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Molecular epidemiological analysis of *Japanese encephalitis virus* in China

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Sixty-two new Japanese encephalitis virus (JEV) isolates were obtained from mosquitoes, biting midges, human cerebrospinal fluid and human blood samples in China during 2002–2005. The E and prM genes were sequenced and phylogenetic analyses were performed with 38 JEV other isolates from China and 36 JEV strains from other countries. Phylogenetic trees based on the E and prM gene sequences were similar. The results indicate that: (i) recent JEV isolates from China are divided into two genotypes, genotype 1 and genotype 3; (ii) recent JEV isolates from China are grouped into the same clusters within genotypes 1 and 3; and (iii) genotype 1 JEV strains have been isolated in China since 1979, whilst genotype 3 JEV strains were isolated before the 1970s. The results suggest that genotype 1 JEV was introduced to China around 1979 and that JEV strains belonging to genotypes 1 and 3 circulate in China.

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INTRODUCTION

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that causes severe encephalitis in humans (Burke & Leake, 1988; Vaughn & Hoke, 1992). Japanese encephalitis (JE) is the most important cause of epidemic viral encephalitis in the South-East Asian and western Pacific regions, the Indian subcontinent and China, with an estimated 35 000–50 000 reported cases and 10 000 deaths annually. Of JE cases, around 25 % are fatal, and nearly 50 % of survivors, especially young children, display persistent neurological and/or psychological sequelae (Burke & Leake, 1988). JEV exists in a zoonotic cycle between mosquitoes and pigs and/or water birds (Monath & Heinz, 1996). Since the first report in 1949, JE epidemics have occurred in China for over 50 years. JEV is active in all of the provinces in China except for Tibet, Xinjiang and Qinhai provinces, and nearly 80 % of globally reported cases occur in China. In China, the most important vector is *Culex tritaeniorhynchus*.

Phylogenetic analyses of JEV first focused on the prM region with limited sequence information (Chen *et al.*, 1990, 1992; Huong *et al.*, 1993; Ali & Igarashi, 1997), and JEV strains were divided into four genotypes. Then, the E region was targeted for JEV phylogenetic analyses and JEV strains were divided into five genotypes, with one isolate from Singapore (Ni & Barrett, 1995; Paranjpe & Banerjee, 1996; Mangada & Takegami, 1999; Williams *et al.*, 2000; Solomon *et al.* 2003; Nga *et al.* 2004). Molecular biological research of JEV strains isolated in China is important for understanding the

characteristics of JEV circulating in Asia. So far, there has been only limited information about the genetic difference among JEV strains isolated throughout China. It was reported that JEV isolated between 1949 and 1987 belonged to genotype 3 (Li *et al.*, 2004). It was reported recently that JEV strains isolated in Shanghai in 2001 belonged to genotype 1 (Wang *et al.*, 2004) and that those isolated in Fujian Province in 2002 belonged to genotype 3 (Chen *et al.*, 2005).

In the present study, JEV strains isolated in different areas of China during the period 1949–2005 were analysed and compared with a large group of previously published JEV strains, especially those from China. The study will provide information necessary for understanding JEV molecular epidemiology in Asia.

METHODS

Sixty-two JEV isolates were obtained from mosquitoes, biting midges, human cerebrospinal fluid and human blood samples in China during 2002–2005. These included two isolates from mosquitoes and two from biting midges in Heilongjiang Province, two from mosquitoes in Liaoning Province, two from human blood samples and one from a cerebrospinal fluid sample in Fujian Province in 2002; nine from mosquitos in Shanghai, one from a mosquito in Yunnan Province and 11 from human blood samples in Fujian Province in 2003; three from mosquitoes in Henan Province, three from mosquitoes in Shanghai, six from mosquitoes in Sichuan Province, seven from mosquitoes in Guizhou Province and 12 from mosquitoes in Yunnan Province in 2004; and one from a mosquito in Shanghai in 2005. The locations of the provinces are shown in Fig. 1. Among them, 56 virus isolates were obtained by inoculation onto C6/36 cells and six by inoculation into mouse brains. One isolate from 1979 and seven isolates from the 1980s from Yunnan Province were newly identified by RT-PCR and included in the analysis (Table 1).

Viral RNA was extracted from a JEV-infected C6/36 cell culture by using a QIAamp viral RNA extraction kit (Qiagen). Briefly, purified RNA was used as template for cDNA synthesis using Ready-to-Go You-Prime First-Strand beads (Amersham Biosciences). The prM gene was amplified by RT-PCR with a pair of primers, JEV-prMf [5′-CGTTCTTCAAGTTTACAGCATTAGC-3′ (251–275)] and JEV-prMr [5′-CCYRTGTTYCTGCCAAGCATCCAMCC-3′ (901–925)], and the complete E gene was amplified with JEV-Ef [5′-TGYTGGTCGCT-CCGGCTTA-3′ (955–973)] and JEV-Er [5′-AAGATGCCACTTC-CACAYCTC-3′ (2516–2536)]. RT-PCR products were purified by using an ExoSAP-IT PCR purification kit (USB Corp.). The sequences of the purified DNA products were determined with an ABI PRISM 3100 DNA sequencer. Multiple alignments and phylogenetic analysis were performed by the neighbour-joining (NJ) method using the CLUSTAL_X program (Thompson *et al.*, 1997). The bootstrap probabilities of each node were calculated using 1000 replicates. A bootstrap values $>70\,\%$ was defined as the criterion for phylogenetic grouping (Hillis & Bull, 1993). All of the phylogenetic trees were drawn by using TreeView software (Page, 1996). A list of the JEV isolates used for analyses is shown with origin and years of isolation in Tables 1 and 2.

The genetic relationships of the new isolates were analysed along with the selected previous Chinese isolates and those representing five JEV genotypes by NJ (Fitch & Margoliash, 1967).

