RNA amplification and sequencing

Nucleotide sequences in the E2 and NS5B regions were amplified by RT-PCR from cultured virus suspension. Viral RNA was extracted from 140 μl of cultured suspension using QIAamp Viral RNA Mini Kit (QIAGEN) by the method recommended by the manufacturers. For E2 amplification, viral RNA was reverse-transcribed using the antisense primer (5′-TGTCTCATTTGCCAAGATGCACTT-3′, position 3134–3111) and then amplified for 30 cycles (denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s) using the sense primer (5′-TGAGGGATTTRACYAGRGTCTGGA-3′, position 2317–2337). For NS5B amplification, the sense primer (5′-TGACCATGCACATGTCAGAAGTACC-3′, position 11,053–11,077) and the antisense primer (5′-TATCCTTCTAATCAGTGGGTTCCAG-3′, position 11,576–11,600) were used. The amplified product was purified using the QIAquick PCR Purification Kit (QIAGEN). Subsequently, DNA fragments were sequenced by the direct sequencing method, using primers as in the PCR amplification and using BigDye™ Terminator Cycle Sequencing Kit. The samples were loaded on an Applied Biosystems 3100 sequencer (Foster City, CA).

2.3. Phylogenetic analysis

Phylogenetic analysis was initially carried out on the *E2* gene a 190 nt region encompassing nucleotides 2518–2707 (Lowings et al., 1996; Paton et al., 2000) and the *NS5B* gene, a 409 nt region spanning nucleotides 11,158–11,566 (Bjorklund et al., 1999;

