Letter to the Editor

Identification of a Newly Isolated Getah Virus in the China-Laos Border, China*

LI Yuan Yuan[1,2,3,&], FU Shi Hong[2,3,&], GUO Xiao Fang[4], LEI Wen Wen[2,3], LI Xiao Long[2,3], SONG Jing Dong[2,3], CAO Lei[2,3], GAO Xiao Yan[2,3], LYU Zhi[2,3], HE Ying[2,3], WANG Huan Yu[2,3], REN Xiao Jie[1,2,3], ZHOU Hong Ning[4], WANG Gui Qin[1,#], and LIANG Guo Dong[2,3,#]

In this study, we isolated a virus strain (YN12031) from specimens of *Armigeres subalbatus* collected in the China-Laos border. BHK-21 cells infected with YN12031 exhibited an evident cytopathic effect (CPE) 32 h post-infection. The virus particles were spherical, 70 nm in diameter, and enveloped; they also featured surface fibers. Molecular genetic analysis revealed that YN12031 was closely related to alpha viruses such as Chikungunya virus and Sindbis virus, and located in the same clade as MM2021, the prototype of Getahvirus (GETV) isolated in Malaysia in 1955. Phylogenetic analysis of the E2 and capsid genes further revealed that YN12031 was located in the same clade as the Russian isolate LEIV/16275/Mag. Analysis of the homology of nucleotides and amino acids in the coding area and E2 gene demonstrated that the YN12031 isolated from the China-Laos border (tropical region) was related closest to the LEIV/16275/Mag isolate obtained in Russia (North frigid zone area) among other isolates studied. These results suggest that GETV can adapt to different geographical environments to propagate and evolve. Thus, strengthening the detection and monitoring of GETV and its related diseases is very crucial.

Key words: Getah virus; China-Laos border; Phenotypic characteristics; Molecular evolution

Getah virus (GETV) was first isolated from *Culex* samples collected in Malaysia in 1955. The prototype virus strain was MM2021[1]. GETV belongs to the genus *Alphavirus* of the family Togaviridae and is a mosquito-transmitted arbovirus[2]. To date, GETV has been identified in about 10 countries or regions, including Australia[2], Malaysia[1], Japan[3], China[4], Mongolia[5], and Russia[5]. GETV can cause fever, body rashes, and leg edema in horses[3], as well as fetal death and reproduction disorders in pigs[6]. Thus, GETV is an important animal pathogen. Although antibodies neutralizing GETV have been identified in human serum samples in Malaysia, northern Australia, and Hainan Province in China[4,7], GETV has not been reported to cause human diseases.

An arbovirus investigation was conducted in the China-Laos border region (longitude 100°5'E, latitude 21°69'N) in August 2012. Specimens of *Armigeres subalbatus* collected during this investigation were ground and centrifuged. The obtained supernatant was utilized to inoculate BHK cells and the resulting cytopathic effect (CPE) was monitored[8]. After 32 h, BHK-21 cells infected with YN12031 exhibited an obvious CPE, including rounding up, aggregation, and exfoliation (Figure 1A). This CPE progressed rapidly to the '+++' level (i.e., 75% of the cells became cytopathic) about 48 h post-infection.

BHK cells inoculated with YN12031 were visualized by transmission electron microscopy (TEM)[8]. YN12031 exhibited a typical alphavirus morphology; the virus particles were spherical, 0-70 nm in diameter, enveloped, and featured surface fibers (Figure 1B1). After infection, the BHK cells were centrifuged, sectioned, and visualized by TEM. Virus particles were evident, and the majority were located in cytoplasmic vesicles. Virus particles contained a core with a high electron density (Figure 1B2).