RESULTS

A phylogenetic tree was prepared based on the E gene sequences of 135 JEV isolates, including 65 isolates from

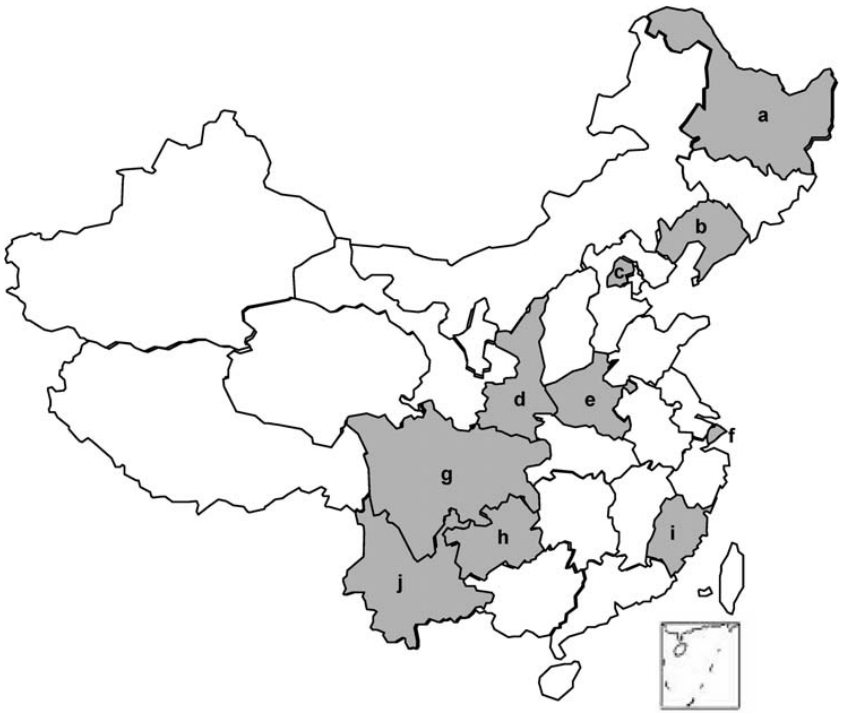

Fig. 1. Geographical locations of all JEV isolates used in this study, covering a time span of 57 years (1949–2005) in China. (a) Heilongjiang Province (HLJ), 1950s/one strain, 1960s/one strain, 2002/four strains; (b) Liaoning Province (LN), 1971/one strain, 2002/two strains; (c) Beijing (BJ), 1949/two strains, 1960s/one strain; (d) Shaanxi Province (SX), 1960/one strain, vaccine/one strain; (e) Henan Province (HN), 2004/three strains; (f) Shanghai (SH), 1987/one strain, 2001/seven strains, 2003/nine strains, 2004/three strains, 2005/one strain; (g) Sichuan Province (SC), 1957/one strain, 2004/six strains; (h) Guizhou Province (GZ), 2004/seven strains; (i) Fujian Province (FJ), 1954/three strains, 1955/five strains, 1957/one strain, 2002/six strains, 2003/11 strains; (j) Yunnan Province (YN), 1954/one strain, 1979/one strain, 1980s/seven strains, 2003/one strain, 2004/12 strains.

Table 1. Details of JEV strains from China used for analysis in this study

IU, Information unavailable. MVEV was used as an outgroup in all phylogenetic analyses.

Strain	Year	Geographical location	Source	Genotype	GenBank accession no.	
					prM gene	E gene
47	1950s	China, Heilongjiang	Human brain	3	AY243810	AY243827
Ha-3	1960s	China, Heilongjiang	Human brain	3	AY243806	AY243842
HLJ02-134*	2002	China, Heilongjiang	*Genus culicoides*	3	DQ40414	DQ404081
HLJ02-136*	2002	China, Heilongjiang	*Genus culicoides*	3	DQ40415	DQ404082
HLJ02-144*	2002	China, Heilongjiang	*Aedes vexans*	3	DQ40416	DQ404083
HLJ02-170*	2002	China, Heilongjiang	*Aedes vexans*	3	DQ40417	DQ404084
TLA	1971	China, Liaoning	Human brain	3	AY243808	AY243832
LN02-102*	2002	China, Liaoning	*Culex modestus*	1	DQ40418	DQ404085
LN02-104*	2002	China, Liaoning	*Culex pipiens pallens*	1	DQ40419	DQ404086
A2	1949	China, Beijing	Human brain	3	AY243809	AY243843
P3	1949	China, Beijing	Human brain	3	AY243805	AY243844
GSS	1960s	China, Beijing	Human brain	3	AY243816	AY243845
HN04-11*	2004	China, Henan	*Culex*	1	DQ40420	DQ404087
HN04-21*	2004	China, Henan	*Culex*	1	DQ40421	DQ404088
HN04-40*	2004	China, Henan	*Culex*	1	DQ40422	DQ404089
SA14	1960	China, Shanxi	Mosquito pool	3	AY243807	AY243850
SA14-14-2		China	Vaccine	3	AF315119	AF315119
CH-13	1957	China, Sichuan	Human brain	3	AY243813	AY243835
SC04-12*	2004	China, Sichuan	*Culex*	1	DQ40423	DQ404090
SC04-15*	2004	China, Sichuan	*Culex tritaeniorhynchus*	1	DQ40424	DQ404091
SC04-16*	2004	China, Sichuan	*Armigeres*	1	DQ40425	DQ404092
SC04-17*	2004	China, Sichuan	*Culex tritaeniorhynchus*	1	DQ40426	DQ404093
SC04-25*	2004	China, Sichuan	*Culex*	1	DQ40427	DQ404094
SC04-27*	2004	China, Sichuan	*Culex*	1	DQ40428	DQ404095
SH-3	1987	China, Shanghai	Human brain	3	AY243819	AY243826
SH-53	2001	China, Shanghai	*Culex tritaeniorhynchus*	1	AY555746	AY555757
SH-80	2001	China, Shanghai	*Culex tritaeniorhynchus*	1	AY243823	AY243841
SH-81	2001	China, Shanghai	*Culex tritaeniorhynchus*	1	AY555747	AY555758
SH-83	2001	China, Shanghai	*Culex tritaeniorhynchus*	1	AY555748	AY555759
SH-90	2001	China, Shanghai	*Culex tritaeniorhynchus*	1	AY243822	AY243835
SH-96	2001	China, Shanghai	*Culex tritaeniorhynchus*	1	AY555749	AY555760
SH-101	2001	China, Shanghai	*Culex tritaeniorhynchus*	1	AY555750	AY555761
SH03-103*	2003	China, Shanghai	*Culex tritaeniorhynchus*	1	DQ40429	DQ404096
SH03-105*	2003	China, Shanghai	*Culex tritaeniorhynchus*	1	DQ40430	DQ404097
SH03-109*	2003	China, Shanghai	*Culex tritaeniorhynchus*	1	DQ40431	DQ404098
SH03-115*	2003	China, Shanghai	*Culex tritaeniorhynchus*	1	DQ40432	DQ404099
SH03-124*	2003	China, Shanghai	*Culex tritaeniorhynchus*	1	DQ40433	DQ404100
SH03-127*	2003	China, Shanghai	*Culex tritaeniorhynchus*	1	DQ40434	DQ404101
SH03-128*	2003	China, Shanghai	*Culex tritaeniorhynchus*	1	DQ40435	DQ404102
SH03-129*	2003	China, Shanghai	*Culex tritaeniorhynchus*	1	DQ40436	DQ404103
SH03-130*	2003	China, Shanghai	*Culex tritaeniorhynchus*	1	DQ40437	DQ404104
SH04-3*	2004	China, Shanghai	*Culex tritaeniorhynchus*	3	DQ40438	DQ404105
SH04-5*	2004	China, Shanghai	*Culex tritaeniorhynchus*	3	DQ40439	DQ404106
SH04-10*	2004	China, Shanghai	*Culex tritaeniorhynchus*	3	DQ40440	DQ404107
SH05-24*	2005	China, Shanghai	*Culex tritaeniorhynchus*	1	DQ40441	DQ404108
GZ04-2*	2004	China, Guizhou	*Armigeres*	3	DQ40442	DQ404109
GZ04-4*	2004	China, Guizhou	*Armigeres*	3	DQ40443	DQ404110
GZ04-29*	2004	China, Guizhou	*Culex*	3	DQ40444	DQ404111
GZ04-36*	2004	China, Guizhou	*Culex*	3	DQ40445	DQ404112
GZ04-43*	2004	China, Guizhou	*Culex*	3	DQ40446	DQ404113
GZ04-71*	2004	China, Guizhou	*Armigeres*	3	DQ40447	DQ404114