Table 1
CSFV isolates obtained from field outbreaks in Taiwan during 1993–2001

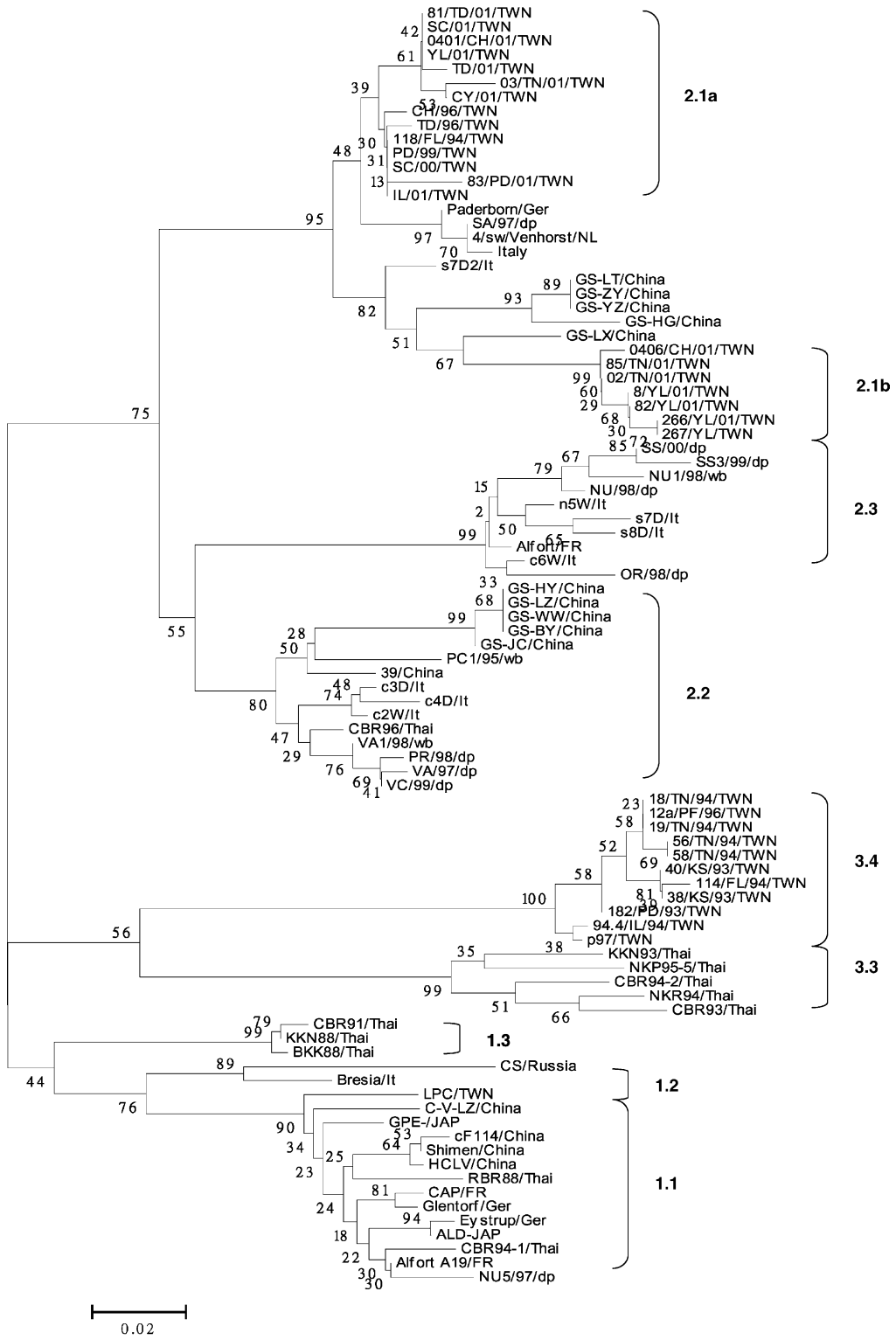
Virus isolate	Region of isolation (prefecture)	Year of isolation	Subgroup	GenBank accession no.	
				E2	*NS5B*
38/KS/93	Kaoshiong	1993	3.4	AY571988	AY571129
40/KS/93	Kaoshiong	1993	3.4	AY571089	AY571130
182/PD/93	Pindon	1993	3.4	AY571084	AY571125
p97/FL/94	Farlin	1994	3.4	L49347	L49347
94.4/IL/94	Ilan	1994	3.4	AY571097	AY571138
18/TN/94	Tainan	1994	3.4	AY571090	AY571131
19/TN/94	Tainan	1994	3.4	AY571091	AY571132
56/TN/94	Tainan	1994	3.4	AY571092	AY571133
58/TN/94	Tainan	1994	3.4	AY571093	AY571134
114/FL/94	Farlin	1994	3.4	AY571087	AY571128
118/FL/94	Farlin	1994	2.1a	AY571086	AY571127
12A/PF/96	Pinfu	1996	3.4	AY571065	AY571106
nh/TN/96	Tainan	1996	3.4	AY571103	AY571144
CH/96	Chanhua	1996	2.1a	AY571063	AY571104
c/TN/96	Tainan	1996	2.1a	AY571102	AY571143
TD/96	Taidon	1996	2.1a	AY571064	AY571105
32/IL/96	Ilan	1996	2.1a	AY571099	AY571140
PD/98	Pindon	1998	2.1a	AY571067	AY571108
PD/99	Pinton	1999	2.1a	AY571066	AY571107
SC/00	Sinchu	2000	2.1a	AY571068	AY571109
CY/01	Chiayi	2001	2.1a	AY571069	AY571110
83/PD/01	Pindon	2001	2.1a	AY571080	AY571121
03/TN/01	Tainan	2001	2.1a	AY571083	AY571124
0401/CH/01	Chanhua	2001	2.1a	AY571074	AY571115
IL/01	Ilan	2001	2.1a	AY571072	AY571113
TD/01	Taidon	2001	2.1a	AY571071	AY571112
SC/01	Sinchu	2001	2.1a	AY571070	AY571111
81/TD/01	Taidon	2001	2.1a	AY571078	AY571119
YL/01	Yunlin	2001	2.1a	AY571073	AY571114
0406/CH/01	Chanhua	2001	2.1b	AY571075	AY571116
85/TN/01	Tainan	2001	2.1b	AY571081	AY571122
82/YL/01	Yunlin	2001	2.1b	AY571079	AY571120
02/TN/01	Tainan	2001	2.1b	AY571082	AY571123
8/YL/01	Yunlin	2001	2.1b	AY571085	AY571126
266/YL/01	Yunlin	2001	2.1b	AY571076	AY571117
267/YL/01	Yunlin	2001	2.1b	AY571077	AY571118