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1. Shanxi Medical University, Taiyuan 030001, Shanxi, China; 2. State Key Laboratory of Infectious Disease Prevention and Control, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; 3. Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou 310058, Zhejiang, China; 4. Yunnan Institute of Parasitic Diseases, Pu'er 665000, Yunnan, China

To understand the plaque morphology and proliferation of YN12031 in tissue culture cells, we first observed the formation of virus plaques in BHK-21 cells and then detected dynamic changes in virus multiplication by plaque assay[9]. The plaques were 1.09 mm (1.15 ± 0.35 mm, *n* = 10, 2d) in diameter and regular in shape with distinct edges (Figure 1C). Following infection of BHK-21 cells at multiplicity of infeetion (MOIs) of 1 and 2, YN12031 proliferated rapidly about 8-24 h post-infection and reached peak titers of $1 \times 10^{7.69}$ and $1 \times 10^{7.55}$ pfu/mL, respectively. These titers decreased thereafter, reaching minima of $1 \times 10^{6.77}$ and $1 \times 10^{5.95}$ pfu/mL, respectively, at 72 h. Following infection at an MOI of 0.5, the YN12031 titer increased rapidly from 8 h to 16 h. The titer increased slowly from 16 h to 32 h, at which point it peaked ($1 \times 10^{7.86}$ pfu/mL). The YN12031 titer decreased slowly afterward, reaching a level comparable with that following infection at an MOI of 1 ($1 \times 10^{6.76}$ pfu/mL) at 72 h. These results are shown in Figure 1D.

BHK-21 cells infected with GETV exhibited an obvious CPE 32 h post-infection, and the CPE level reached '+++' after 48 h. The virus titer peaked 32 h post-infection [$1 \times 10^{7.86}$ pfu/mL (MOI = 1/2)] and then decreased gradually (Figure 1D). Previous studies have reported that BHK-21 cells infected with Sindbis virus (YN87448), the model virus of alphavirus, can exhibit an obvious CPE at 24 h and that the virus titer peaks ($1 \times 10^{9.5}$ pfu/mL) at 36 h[10]. By comparison, the CPE caused by infection with Japanese encephalitis virus, a flavivirus with linear positive-sense single-stranded RNA, appears at 48-72 h, and the highest viral titer could reach $1 \times 10^{7.1}$ pfu/mL[9]. These findings reveal that alphaviruses can cause CPEs in tissue culture cells faster than other virus types can.

Whole-genome amplification of YN12031 was performed using the GETV gene amplification primers described in Table 1. The amplified products were examined by agarose gel electrophoresis, purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA USA), and then sequenced directly. The sequences obtained were assembled, edited, and corrected using SeqMan in DNASTAR[8]. The coding region of YN12031 is 11,166 nt in length and encodes 3,720 amino acids. The length of the non-structural gene is 7,404 nt and located in the region between 79 and 7,482 nt. The non-structural gene codes four non-structural proteins (NSP1-NSP4) with a total of 2,467 amino acids. The structural gene is 3,762 nt in length and located in the region between 7,527 and 11,288 nt. This gene encodes a variety of structural proteins (E1, E2, E3, 6K, and capsid protein) with a total of 1,253 amino acids.