Table 1. cont.

Strain	Year	Geographical location	Source	Genotype	GenBank accession no.	
					prM gene	E gene
GZ04-89*	2004	China, Guizhou	*Armigeres*	3	DQ40448	DQ404115
G35	1954	China, Fujian	Mosquito pool	3	AY243815	AY243831
CBH	1954	China, Fujian	Human brain	3	AY243812	DQ404116
CZX	1954	China, Fujian	Human brain	3	AY243811	AY243828
YLG	1955	China, Fujian	Human brain	3	AY243824	AY243837
ZSZ	1955	China, Fujian	Human brain	3	AY243825	AY243839
LFM	1955	China, Fujian	Human blood	3	AY243817	AY243833
CTS	1955	China, Fujian	Human brain	3	AY243814	AY243830
ZMT	1955	China, Fujian	Human brain	3	AY243821	AY243840
LYZ	1957	China, Fujian	Human brain	3	AY243818	AY243834
02-29*	2002	China, Fujian	Human cerebrospinal fluid	3	AY555751	AY555762
02-41	2002	China, Fujian	Human blood	3	AY555752	AY555763
02-43	2002	China, Fujian	Human blood	3	AY555753	AY555764
02-76*	2002	China, Fujian	Human blood	3	AY555754	AY555765
02-84*	2002	China, Fujian	Human blood	3	AY555755	AY555766
02-102	2002	China, Fujian	Human blood	3	AY555756	AY555767
FJ03-31*	2003	China, Fujian	Human blood	3	DQ40449	DQ404117
FJ03-35*	2003	China, Fujian	Human blood	3	DQ40450	DQ404118
FJ03-39*	2003	China, Fujian	Human blood	3	DQ40451	DQ404119
FJ03-46*	2003	China, Fujian	Human blood	3	DQ40452	DQ404120
FJ03-56*	2003	China, Fujian	Human blood	3	DQ40453	DQ404121
FJ03-66*	2003	China, Fujian	Human blood	3	DQ40454	DQ404122
FJ03-67*	2003	China, Fujian	Human blood	3	DQ40455	DQ404123
FJ03-68*	2003	China, Fujian	Human blood	3	DQ40456	DQ404124
FJ03-69*	2003	China, Fujian	Human blood	3	DQ40457	DQ404125
FJ03-94*	2003	China, Fujian	Human blood	3	DQ40458	DQ404126
FJ03-97*	2003	China, Fujian	Human blood	3	DQ40459	DQ404127
YN	1954	China, Yunnan	Human brain	3	AY243820	AY243838
YN79-Bao83*	1979	China, Yunnan	*Culex tritaeniorhynchus*	1	DQ40460	DQ404128
YN82-BN8219*	1982	China, Yunnan	*Culex*	1	DQ40461	DQ404129
YN83-Meng83-54*	1983	China, Yunnan	*Forcipomyia taiwana* Shiraki	1	DQ40462	DQ404130
YN83-83199*	1983	China, Yunnan	*Culex*	1	DQ40463	DQ404131
YN85-L86-99*	1985	China, Yunnan	*Culex*	1	DQ40464	DQ404132
YN86-B8639*	1986	China, Yunnan	*Culex tritaeniorhynchus*	1	DQ40465	DQ404133
YN86-86266*	1986	China, Yunnan	IU	1	DQ40466	DQ404134
YN-Xiang JE*	IU	China, Yunnan	Human blood	1	DQ40467	DQ404135
YN98-151*	1998	China, Yunnan	Mosquitoes	3	DQ40468	DQ404136
YNDL04-1*	2004	China, Yunnan	*Culex tritaeniorhynchus*	3	DQ40469	DQ404137
YNDL04-6*	2004	China, Yunnan	*Culex pipiens quinquef ascitatus*	3	DQ40470	DQ404138
YNDL04-29*	2004	China, Yunnan	*Culex theileri*	3	DQ40471	DQ404139
YNDL04-31*	2004	China, Yunnan	*Culex theileri*	3	DQ40472	DQ404140
YNDL04-37*	2004	China, Yunnan	*Anopheles sinensis*	3	DQ40473	DQ404141
YNDL04-39*	2004	China, Yunnan	*Culex tritaeniorhynchus*	3	DQ40474	DQ404142
YNDL04-42*	2004	China, Yunnan	*Armigeres subalbatus*	3	DQ40475	DQ404143
YNDL04-44*	2004	China, Yunnan	*Culex theileri*	3	DQ40476	DQ404144
YNDL04-45*	2004	China, Yunnan	Mosquito pool	3	DQ40477	DQ404145
YNJH04-18*	2004	China, Yunnan	Mosquito pool	3	DQ40478	DQ404146
YNJH04-19*	2004	China, Yunnan	Unclassified *Culex*	3	DQ40479	DQ404147
YNJH04-25-3*	2004	China, Yunnan	*Culex*	3	DQ40480	DQ404148