Paton et al., 2000). A total of 36 Taiwanese isolates (Table 1) and 59 reference sequences (Fig. 1) retrieved from the GenBank database were used in this study. Multiple and pairwise sequence alignments were constructed using the clustal algorithm of the computer program MegAlign from LaserGene Biocomputing Software Package (DNASTAR, 1997). Phylogenetic trees were constructed using the programs minimum-evolution and neighbor-joining in the MEGA software version 2.1 (Saitou and Nei, 1987; Kumar et al., 2001). The robustness of the groupings in the neighbor-joining analysis was assessed with 1000 bootstrap resampling.

3. Results

3.1. Virus isolates

Pairwise comparison of the 36 Taiwanese viruses (Table 1) has divided them into three distinct


81/TD/01/TWN
SC/01/TWN
0401/CH/01/TWN
YL/01/TWN
TD/01/TWN
03/TN/01/TWN
CY/01/TWN
CH/96/TWN
TD/96/TWN
118/FL/94/TWN
PD/99/TWN
SC/00/TWN
83/PD/01/TWN
IL/01/TWN
2.1a
Paderborn/Ger
SA/97/dp
4/sw/Venhorst/NL
Italy
s7D2/It
GS-LT/China
GS-ZY/China
GS-YZ/China
GS-HG/China
GS-LX/China
0406/CH/01/TWN
85/TN/01/TWN
02/TN/01/TWN
8/YL/01/TWN
82/YL/01/TWN
266/YL/01/TWN
267/YL/TWN
2.1b
SS/00/dp
SS3/99/dp
NU1/98/wb
NU/98/dp
n5W/It
s7D/It
s8D/It
Alfort/FR
c6W/It
OR/98/dp
2.3
GS-HY/China
GS-LZ/China
GS-WW/China
GS-BY/China
GS-JC/China
PC1/95/wb
39/China
c3D/It
c4D/It
c2W/It
CBR96/Thai
VA1/98/wb
PR/98/dp
VA/97/dp
VC/99/dp
2.2
18/TN/94/TWN
12a/PF/96/TWN
19/TN/94/TWN
56/TN/94/TWN
58/TN/94/TWN
40/KS/93/TWN
114/FL/94/TWN
38/KS/93/TWN
182/PD/93/TWN
94.4/IL/94/TWN
p97/TWN
3.4
KKN93/Thai
NKP95-5/Thai
CBR94-2/Thai
NKR94/Thai
CBR93/Thai
3.3
CBR91/Thai
KKN88/Thai
BKK88/Thai
1.3
CS/Russia
Bresia/It
1.2
LPC/TWN
C-V-LZ/China
GPE-/JAP
cF114/China
Shimen/China
HCLV/China
RBR88/Thai
CAP/FR
Glentorf/Ger
Eystrup/Ger
ALD-JAP
CBR94-1/Thai
Alfort A19/FR
NU5/97/dp
1.1
0.02

genotypes. Twelve out of the 36 Taiwanese isolates collected from 1993 to 1996 including the reference strains, p97/FL/94 and 94.4/IL/94, were historical strains in Taiwan, which have been classified into subgroup 3.4 by previous investigators (Paton et al., 2000). The 3.4 viruses did not appear in field outbreaks after 1996. Nevertheless, two exotic strains differing in genotypes belonging to subgroup 2.1 have been separately introduced into Taiwan in 1994 and 2001. Therefore, based on the similarities of nucleotide sequences the viruses correlated to the 1994 outbreak were named subgroup 2.1a to differentiate from the strains (2.1b) obtained in 2001. The 2.1a viruses have continuously caused outbreaks and dominated the field infection after 1996.