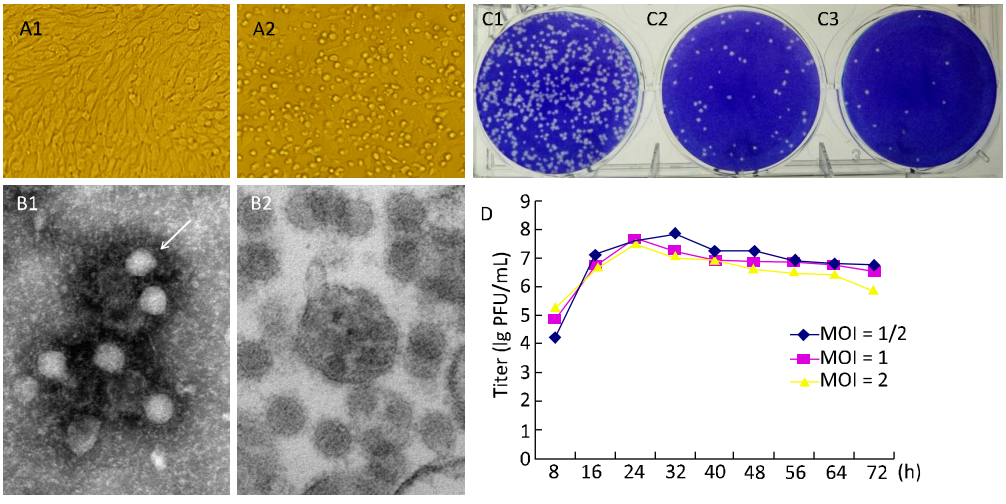

Figure 1. Biological characteristics of YN12031. (A) Cytopathic effect of YN12031 in BHK-21 cells (200 × magnification). (A1) Control (uninfected BHK-21 cells; 48 h). (A2) Infected BHK-21 cells 48 h post-infection showing rounding and exfoliation. (B) Electron micrographs of YN12031 particles negatively stained with 2% potassium phosphotungstate. (B1) Black arrow indicates an intact particle. (B2) Morphology of virus particles. (C) Plaques formed after inoculation of (C1) 10^{-4}, (C2) 10^{-5}, and (C3) 10^{-6} dilutions of YN12031. (D) Growth curve of YN12031 in BHK-21 cells.

Nucleotide and amino acid homology analyses of the GETV coding region (excluding 5' and 3' non-coding sequences) were conducted using MegAlign in DNASTAR (version 5.00) and BioEdit (version 7.0.5.3), respectively[8]. The nucleotide homology of YN12031 with 13 other GETVs obtained from GenBank ranged from 95.9% (Korean isolate South Korea) to 97.7% (Russian isolate LEIV/16275/Mag), and its amino acid homology ranged from 95.4% (Korean isolate South Korea) to 97.0% (Russian isolate LEIV/16275/Mag). The coding region of GETV was clearly relatively conserved. Both the nucleotide and amino acid homology between various strains varied between 95% and 98%, and YN12031 showed the closest relationship with the LEIV/16275/Mag isolate obtained in Russia among other GETV strains studied.

Twenty-six GETV strains isolated from both China and abroad were selected for homology analysis of the nucleotides and amino acids of the E2 gene. The nucleotide homology between YN12031 and 25 other strains ranged from 93.8% (Malaysia prototype strain MM2021) to 97.4% (Russian isolate LEIV/16275/Mag), and its amino acid homology ranged from 96.0% (Malaysia prototype strain MM2021) to 98.1% (Russian isolate LEIV/16275/Mag). The homology of YN12031 with the Russian isolate LEIV/16275/Mag was the closest found. GETV isolates used in this study and the sequence identities of the E2 gene are provided in Supplementary Tables 1 and 2, respectively, which are available in BES online.

