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Emergence of Genotype I of Japanese Encephalitis Virus as the Dominant Genotype in Asia▽

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Japanese encephalitis virus (JEV), a mosquito-borne zoonotic pathogen, is one of the major causes of viral encephalitis worldwide. Previous phylogenetic studies based on the envelope protein indicated that there are four genotypes, and surveillance data suggest that genotype I is gradually replacing genotype III as the dominant strain. Here we report an evolutionary analysis based on 98 full-length genome sequences of JEV, including 67 new samples isolated from humans, pigs, mosquitoes, midges. and bats in affected areas. To investigate the relationships between the genotypes and the significance of genotype I in recent epidemics, we estimated evolutionary rates, ages of common ancestors, and population demographics. Our results indicate that the genotypes diverged in the order IV, III, II, and I and that the genetic diversity of genotype III has decreased rapidly while that of genotype I has increased gradually, consistent with its emergence as the dominant genotype.

Japanese encephalitis virus (JEV), a member of the genus *Flavivirus* in the family *Flaviviridae*, is a major cause of viral encephalitis and is endemic in several regions of Asia and the Pacific (4, 13), causing an estimated 35,000 to 50,000 infections and 10,000 to 15,000 deaths annually (4, 13, 27). Fifty percent of survivors suffer from lingering neurological effects (7, 27, 30). Japanese encephalitis (JE) was first reported in Japan in 1924, and JE cases were subsequently reported in many other Asian countries (4, 6, 7, 13, 22, 27, 30). JE was first reported in Australia in 1995 (8, 9, 31). Thus, JE has become a major cause of mosquito-transmitted viral encephalitis on two continents (15, 16, 25).

JEV, the pathogen of JE, has a genome comprising a positive-sense, single-stranded RNA molecule of approximately 11 kb that is capped at the 5′ end and is not polyadenylated at the 3′ end. It carries a single open reading frame (ORF) encoding a polyprotein that is processed into three structural (C, M, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins, flanked by 5′ and 3′ nontranslated regions (NTRs) (13).

Until the latter part of the 20th century, studies indicated that the predominant genotype was genotype III. Since then, there have been multiple reports of genotype I displacing genotype III in many regions (12, 18, 19, 20, 24, 32, 34, 35), and in many areas genotype I is now recognized as the dominant strain.

As part of a national encephalitis surveillance program, we collected samples from a variety of vectors (mosquitoes and midges), host animals (bats and pigs), and patients with cases of encephalitis in areas where the disease is epidemic, and we isolated viruses from a selection of the JEV-positive samples and sequenced their full genomes. We combined these sequences with other, publicly available full-length genome sequences for a final set of 98 genome sequences. With this set we performed the first detailed evolutionary analysis of JEV based on full-length genome sequences and investigated the epidemiology of genotype I relative to that of genotype III.

MATERIALS AND METHODS

Sample collection and genome sequencing. As part of a national encephalitis surveillance program, samples were collected from around China. Sixty-seven of these samples were selected for isolation, identification, and full-genome sequencing.

The samples were amplified as described elsewhere (12, 17, 28, 32, 33, 34, 35). Briefly, viral RNA was isolated using the Viral RNA Mini kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using the Ready-To-Go kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Whole genomes were amplified using primer sets described previously (32, 33); PCR products were recovered using a gel purification kit (Qiagen, Valencia, CA); and sequences were deter-

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TABLE 1. JEV isolates analyzed in this study