3.2. Phylogenetic analysis

To define the evolutionary relationships among viruses, phylogenetic analysis of the virus based on the E2 was undertaken. In the E2 tree, the viruses segregated into three major groups (Groups 1–3) and their subgroups, which correlated precisely to previous studies by other investigators (Lowings et al., 1996; Paton et al., 2000). However, the Taiwanese isolates were divided into two major clusters (Fig. 1). A cluster containing the strains p97/FL/94, 94.4/IL/94, 182/PD/93, 40/KS/93, 114/FL/94, 38/KS/93, 58/TN/94, 56/TN/94, 12A/PF/96, 18/TN/94 and 19/TN/94 were jointed together with the Kanagawa strain from Japan and classified into subgroup 3.4 (Sakoda et al., 1999; Paton et al., 2000). The 3.4 viruses have been reported since the 1920s in Taiwan. In pairwise comparisons, the minimum nucleotide similarity among isolates from 1993 to 1996 was 96.8% for the E2 and 97.7% for the NS5B (data not shown). Therefore, the 3.4 viruses did not diverse to any further subdivisions in the phylogenetic tree.

The Taiwanese strains of subgroup 2.1 were further segregated into two discriminated genotypes (termed 2.1a and 2.1b) (Fig. 1). Subgroup 2.1a containing the viruses 83/PD/01, 118/FL/94, SC/00, PD/99, IL/01, TD/96, CH/96, CY/01, 03/TN/01, TD/01, 81/TD/01, SC/01, YL/01, and 0401/CH/01 were isolated from 1994 to 2001. These 2.1a viruses were found to be more closely related to the strains that caused outbreaks in Europe such as the Paderborn, SA/97/dp, 4/sw/Venhorst, and the Italy strains (Fig. 1). In pairwise comparisons, the nucleotide identities for E2 between the 2.1a and the Paderborn strains were approximately 96.9%, for NS5B was 97% and for the total genomic sequence was 97% (data not shown). Tree constructed with the NS5B sequences also indicated that the 2.1a viruses were related most closely to the European strains such as the Paderborn, MP104, and V3 (data not shown). The minimum similarity of the 2.1a viruses in the *E2* gene from 1994 to 2001 was 96.4% and in the *NS5B* gene was 96.6% (data not shown).

However, the 2.1b strains including the 0406/CH/01, 85/TN/01, 02/TN/01, 8/YL/01, 82/YL/01, 266/YL/01 and 267/YL/01 were only obtained in 2001. So far, the 2.1b viruses only clustered together with the strains GS-LT, GS-YZ, GS-ZY and GS-LX from China and the s7D2 strain from Italy (Fig. 1). The closest distance for the *E2* gene to the 2.1b viruses in the GenBank was the GS-LX strain with about 97% nucleotide identity. In addition, the minimum similarity of the 2.1b viruses for the E2 was 97.8% and for the NS5B was 98.5%.