*Sequences used in the present study.

Table 2. Details of JEV strains used for analysis in this study

NA, Not available in GenBank; IU, information unavailable. MVEV was used as an outgroup in all phylogenetic analyses.

Strain	Year	Geographical location	Source	GenBank accession no.	
				prM gene	E gene
MVE-1-51	1951	Australia	Human brain	NC_000943	NC_000943
FU	1995	Australia	Human serum	AF217620	AF217620
TS5313	2000	Australia	Mosquito pool	AY251615	NA
M859	1967	Cambodia	Mosquito	D00984	U70410
P20778	1958	India	Human brain	AF080251	AF080251
GP78	1978	India	Human brain	AF075723	AF075723
JKT1724	1979	Indonesia, Java	*Culex tritaeniorhynchus*	D00995	U70404
JKT5441	1981	Indonesia, Bali	*Anopheles vagus*	L42164	U70406
JKT6468	1981	Indonesia, Flores	*Culex tritaeniorhynchus*	AY184212	AY184212
Nakayama-RFVL	1935	Japan, Nakayama	Human brain	S75726	S75726
JaGAr01	1959	Japan, Gunma	*Culex tritaeniorhynchus*	AF069076	AF069076
Kamiyama	1966	Japan	Human brain	Hasegawa *et al.* (1994)	S47265
JaOArS982	1982	Japan, Osaka	*Culex tritaeniorhynchus*	M18370	M18370
Ishikawa	1994	Japan, Ishikawa	Swine mononuclear cells	AB051292	AB051292
JaNAr0102	2002	Japan, Nagasaki	Mosquito	AB178127	AY377577
K87P39	1987	Korea	Mosquito	AY585242	AY585242
K94P05	1994	Korea	Mosquito	AF045551	AF045551
KV1899	1999	Korea	Pig serum	AY316157	AY316157
Sarawak	1965	Malaysia	Mosquito	AY184214	AY184214
WTP/70/22	1970	Malaysia, Kuala Lumpur	Mosquito pool	D00998	U70421
CNS138-11	1999	Malaysia	Human brain	NA	AY184213
B2524	1985	Nepal	Human cerebrospinal fluid	D00956	U70392
DH20	1985	Nepal	Human brain	U03690	U03690
PhAn1242	1984	Philippine, Santo Cristo	Pig serum	D00982	U70417
Muar	1952	Singapore	Human brain	Hasegawa *et al.* (1994)	Hasegawa *et al.* (1994)
691004	1969	Sri Lanka	Human blood	D00965	Z34097
H-49778	1987	Sri Lanka	Human brain	D00981	U70395
Ling	1965	Taiwan	Human brain	L78128	L78128
HK 8256	1972	Taiwan	*Culex annulus*	D00963	U70396
RP-9	1985	Taiwan	Mosquito	AF014161	AF014161
T1P1	1997	Taiwan	*Armigeres subalbatus*	AF254453	AF254453
Th2372	1979	Thailand	Human brain	D76424	U70401
B-1065	1983	Thailand, Chumporn	Pig blood	D00996	U70388
ThCMAr6793	1993	Thailand	*Culex tritaeniorhynchus*	D45361	D45363
VN-118	1979	Vietnam, Ho Chi Minh City	*Culex fatigans*	D00975	U70420
01VN88	2001	Vietnam	Pig blood	NA	AY376464
02VN78	2002	Vietnam	Mosquito	NA	AY376467

2002–2005, seven isolates from 2001, 28 isolates from 1949–1986 in China, 35 isolates from other countries and *Murray Valley encephalitis virus* (MVEV) (Fig. 2). The JEV isolates from 2001–2005 in China were divided into two genotypes, genotype 1 and genotype 3. Thirty-six isolates in Shanghai (2001, 2003, 2005), Liaoning (2002), Henan (2004) and Sichuan (2004) provinces were clustered in genotype 1, along with those isolated in China during 1979–1986 and two isolates from Japan in 1994 and 2002. Forty-eight isolates in Heilongjiang (2002), Fujian (2002, 2003), Yunnan (2003, 2004) and Guizhou (2004) provinces and Shanghai (2004) were clustered in genotype 3. Sixteen isolates from 1949–1971 and one in 1987 from China were grouped into the other cluster in genotype 3, along with those isolated in neighbouring countries from the 1930s to the 1980s.