Table 1. Primers Used in This Study

Primer	Amplity Region	Orientation	Sequence (5'-3')	Site in Genome
GETV-F1	5'UTR-Nsp1	Sense	ATGGCGGACGTGTGACATCAC	1-21
GETV-R1		Antisense	GTAACCTTCGCATGACACCACC	909-930
GETV-F2	Nsp1-Nsp2	Sense	GGCATTTACCTTCCGTGTTTC	848-868
GETV-R2		Antisense	TGTGCTTGCGGTGTAACCTTC	1710-1730
GETV-F3	Nsp2	Sense	TAGTGAGCGGCTCTTGTGCTG	1610-1630
GETV-R3		Antisense	CCGCACAGTACTACCTTACCTGAC	2493-2516
GETV-F4	Nsp2	Sense	GATGAGGCGTTCGCGTGTCACT	2434-2455
GETV-R4		Antisense	GGTAACCGACGATTGGATGGGACT	3480-3503
GETV-F5	Nsp2-Nsp3	Sense	TCTACGTGGCAACATGAACTCG	3399-3420
GETV-R5		Antisense	CGTGAATAAGTGGTTCAAGGACTGC	4440-4464
GETV-F6	Nsp3	Sense	GCTGTGGCTAGCATAATTAGTACC	4345-4368
GETV-R6		Antisense	TGGGATAGCGCGTATGTCTGT	5308-5328
GETV-F7	Nsp3-Nsp4	Sense	GTCGCCCAACTTAGACAGG	5212-5230
GETV-R7		Antisense	TGGTTGGTGGTATGCGTGG	6171-6189
GETV-F8	Nsp4	Sense	CCGATGAGTATGACGCTTATCTGG	6071-6094
GETV-R8		Antisense	ACTTCCATGTTGACCCAACTC	7098-7118
GETV-F9	Nsp4-C Protein	Sense	CGCTGCTGAACATTGTCATAG	6965-6985
GETV-R9		Antisense	GGTTGTCGATCACACCTTTG	7967-7986
GETV-F10	C Protein-E2	Sense	GATTGCATCTTCGAGGTCAAGC	7881-7922
GETV-R10		Antisense	GTGCGTGTTTGTACTGCACTTTG	8897-8919
GETV-F11	E2 -6K Protein	Sense	TGCGCTATTCGAGGCACGAT	8793-8812
GETV-R11		Antisense	ATGATTATGGCAGCGAGCGG	9861-9880
GETV-F12	E2-E1	Sense	CCGGTAACACTAGGAGTACTATGC	9744-9767
GETV-R12		Antisense	TTGTCATTCAGCGACGTGCCT	10712-10732
GETV-F13	E1-3'UTR	Sense	CCTCAAGTTGTCAAGACCTTCGTC	10625-10648
GETV-R13		Antisense	GTAAAATATTAAAAAAACAAATTAGACGCC	11661-11690

We constructed a phylogenetic tree using the coding region sequences of YN12031 and 10 other alphaviruses-Western equine encephalitis virus, Eastern equine encephalitis virus, Sindbis virus, Chikungunya virus, several Maya Luo virus, Barmah Forest virus, O'Nyong-nyong virus, Semliki fo rest virus, Ross River virus and GETV-using the neighbor-joining method (Figure 2A). YN12031 and MM2021, the prototype strain of GETV first isolated in Malaysia in 1955, were included in the same clade, which means YN12031 could be a GETV.

Further phylogenetic analysis of the E2 gene sequences of GETV isolates from Malaysia, Japan, South Korea, Russia, and China demonstrated that the Malaysia prototype strain MM2021 was located at the root of phylogenetic tree. The newly isolated strain YN12031 was identified in the same clade as the LEIV/16275/Mag isolate obtained in Russia (Figure 2B). Phylogenetic analysis of the GETV capsid gene sequences of these strains yielded similar results (Figure 2C).

In this study, the structural gene (E2) nucleotide and amino acid homologies between the GETV prototype strain isolated in Malaysia in 1955 and other GETVs isolated during the next 60 years were 93.8%-100% and 96.0%-100%, respectively, thus suggesting that the GETV gene is conserved. Phylogenetic analysis of E2 and capsid genes showed that GETVs isolated from different mosquito vectors, pigs, and horses in different countries (or regions), such as China, Japan, Korea, Mongolia, and Russia, from 1964 to 2014 were clustered in the same evolutionary clade. All of the GETVs isolated in Russia (60°N latitude) and Hainan province in China (19°N latitude) where thousands of kilometers apart from each other, and no matter GETVs isolated from specimens of mosquito or host animals (horses, pigs), they were all located at the different positions in the evolutionary branches. No vector, host specificity and geographic distribution characteristic were observed in the distribution of GETVs in the phylogenetic tree.