Fig. 1. Phylogenetic tree based on E2 (190 nt) region showing the genetic relatedness of CSFV strains. Bootstrap values were estimated for this tree using the neighbor-joining model for 1000 replicates. The Taiwanese strains were generated in this study (Table 1). Other sequences were retrieved from the GenBank database with the accession no. as: Paderborn (AY072924), SA/97/dp (AJ312882), 4/sw/Venhorst (AF084050), Italy (AY027672), s7D2 (L36171), GS-YZ (AF143089), GS-ZY (AF143088), GS-LT (AF143091), GS-HG (AF143090), GS-LX (AF143087), VA/97/dp (AJ312862), VC/99/dp (AJ312870), PR/98/dp (AJ312876), VA1/98/wb (AJ312861), CBR96/Thai (AF241626), c2W (L36165), c3D (L36166), c4D (L36167), 39 (AF407339), PC1/95/wb (AJ312866), GS-JC (AF143082), GS-BY (AF143085), GS-WW (AF143084), GS-HY (AF143086), GS-LZ (AF143082), s7D (L36170), s8D (L36172), n5W (L36169), c6W (L36168), OR/98/dp (AJ312857), Alfort (J04358), NU/98/dp (AJ312855), NU1/98/wb (AJ312853), SS/00/dp (AJ312873), SS3/99/dp (AJ312860), CBR91(AF241621), KKN88 (AF241623), BKK88 (AF134209), CS (AF099102), Bresia (AF091661), C-V-LZ (AF143092), GPE$^-$ (D49533), RBR (AF241618), cF114 (AF333000), Shimen (AF092448), HCLV (AF091507), CAP (X96550), Glentorf (U45478), Eystrup (AF326963), CBR94-1 (AF241615), Alfort A19 (U90951), NU5/97/dp (AJ312877), NKR94 (AF241631), CBR93 (AF241628), CBR94-2 (AF241630), KKN93 (AF241632), NKP95-5 (AF241635), p97 (L49347).

4. Discussion

Our analyses based on the E2 nucleotide sequences of CSFV have uncovered three distinct virus populations in Taiwan during 1993–2001. These viruses include one historical and two exotic strains. The historical strains were classified into subgroup 3.4, as have been previously suggested by other investigators (Paton et al., 2000), whereas, the two exotic strains differing in genotypes were classified into subgroup 2.1. More importantly, the 2.1 virus strains were first isolated in 1994 and further outbreaks occurred thereafter. These results are therefore precious to understand the evolution of subgroup 2.1 virus in a single geographical area.

Comparison of the E2 and 5′-end nucleotides, previous studies (Sakoda et al., 1999; Paton et al., 2000) reveal that two Taiwanese strains, p97/FL/94 and 94.4/IL/94, are the most distinct variants of CSFV. Tracing of epidemiology also indicated that the 3.4 viruses have been present in Taiwan since the 1920s. Similar viruses to the p97/FL/94 strain such as the Kanagawa/74, Okinawa/86, Okinawa/86-2 and CBR/93 strains were also identified in Japan and Thailand (Sakoda et al., 1999). These investigations indicate that they only appear in the regions of Asia from southern Japan to Thailand. It is a noteworthy feature in epidemiology that the 3.4 strains were investigated to prevail in the fields only prior to 1996 in Taiwan. In Japan, they were only obtained before 1986 (Sakoda et al., 1999). Therefore, the 3.4 viruses may have become subclinical strains in those regions.

Epidemiological analysis in Taiwan has showed that there has been a switch in virus populations from subgroup 3.4 to 2.1 after 1996. The first 2.1 strain (118/FL/94) was obtained in 1994, which have caused epidemic disease and dominated the field infections since 1996. The reasons for the change of different virus populations in natural situations are not clear. The possible explanations include: first, the 2.1 strains may possess higher replication rate than the 3.4 strains in pigs; second, the 2.1 viruses may contain higher affinity to compete the cellular receptors. In addition, the attenuated lapinized live vaccine (LPC; Group 1) has been used to protect pigs against the 3.4 strains since the 1950s in Taiwan. A study (Uttenthal et al., 2001) indicates that vaccines based on the recombinant E2 protein produced from the Bresia and Alfort-Tubingen strains (Group 1) do not protect pigs perfectly against possibility of infection from the Paderborn strain (subgroup 2.1). Whether the 2.1 strains contain higher ability than the 3.4 strains to escape from antibody neutralization will require additional investigation. Similar situations of the virus switch in the fields were also observed in China and Europe. In China, 94% of the virus strains attained from field outbreaks during 1986–1999 have changed from Groups 1 to 2 (Tu et al., 2001).