A phylogenetic tree was also prepared based on the prM gene sequences of 133 isolates, including 65 isolates from 2002–2005, seven isolates from 2001, 28 isolates from 1949–1986 in China, 33 isolates from other countries and MVEV (Fig. 3). The JEV isolates from 2001–2005 in China were divided into two genotypes, genotype 1 and genotype 3. Thirty-six isolates in Shanghai (2001, 2003, 2005), Liaoning (2002), Henan (2004) and Sichuan (2004) provinces were clustered in genotype 1, along with those isolated in China


MVEV
JKT6468 (Indonesia, 1981)
GIV
WTP/70/22 (Malaysia, 1970)
FU (Australia, 1995)
B-1065 (Thailand, 1983)
GII
JKT1724 (Indonesia, 1979)
JKT5441 (Indonesia, 1981)
M-859 (Cambodia, 1967)
HN04-40 (China-HN, 2004)*
SH-81 (China-SH, 2001)
TS5313 (Australia, 2000)
Th2372 (Thailand, 1979)
GI
SH03-130 (China-SH, 2003)*
Ishikawa (Japan, 1994)
JaNAr0102 (Japan, 2002)
SH-53 (China-SH, 2001)
K94P05 (Korea, 1994)
SH03-128 (China-SH, 2003)*
SH03-124 (China-SH, 2003)*
SH-83 (China-SH, 2001)
SH03-109 (China-SH, 2003)*
SC04-17 (China-SC, 2004) *
HN04-11 (China-HN, 2004)*
SC04-27 (China-SC, 2004) *
SC04-16 (China-SC, 2004)*
SC04-12 (China-SC, 2004)*
SH05-24 (China-SH, 2005) *
SC04-25 (China-SC, 2004) *
HN04-21 (China-HN, 2004)*
SC04-15 (China-SC, 2004)*
SH-101 (China-SH, 2001)
SH03-129 (China-SH, 2003)*
SH03-127 (China-SH, 2003)*
YN86-B8639 (China-YN, 1986)*
SH03-105 (China-SH, 2003) *
YN-Xiang-JE (China-YN)*
YN86-86266 (China-YN, 1986) *
YN85-L86-99 (China-YN, 1985)*
YN83-83199 (China-YN, 1983) *
YN83-Meng83-54 (China-YN, 1983)*
YN82-BN8219 (China-YN, 1982)*
YN79-Bao83 (China-YN, 1979) *
SH03-103 (China-SH, 2003)*
LN02-102 (China-LN, 2002) *
LN02-104 (China-LN, 2002) *
ThCMAr6793 (Thailand, 1993)
SH-96 (China-SH, 2001)
SH-80 (China-SH, 2001)
SH-90 (China-SH, 2001)
KV1899 (Korea,1999)
SH03-115 (China-SH, 2003)*
Muar (Singapore, 1952)
GP78 (India, 1978)
H49778 (Sri Lanka, 1987)
Kamiyama (Japan, 1966)
JaOArS982 (Japan, 1982)
GIII
JaGAr-01 (Japan, 1959)
PhAn1242 (Philippine, 1984)
HK8256 (Taiwan, 1972)
YLG (China-FJ, 1955)
RP-9 (Taiwan, 1985)
T1P1 (Taiwan, 1997)
ZMT (China-FJ, 1955)
B-2524 (Nepal, 1985)
CTS (China-FJ, 1955)
CH-13 (China-SC, 1957)
GSS (China-BJ, 1960s)
LYZ (China-FJ, 1957)
ZSZ (China-FJ, 1955)
47 (China-HLJ, 1950s)
SA14-14-2 (China)
K87P39 (Korea, 1987)
Ha-3 (China-HLJ, 1960s)
SH-3 (China-SH, 1987)
CZX (China-FJ, 1954)
P-20778 (India, 1958)
TLA (China-LN, 1971)
YN (China-YN, 1954)
VN-118 (Vietnam, 1979)
Ling (Taiwan, 1965)
SA14 (China-SX, 1960)
691004 (Sri Lanka, 1969)
DH20 (Nepal, 1985)
Beijing-1/A2 (China-BJ, 1949)
Nakayama-RFVL (Japan, 1935)
Sarawak (Malaysia, 1965)
P3 (China-BJ, 1949)
YNDL04-45 (China-YN, 2004)*
YN98-151 (China-YN, 1998) *
LFM (China-FJ, 1955)
FJ03-66 (China-FJ, 2003) *
G35 (China-FJ, 1954)
02-41 (China-FJ, 2002)
CBH (China-FJ, 1954)
GZ04-43 (China-GZ, 2004) *
YNDL04-44 (China-YN, 2004) *
YNDL04-29 (China-YN, 2004) *
YNJH04-19 (China-YN, 2004) *
YNJH04-25-3 (China-YN, 2004) *
YNJH04-18 (China-YN, 2004) *
YNDL04-42 (China-YN, 2004)*
YNDL04- 39(China-YN, 2004) *
YNDL04-37 (China-YN, 2004) *
YNDL04-31 (China-YN, 2004) *
YNDL04-6 (China-YN, 2004)*
YNDL04-1 (China-YN, 2004)*
FJ03-97 (China-FJ, 2003)*
FJ03-94 (China-FJ, 2003)*
FJ03-69 (China-FJ, 2003)*
FJ03-68 (China-FJ, 2003)*
FJ03-67 (China-FJ, 2003)*
FJ03-56 (China-FJ, 2003)*
FJ03-46 (China-FJ, 2003)*
FJ03-39 (China-FJ, 2003)*
HLJ02-134 (China-HLJ, 2002)*
HLJ02-136 (China-HLJ, 2002)*
HLJ02-144 (China-HLJ, 2002)*
HLJ02-170 (China-HLJ, 2002)*
SH04-3 (China-SH, 2004)*
SH03-5 (China-SH, 2004)*
SH04-10 (China-SH, 2004)*
GZ04-2 (China-GZ, 2004)*
GZ04-4 (China-GZ, 2004)*
GZ04-29 (China-GZ, 2004)*
02-29 (China-FJ, 2002)*
GZ04-71 (China-GZ, 2004)*
GZ04-89 (China-GZ, 2004)*
02-29 (China-FJ, 2002)*
02-43 (China-FJ, 2002)
02-76 (China-FJ, 2002)*
02-84 (China-FJ, 2002)*
02-102 (China-FJ, 2002)
FJ03-35 (China-FJ, 2003)*
FJ03-31 (China-FJ, 2003)*
0.1

during 1979–1986 and those isolated in neighbouring countries, Japan, Korea, Vietnam and Thailand, in the 1990s and 2000s. Forty-eight isolates in Heilongjiang (2002), Fujian (2002, 2003), Yunnan (2003, 2004) and Guizhou (2004) provinces and Shanghai (2004) were clustered in genotype 3. The isolates from China from the 1940s to the 1980s were grouped into the other cluster within genotype 3, along with those isolated in other Asian countries from the 1950s to the 1980s.