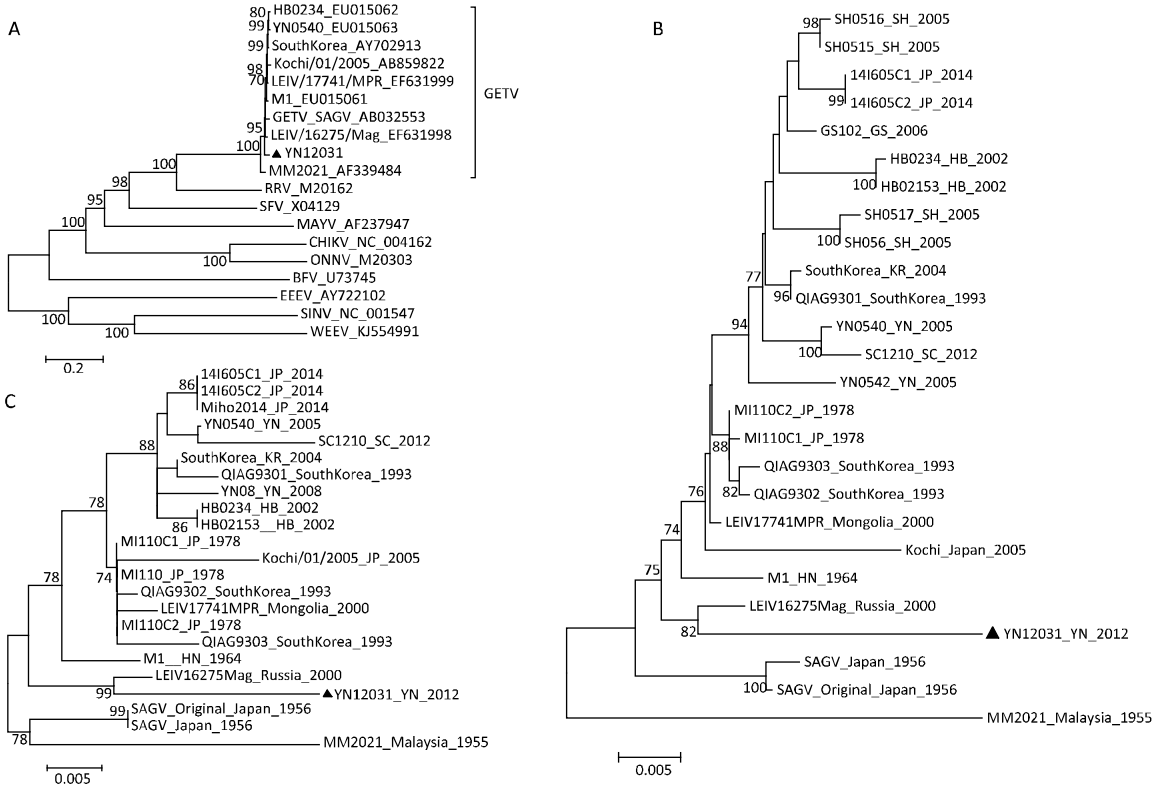

Figure 2. Phylogenetic analysis of YN12031. (A) Phylogenetic tree constructed using the coding region sequences of YN12031 and 10 other alphavirus strains. (B) Phylogenetic analysis of the E2 gene sequences of GETV isolates from Malaysia, Japan, South Korea, Russia, and China. (C) Phylogenetic tree constructed using the nucleotide sequences of the GETV capsid protein gene.

GETV was previously isolated form *A. subalbatus* in Yunnan Province[7], but the newly isolated strain (YN12031) is not located in the same evolutionary branch as two other stains (YN0540, YN0542) isolated from samples of *A. subalbatus* in the same province (Figure 2B-C), this finding suggests that diverse GETV populations with different evolutionary states exist in *A. subalbatus* in Yunnan Province. The YN12031 and LEIV/16275/Mag isolates obtained from mosquito specimens in Russia (60°N latitude) are in the same evolutionary branch, and homology analysis of the GETV coding region and E2 gene revealed the very close relationship between YN12031 and LEIV/16275/Mag. These results suggest that GETV could adapt to both tropical environments (Yunnan, China) and frigid zone environments (Russia) to survive and evolve; this characteristic could enable the spread of equine diseases[11] endemic in tropical regions to a wider range or higher latitude (such as Russia). Therefore, strengthening the detection and monitoring of GETV and its infections in temperate and northern frigid zones is essential.