Phylogenetic tree has further discriminated the Taiwanese isolates of subgroup 2.1 into two different genotypes (2.1a and 2.1b), whether using either the E2 (Fig. 1) or the NS5B sequence data. Our molecular analysis correlates precisely with the epidemiology, indicating that the 2.1a virus caused initial infection in 1994 and then it continuously caused outbreaks after 1996. However, the 2.1b virus was only obtained in 2001. Several indicators support that the 2.1b strains are not the mutants of 2.1a strains but from a separate introduction. First, the 2.1a viruses from 1994 to 2001 maintain $\geqq$97.4% identities in either the *E2* or *NS5B* gene. While the 2.1b viruses only shared approximately 94.1–95.1% identities to the 2.1a viruses in the E2, NS5B and the total genomic sequence (unpublished data). Second, the closest relationship to the 2.1a strains in the GenBank database is the Paderborn strain (Oleksiewicz et al., 2003) from Germany having 96.8% identities common to the E2. However, the closest relationship to the 2.1b cluster is with the viruses from China including the GX-LX, GS-LT, GS-YZ and GS-ZY strains (Tu et al., 2001) with about 96–97% identities in the E2. Third, there is no intertypic strain obtained from fields. In addition, the 2.1b strains cluster together with the GS-LX, GS-LT, GS-YZ and GS-ZY strains from China and the s7D2 strain from Italy in the phylogenetic analysis. A previous investigation (Tu et al., 2001) has also clustered the s7D2 strain with the subgroup 2.1 viruses from China. Based on these analyses, we conclude that the 2.1b strains are new strains in Taiwan but not the mutants of 2.1a strains, suggesting that they are discriminated by the genotypes.

The earliest strain (VRI 2277 or VRI 4425) of subgroup 2.1 appeared in 1986 in Malaysia and it reappeared in the 1990s in Europe and in China. In Europe, it caused outbreaks in Germany (1996), The Netherlands (1997), Italy (1994) and United Kingdom

(2000). In China, the earliest strain was detected in 1993 (Tu et al., 2001). Previous studies have showed that the VRI 2277 or VRI 4425 strain may be the recent precursor of subgroup 2.1. Our analyses do support such a hypothesis. The phylogenetic tree based on the NS5B appeared to have the deepest lineage in the VRI 2277 and VRI 4425 strains (data not shown). In addition, we have compared the NS5B sequences among the 2.1a, 2.1b and VRI 2277 viruses, finding that they were virtually equidistant with approximately 94.6% identities no matter between the VRI 2277 and the 2.1a or between the VRI 2277 and the 2.1b viruses. From these observations, we consider that our tree documents the gradual dispersal of the 2.1 virus from the common evolutionary origin. Although these studies would not predict where the 2.1a and 2.1b viruses directly originated from, they have revealed the genetic relationships among virus strains from different regions. Our studies also reveal that the subgroup 2.1 viruses have increased in mutation rate recently and diverged into differentiated virus strains.

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台灣分離豬瘟病毒株之演化樹分析

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摘要

分析 1993 至 2001 年間台灣分離之豬瘟病毒株 E2 基因片段，可以明顯地區分出三個不同基因型的病毒株，分別為一個古典型（3.4 基因亞型）及兩個外來型（2.1 基因亞型）。其中 2.1 基因亞型病毒株首先於 1994 年被分離到並於 1996 年開始散播。而利用演化樹分析豬瘟病毒 E2 基因片段可以成功將台灣分離的 2.1 基因亞型病毒再區分為兩個不同的基因型（分別命名為 2.1a 及 2.1b）。而 2.1b 病毒只於 2001 年間被分離到，且與 2.1a 病毒株間只有 94.8%的核酸相似度。這樣的結果說明 2.1a 及 2.1b 病毒株間可能來自不同的來源。

關鍵詞：豬瘟病毒、瘟疫病毒鼠、流行病學、演化樹分析

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