Phylogenetic trees based on the E and prM gene sequences provided similar topology, with differences confined predominantly to bootstrap support. The results indicate that: (i) recent JEV isolates from China are divided into two genotypes, genotype 1 and genotype 3; (ii) recent JEV isolates from China are grouped into same clusters within genotypes 1 and 3; (iii) genotype 1 JEV strains have been isolated in China since 1979, whilst genotype 3 JEV strains have been isolated since the 1940s. Interestingly, the genotypes of JEV isolated in Shanghai differed from year to year: genotype 1 in 2001, 2003 and 2005 and genotype 3 in 2004.

Phylogenetic analyses of the JEV isolates in different geographical areas of the world for long periods of time were done in several studies. Early analyses were based on short, limited sequence of the prM gene (Chen *et al.*, 1990, 1992; Ni & Barrett, 1995; Nam *et al.*, 1996; Paranjpe & Banerjee, 1996; Ali & Igarashi, 1997; Tsuchie *et al.*, 1997; Wu *et al.*, 1998; Mangada & Takegami, 1999). However, it is possible that analysis based on short sequences leads to somewhat unclear and unreliable results (Westaway & Blok, 1997; Kuno *et al.*, 1998). The E gene has mainly been used recently (Ni & Barrett, 1995; Paranjpe & Banerjee, 1996; Mangada & Takegami, 1999; Williams *et al.*, 2000; Solomon *et al.* 2003; Nga *et al.* 2004).

There have been limited reports on the molecular epidemiology of JEV in China. Some studies analysed only a few Chinese JEV strains, such as Beijing-1, P3 and SA14 (including the vaccine strains, SA14-2-8 and SA14-14-2), and these strains were isolated before 1960 and belong to genotype 3. Li *et al.* (2004) recently reported a phylogenetic analysis of 19 JEV isolates from seven provinces in China, covering a time span of 38 years (1949–1987). In this study, the Chinese isolates were examined in the prM region and they were related closely to the Nakayama strain, which was isolated in Japan and belonged to genotype 3. Wang *et al.* (2004) isolated seven JEV strains in Shanghai and analysed these isolates. They belonged to genotype 1, and this was the first report of genotype 1 in China. Chen *et al.* (2005) reported JEV isolates in Fujian Province that were obtained from human blood and belonged to genotype 3. Nevertheless, these analyses were based on the prM gene and only limited numbers of isolates were included. Thus, entire features of JEV strains in China were not presented. In the present study, we obtained a total of 62 isolates from mosquitoes, biting midges, human cerebrospinal fluid and blood samples from 2002–2005. We also included seven JEV isolates from 2001 and 28 isolates from 1949–1986 in China, and 37 strains that were isolated in other countries and represent five JEV genotypes were included in the analyses. We sequenced both the prM and E genes. For comparison with E gene phylogeny, the prM gene sequence was also sequenced and analysed. The prM phylogenetic tree was similar to those from previous reports (Chen *et al.*, 1990, 1992; Huong *et al.*, 1993; Ali & Igarashi, 1997) and also to those from studies in China (Li *et al.*, 2004; Wang *et al.*, 2004; Chen *et al.*, 2005). The new isolates represented eight provinces in China: from Heilongjiang Province in the north to Yunnan Province in the south (Fig. 1). The analyses included the first JE isolate in China (Beijing-1), as well as the new isolate from 2005 (SH05-24). Before the 1980s, genotype 3 JEV was predominant in China, but genotype 1 JEV was detected after 1979 and became predominant in some areas, including Shanghai.

Solomon *et al.* (2003) suggested that JEV genotype 1 was transported from the South-East Asian region, especially Indonesia and Malaysia, to the north. Factors may include water-bird migration, new irrigation projects and enhancing animal husbandry, mosquito dispersal, natural conditions such as south-to-north wind or typhoon, and transport by ships and aeroplanes (Chen *et al.*, 1992; Ming *et al.*, 1993; Tsuchie *et al.*, 1997; Williams *et al.*, 2000; Nga *et al.*, 2004). It is possible that the intermediate hosts, birds infected with genotype 1 JEV, migrated and spreaded this genotype of JEV in the western Pacific regions. The introduction of JEV belonging to genotype 1 was reported recently in Japan (Takegami *et al.*, 2000), Vietnam (Nga *et al.*, 2004), Korea (Nam *et al.*, 2001) and China (Wang *et al.*, 2004). The first JEV strain belonging to genotype 1 in this region was reported in 1994 (Takegami *et al.*, 2000). All previous isolates in these countries belonged to genotype 3.

Previous studies reported two distribution patterns of JEV. In the northern temperate regions, JEV strains belonged to genotype 1 and genotype 3, and were active and epidemic. Near equatorial areas, genotypes 2 and 4 were active and endemic (Burke & Leake, 1988; Chen *et al.*, 1992; Huong

Fig. 2. Phylogenetic analysis of JEV strains predicted from the E gene sequences. The neighbour-joining tree was generated by using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic groups are given on the left of each tree, indicated according to Uchil & Satchidanandam (2001). The tree was rooted by using Murray Valley encephalitis virus (MVEV) sequence information. Bootstrap confidence limits for 100 replicates are indicated above each branch. Horizontal branch lengths are proportional to genetic distance and vertical branch lengths have no significance. The scale indicates the number of nucleotide substitutions per site. Isolates presented in this study are indicated by an asterisk; the Chinese strains used in this paper are indicated in bold type.