GETV is an important animal pathogen, and epidemics of GETV infection among horses and pigs have emerged several times across Asia[3,6,11]. A number of GETV strains in China have been geographically isolated across the latitudes 19°N (Hainan Province) to 42°N (Liaoning Province) and longitudes 97°E (Gansu Province) to 124°E (Liaoning Province). GETV can be isolated from *Culex*[1,7], *Aedes*[1], *Armigeres*[7], and *Anopheles*[1]. Considering this prevalence, GETV and GETV infections present an increasing threat to animal health, particularly horses and pigs, and strengthening the detection and monitoring of GETV in animals is necessary to prevent development of the related animal diseases in China and reduce economic losses.

[&]LI Yuan Yuan and FU Shi Hong contributed equally to this work.

[#]Correspondence should be addressed to: LIANG Guo Dong, MD, Tel & Fax: 86-10-63510124, E-mail: gdliang@hotmail.com; WANG Gui Qin, MD, Tel & Fax: 86-13754830795, E-mail: guiqinwang321@163.com

Biographical notes of the first authors: LI Yuan Yuan, female, born in 1989, MS, majoring in Japanese encephalitis and other arboviruses; FU Shi Hong, female, born in 1967, Associate Chief Technician, majoring in Japanese encephalitis and other arboviruses.

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Table 1. GETV Isolates Analyzed in This Study

Strain	Date	Country	Host	Genebank Accession No		
				complete sequences	E2	Capsid
MM2021	1955	Malaysia	C.gelidus	-	AF339484	AF339484
GETV-SAGV	1956	Japan	mosquito	AB032553	AB032553	AB032553
GETV-SAGV-Original	1956	Japan	-	-	AF339483	AF339483
Kochi/01/2005	2005	Japan	Sus scrofa	AY702913	AB859822	AB859822
14-I-605-C2	2014	Japan	Equus caballus	LC079089	LC079089	LC079089
14-I-605-C1	2014	Japan	Equus caballus	LC079088	LC079088	LC079088
MI-110-C2	1978	Japan	Equus caballus	LC079087	LC079087	LC079087
MI-110-C1	1978	Japan	Equus caballus	LC079086	LC079086	LC079086
South Korea	2004	South Korea	swine	AY702913	AY702913	AY702913
LEIV 16275 Mag	2000	Russia	Aedes sp	EF631998	EF631998	EF631998
LEIV 17741 MPR	2000	Mongolia	Culex sp	EF631999	EF631999	EF631999
M1	1964	China, Hainan	Culex sp	EU015061	EU015061	EF375826
YN0540	2005	China, Yunnan	Armigeres subalbatus	EU015063	EU015063	EU015063
HB0234	2002	China, Hebei	Culex tritaeniorhynchus Giles	EU015062	EU015062	EF375825
SC1210	2012	China, Sichuan	Armigeres subalbatus	LC107870	LC107870	LC107870
HB0215-3	2002	China, Hebei	Culex tritaeniorhynchus Giles	-	EU015065	EF375824
QIAG9303	1993	South Korea	swine	-	KR081240	KR081240
QIAG9302	1993	South Korea	swine	-	KR081239	KR081239
QIAG9301	1993	South Korea	swine	-	KR081238	KR081238
GS10-2	2006	China, Gansu	Armigeres subalbatus	-	EU015070	-
SH05-17	2005	China, Shanghai	Culex tritaeniorhynchus	-	EU015069	-
SH05-16	2005	China, Shanghai	Culex tritaeniorhynchus	-	EU015068	-
SH05-15	2005	China, Shanghai	Culex tritaeniorhynchus	-	EU015067	-
SH05-6	2005	China, Shanghai	Culex tritaeniorhynchus	-	EU015066	-
YN0542	2005	China, Yunnan	Armigeres subalbatus	-	EU015064	-
YN12031*	2012	China, Yunnan	Armigeres subalbatus			
MI-110	1978	Japan	Equus caballus	-	-	LC012886
Miho2014	2014	Japan	Equus caballus	-	-	LC012884
YN08	2008	China, Yunnan	Aedes albopictus	-	-	JN578104

Note. *First report of the Getah virus in this study. '-': not available in GenBank.