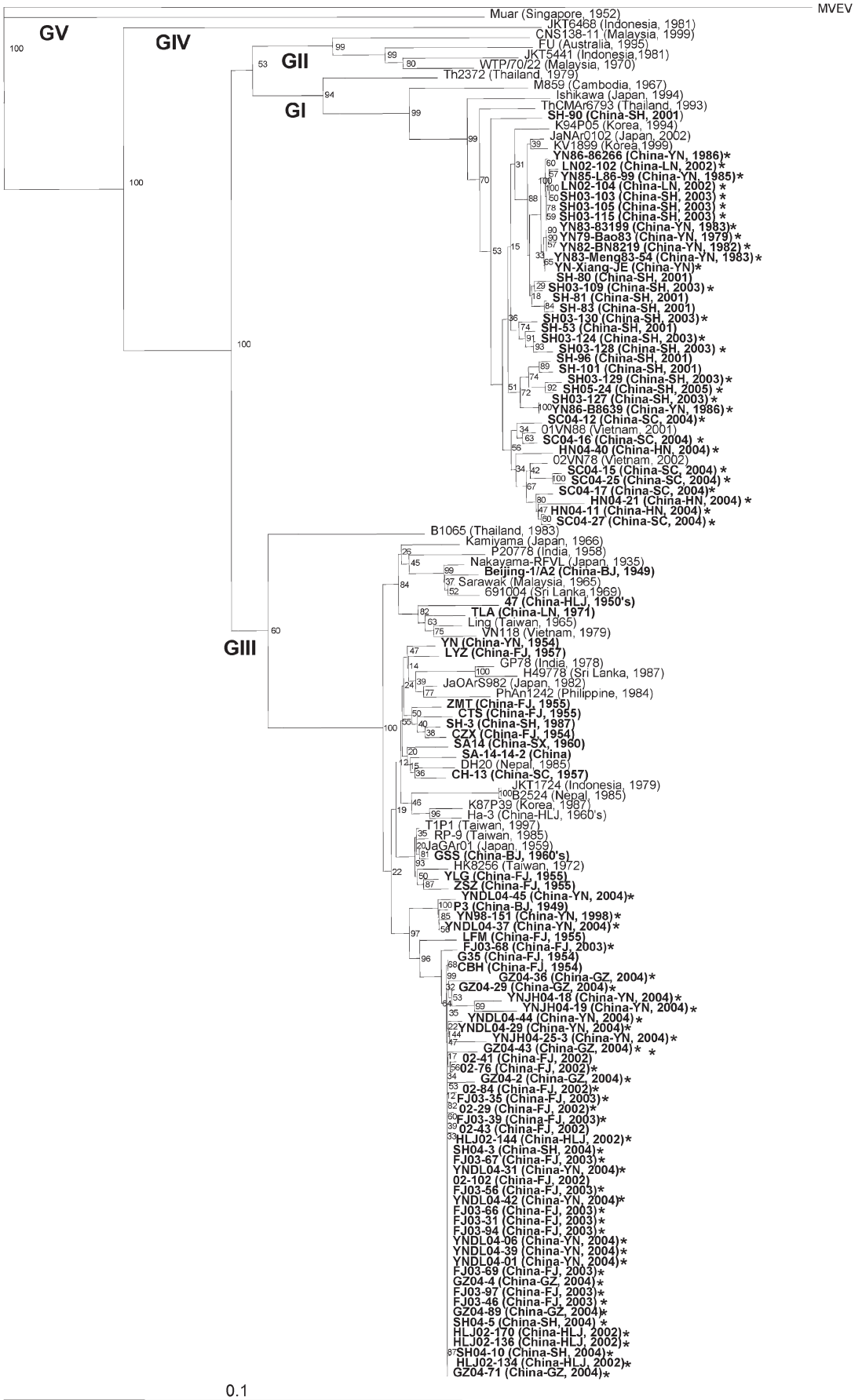
MVEV
Muar (Singapore, 1952)
JKT6468 (Indonesia, 1981)
CNS138-11 (Malaysia, 1999)
FU (Australia, 1995)
JKT5441 (Indonesia,1981)
WTP/70/22 (Malaysia, 1970)
Th2372 (Thailand, 1979)
M859 (Cambodia, 1967)
Ishikawa (Japan, 1994)
ThCMAr6793 (Thailand, 1993)
SH-90 (China-SH, 2001)
K94P05 (Korea, 1994)
JaNAr0102 (Japan, 2002)
KV1899 (Korea,1999)
YN86-86266 (China-YN, 1986)*
LN02-102 (China-LN, 2002)*
YN85-L86-99 (China-YN, 1985)*
LN02-104 (China-LN, 2002) *
SH03-103 (China-SH, 2003) *
SH03-105 (China-SH, 2003) *
SH03-115 (China-SH, 2003) *
YN83-G3199 (China-YN, 1983)*
YN79-Bao83 (China-YN, 1979)*
YN82-BN8219 (China-YN, 1982) *
YN83-Meng83-54 (China-YN, 1983)*
YN-Xiang-JE (China-YN)*
SH-80 (China-SH, 2001)
SH03-109 (China-SH, 2003) *
SH-81 (China-SH, 2001)
SH-83 (China-SH, 2001)
SH03-130 (China-SH, 2003)*
SH-53 (China-SH, 2001)
SH03-124 (China-SH, 2003)*
SH03-128 (China-SH, 2003) *
SH-96 (China-SH, 2001)
SH-101 (China-SH, 2001)
SH03-129 (China-SH, 2003)*
SH05-24 (China-SH, 2005) *
SH03-127 (China-SH, 2003)*
YN86-B8639 (China-YN, 1986)*
SC04-12 (China-SC, 2004)*
01VN88 (Vietnam, 2001)
SC04-16 (China-SC, 2004) *
HN04-40 (China-HN, 2004) *
02VN78 (Vietnam, 2002)
SC04-15 (China-SC, 2004) *
SC04-25 (China-SC, 2004) *
SC04-17 (China-SC, 2004)*
HN04-21 (China-HN, 2004) *
HN04-11 (China-HN, 2004) *
SC04-27 (China-SC, 2004) *
B1065 (Thailand, 1983)
Kamiyama (Japan, 1966)
P20778 (India, 1958)
Nakayama-RFVL (Japan, 1935)
Beijing-1/A2 (China-BJ, 1949)
Sarawak (Malaysia, 1965)
691004 (Sri Lanka,1969)
47 (China-HLJ, 1950's)
TLA (China-LN, 1971)
Ling (Taiwan, 1965)
VN118 (Vietnam, 1979)
YN (China-YN, 1954)
LYZ (China-FJ, 1957)
GP78 (India, 1978)
H49778 (Sri Lanka, 1987)
JaOArS982 (Japan, 1982)
PhAn1242 (Philippine, 1984)
ZMT (China-FJ, 1955)
CTS (China-FJ, 1955)
SH-3 (China-SH, 1987)
CZX (China-FJ, 1954)
SA14 (China-SX, 1960)
SA-14-14-2 (China)
DH20 (Nepal, 1985)
CH-13 (China-SC, 1957)
JKT1724 (Indonesia, 1979)
B2524 (Nepal, 1985)
K87P39 (Korea, 1987)
Ha-3 (China-HLJ, 1960's)
T1P1 (Taiwan, 1997)
RP-9 (Taiwan, 1985)
JaGAr01 (Japan, 1959)
GSS (China-BJ, 1960's)
HK8256 (Taiwan, 1972)
YLG (China-FJ, 1956)
ZSZ (China-FJ, 1955)
YNDL04-45 (China-YN, 2004)*
P3 (China-BJ, 1949)
YN98-151 (China-YN, 1998)*
YNDL04-37 (China-YN, 2004)*
LFM (China-FJ, 1955)
FJ03-68 (China-FJ, 2003)*
G35 (China-FJ, 1954)
CBH (China-FJ, 1954)
GZ04-36 (China-GZ, 2004)*
GZ04-29 (China-GZ, 2004)*
YNJH04-18 (China-YN, 2004)*
YNJH04-19 (China-YN, 2004)*
YNDL04-44 (China-YN, 2004)*
YNDL04-29 (China-YN, 2004)*
YNJH04-25-3 (China-YN, 2004)*
GZ04-43 (China-GZ, 2004)* *
02-41 (China-FJ, 2002)
02-76 (China-FJ, 2002)*
GZ04-2 (China-GZ, 2004)*
02-84 (China-FJ, 2002)*
FJ03-35 (China-FJ, 2003)*
02-29 (China-FJ, 2002)*
FJ03-39 (China-FJ, 2003)*
02-43 (China-FJ, 2002)
HLJ02-144 (China-HLJ, 2002)*
SH04-3 (China-SH, 2004)*
FJ03-67 (China-FJ, 2003)*
YNDL04-31 (China-YN, 2004)*
02-102 (China-FJ, 2002)
FJ03-56 (China-FJ, 2003)*
YNDL04-42 (China-YN, 2004)*
FJ03-66 (China-FJ, 2003)*
FJ03-31 (China-FJ, 2003)*
FJ03-94 (China-FJ, 2003)*
YNDL04-06 (China-YN, 2004)*
YNDL04-39 (China-YN, 2004)*
YNDL04-01 (China-YN, 2004)*
FJ03-69 (China-FJ, 2003)*
GZ04-4 (China-GZ, 2004)*
FJ03-97 (China-FJ, 2003)*
FJ03-46 (China-FJ, 2003) *
GZ04-89 (China-GZ, 2004)*
SH04-5 (China-SH, 2004) *
HLJ02-170 (China-HLJ, 2002)*
HLJ02-136 (China-HLJ, 2002)*
SH04-10 (China-SH, 2004)*
HLJ02-134 (China-HLJ, 2002)*
GZ04-71 (China-GZ, 2004)*
GV
GIV
GII
GI
GIII
0.1

Fig. 3. Phylogenetic analysis of JEV strains predicted from the prM gene sequences. The neighbour-joining tree was generated by using CLUSTAL_X (Thompson *et al*., 1997). Phylogenetic groups are given on the left of each tree, indicated according to Chen *et al*. (1990, 1992). The tree was rooted by using Murray Valley encephalitis virus (MVEV) sequence information. Bootstrap confidence limits for 100 replicates are indicated above each branch. Horizontal branch lengths are proportional to genetic distance and vertical branch lengths have no significance. The scale indicates the number of nucleotide substitutions per site. Isolates in this study are indicated in by an asterisk; the Chinese strains used in this paper are indicated in bold type.

et al., 1993). JE has been a continuously epidemic disease in China since 1949 and the number of JE patients has increased from July to September each year. Recently, there appeared to be two genotypes of JEV in some areas, but not all the provinces. In Heilongjiang, Beijing, Shaanxi, Guizhou and Fujian provinces, we found only genotype 3. The genotype distribution of JEV isolates seems to be changing in various regions. However, there is also a possibility that both genotypes of JEV are active simultaneously in Yunnan and Shanghai.

The results in the present study indicate that: (i) recent JEV isolates in China are divided into two genotypes, genotype 1 and genotype 3; (ii) recent JEV isolates in China are grouped in the same clusters within genotypes 1 and 3; and (iii) genotype 1 JEV strains have been isolated in China since 1979, whilst genotype 3 JEV strains have been isolated since the 1940s. These results suggest that genotype 1 JEV was introduced to China in recent years and that JEV belonging to genotypes 1 and 3 co-circulates. We will continue to isolate new JEV strains, especially from human beings. The study will provide a deep insight into the complex molecular epidemiology of JEV in east Asia, including China.

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