Table 2. Sequence Identities for the E2 Gene of GETV

	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	MM2021		95	95.2	94.5	95.3	94.7	94.5	95.1	95.5	95.4	94.9	94.6	94.9	95	95.1	95.1	94.6	94.6	**93.8**	94.7	94.7	95.3	95.3	95.1	95.2	95.2
2	GETV_SAGV	96.4		99.7	96.8	97.6	97.2	96.9	97.5	97.9	97.9	97.5	97	97	97.2	97.3	97.2	97.4	97	**95.7**	97.1	97.1	97.9	97.9	97.7	97.8	97.6
3	SAGV_Original	96.9	99.5		96.9	97.9	97.4	97.1	97.6	98.2	98.2	97.6	97.2	97.2	97.4	97.5	97.3	97.6	97.2	**96.1**	97.2	97.2	98.2	98.3	98	98.1	97.7
4	Kochi	96.7	97.4	97.9		97.6	97.5	97	97.7	97.4	98.3	97.6	97.1	97.2	97.5	97.6	97.4	97.4	97.2	**95.7**	97.5	97.5	98.2	98.3	98	98.1	97.8
5	M1	96.9	97.6	98.1	97.9		98.2	97.7	98.4	98.6	99.1	98.3	97.8	97.9	98.3	98.4	98.1	98.1	98	**96.8**	98.2	98.2	98.9	99	98.7	98.8	98.5
6	YN0540	97.6	98.3	98.8	99.1	98.8		98.6	99.1	98	99	99	98.7	98.7	98.9	99	98.8	98.7	99.6	**96.2**	98.9	98.9	98.8	98.9	98.7	98.7	99.2
7	HB0234	96.9	97.6	98.1	98.3	98.3	99.3		98.7	97.7	98.5	98.8	99.9	98.5	98.7	98.8	98.7	98.3	98.3	**95.9**	98.7	98.7	98.3	98.4	98.2	98.3	98.7
8	SouthKorea	97.4	98.1	98.6	98.8	98.6	99.8	99.1		98.3	99.2	99.2	98.7	99.1	99.1	99.2	99.2	98.9	98.9	**96.4**	99	99	99.1	99.1	98.9	99	99.9
9	LEIV/16275/Mag	97.9	98.6	99.1	98.8	99.1	99.8	99.1	99.5		98.9	98.1	97.8	97.8	98	98.1	97.9	98.1	97.8	**97.4**	97.9	97.9	98.7	98.8	98.6	98.7	98.3
10	LEIV/17741/MPR	97.6	98.3	98.8	99.1	98.8	100	99.3	99.8	99.8		99.1	98.6	98.7	99	99.1	98.9	98.9	98.7	**97.1**	98.8	98.8	99.7	99.8	99.5	99.6	99.3
11	GS10-2	97.9	98.1	98.6	98.8	98.6	99.8	99.1	99.5	99.5	99.8		98.9	98.9	99.4	99.5	99.1	98.7	98.7	**96.3**	99.3	99.3	99.1	99.1	98.9	99	99.3
12	HB0215-3	96.9	97.6	98.1	98.3	98.3	99.3	100	99.1	99.1	99.3	99.1		98.6	98.8	98.9	98.7	98.4	98.4	**96**	98.8	98.8	98.4	98.5	98.3	98.3	98.8
13	SH05-17	97.2	97.9	98.3	98.6	98.3	99.5	98.8	99.3	99.3	99.5	99.3	98.8		98.8	98.9	99.8	98.4	98.4	**96**	98.7	98.7	98.6	98.7	98.4	98.5	99.1
14	SH05-16	97.4	98.1	98.6	98.8	98.6	99.8	99.1	99.5	99.5	99.8	99.5	99.1	99.3		99.9	99	98.7	98.7	**96.2**	99.4	99.4	98.8	98.9	98.7	98.7	99.2
15	SH05-15	97.6	98.3	98.8	99.1	98.8	100	99.3	99.8	99.8	100	99.8	99.3	99.5	99.8		99.1	98.7	98.7	**96.3**	99.4	99.4	98.9	99	98.7	98.8	99.3
16	SH05-6	97.6	98.3	98.8	99.1	98.8	100	99.3	99.8	99.8	100	99.8	99.3	99.5	99.8	100		98.6	98.6	**96.1**	98.8	98.8	98.7	98.8	98.6	98.7	99.3
17	YN0542	97.6	98.3	98.8	99.1	98.8	100	99.3	99.8	99.8	100	99.8	99.3	99.5	99.8	100	100		98.4	**96.1**	98.5	98.5	98.8	98.9	98.7	98.7	99
18	SC1210	97.1	98.1	98.3	98.6	98.3	99.5	98.8	99.3	99.3	99.5	99.3	98.8	99.1	99.3	99.5	99.5	99.5		**96**	98.7	98.7	98.6	98.7	98.4	98.5	99
19	**YN12031**	**96**	**96.7**	**97.2**	**96.9**	**97.6**	**97.9**	**97.2**	**97.6**	**98.1**	**97.9**	**97.6**	**97.2**	**97.4**	**97.6**	**97.9**	**97.9**	**97.9**	**97.4**		**96.2**	**96.2**	**96.9**	**97**	**96.8**	**96.8**	**96.5**
20	14-I-605-C1	97.6	98.3	98.8	99.1	98.8	100	99.3	99.8	99.8	100	99.8	99.3	99.5	99.8	100	100	100	99.5	**97.9**		100	98.7	98.7	98.5	98.6	99.1
21	14-I-605-C2	97.6	98.3	98.8	99.1	98.8	100	99.3	99.8	99.8	100	99.8	99.3	99.5	99.8	100	100	100	99.5	**97.9**	100		98.7	98.7	98.5	98.6	99.1
22	MI-110-C1	97.4	98.1	98.6	98.8	98.6	99.8	99.1	99.5	99.5	99.8	99.5	99.1	99.3	99.5	99.8	99.8	99.8	99.3	**97.6**	99.8	99.8		99.9	99.7	99.8	99.1
23	MI-110-C2	97.6	98.3	98.8	99.1	98.8	100	99.3	99.8	99.8	100	99.8	99.3	99.5	99.8	100	100	100	99.5	**97.9**	100	100	99.8		99.8	99.8	99.2
24	QIAG9303	97.2	97.9	98.3	98.6	98.3	99.5	98.8	99.3	99.3	99.5	99.3	98.8	99.1	99.3	99.5	99.5	99.5	99.1	**97.4**	99.5	99.5	99.3	99.5		99.8	99
25	QIAG9302	97.4	98.1	98.6	98.8	98.6	99.8	99.1	99.5	99.5	99.8	99.5	99.1	99.3	99.5	99.8	99.8	99.8	99.3	**97.6**	99.8	99.8	99.5	99.8	99.3		99.1
26	QIAG9301	97.6	98.3	98.8	99.1	98.8	100	99.3	99.8	99.8	100	99.8	99.3	99.5	99.8	100	100	100	99.5	**97.9**	100	100	99.8	100	99.5	99.8	

Note. Percentage identity was determined from pairwise comparisons of nucleotide sequences (above the diagonal) and amino acid sequences (below the diagonal) of the E2 gene.