Strain	Date	Country	Host[a]	GenBank accession no.[b]
47	1950s	China, Heilongjiang	CSF	JF706269*
14178	2001	India	—	EF623987
57434	2005	India	—	EF623988
04940-4	2002	India	—	EF623989
B58	1989	China, Yunnan	Bat	FJ185036
Beijing-1	1949	China	Human brain	L48961
BL06-50	2006	China, Guangxi	*Culex tritaeniorhynchus*	JF706270*
BL06-54	2006	China, Guangxi	*Culex tritaeniorhynchus*	JF706271*
CBH	1954	China, Fujian	CSF	JN381860*
CH-13	1957	China, Sichuan	CSF	JN381870*
CH1392	1990	Taiwan	*Culex tritaeniorhynchus*	AF254452
CTS	1955	China, Fujian	CSF	GQ429184
CZX	1954	China, Fujian	CSF	JN381865*
DH107	1989	China, Yunnan	*Aedes lineatopennis*	JN381873*
DL04-29	2004	China, Yunnan	*Culex theileri*	JF706272*
DL04-45	2004	China, Yunnan	*Armigeres subalbatus* and *Mansonia uniformis*	JN381854*
Fj02-29	2002	China, Fujian	CSF	JF706273*
Fj02-76	2002	China, Fujian	Human blood	JN381867*
FJ03-39	2003	China, Fujian	Human blood	JN381859*
FJ03-94	2003	China, Fujian	Human blood	JN381858*
FU	1995	Australia	Human serum	AF217620
G35	1954	China, Fujian	Mosquito pool	GQ429185
GB30	1997	China, Yunnan	*Murina aurata* brain tissue	FJ185037
GP78	1978	India	Human brain	AF075723
GS07-TS11	2007	China, Gansu	*Culex tritaeniorhynchus*	JN381843*
GSBY0801	2008	China, Gansu	*Culex tritaeniorhynchus*	JF706274*
GSBY0804	2008	China, Gansu	*Culex tritaeniorhynchus*	JN381844*
GSBY0810	2008	China, Gansu	*Culex tritaeniorhynchus*	JN381840*
GSBY0816	2008	China, Gansu	*Culex tritaeniorhynchus*	JN381842*
GSBY0827	2008	China, Gansu	*Culex tritaeniorhynchus*	JN381845*
GSBY0861	2008	China, Gansu	*Culex tritaeniorhynchus*	JN381833*
GSS	1960s	China, Beijing	CSF	JF706275*
GX0519	2005	China, Guanxi	*Culex tritaeniorhynchus*	JN381835*
GX0523/44	2005	China, Guanxi	*Culex tritaeniorhynchus*	JN381832*
GZ04-2	2004	China, Guizhou	*Armigeres*	JN381857*
GZ56	2006	China, GuiZhou	CSF	HM366552
Ha-3	1960s	China, Heilongjiang	CSF	JN381872*
HB49	1990	China, Yunnan	*Rousettus leschenaulti* blood	JF706284*
HB97	1990	China, Yunnan	*Rousettus leschenaulti* blood	JF706285*
HLJ02-134	2002	China, Heilongjiang	Genus *Culicoides*	JF706276*
HN04-11	2004	China, Henan	*Culex*	JN381831*
HN04-21	2004	China, Henan	*Culex*	JN381841*
HN06129	2006	China, Henan	*Armigeres*	JF706277*
HN0621	2006	China, Henan	Culex	JN381830*
HN0626	2006	China, Henan	Culex	JN381837*
HVI	1965	Taiwan	Mosquito	AF098735
HYZ	1979	China, Yunnan	Patient blood	JN381853*
Ishikawa	1994	Japan	*Culex tritaeniorhynchus*	AB051292
JaGAr 01	1959	Japan	*Culex*	AF069076
JaOArS982	1982	Japan	Mosquito	M18370
JaOH0566/Japan/1966/human	1966	Japan	Human	AY508813
JEV/sw/Mie/40/2004	2004	Japan	Swine serum	AB241118
JEV/sw/Mie/41/2002	2002	Japan	Swine serum	AB241119
JH04-18	2004	China, Yunnan	*Culex whitmorei* and *Anopheles sinensis*	JN381855*
JKT6468	1981	Indonesia	Mosquito	AY184212
K87P39	1987	South Korea	Mosquito	AY585242
KV1899	1999	Korea	Swine	AY316157
LFM	1955	China, Fujian	Human blood	JN381863*
Ling	1965	Taiwan	Human brain	L78128
LN02-102	2002	China, Liaoning	*Culex modestus*	JF706278*
LN0716	2007	China, Liaoning	*Culex tritaeniorhynchus*	JN381849*
LYZ	1957	China, Fujian	CSF	JN381869*
M28	1977	China, Yunnan	*Culex pseudovishnui*	JF706279*
Nakayama	1935	Japan	Human brain	EF571853
p3	1949	China, Beijing	Human brain	U47032
RP-2ms	1985	Taiwan	Mosquito	AF014160
RP-9	1985	Taiwan	Mosquito	AF014161
SA14	1954	China	Mosquito	U14163
SC04-12	2004	China, Sichuan	*Culex*	JN381839*
SC04-15	2004	China, Sichuan	*Culex tritaeniorhynchus*	JN381838*

Continued on following page

TABLE 1—*Continued*

Strain	Date	Country	Host[a]	GenBank accession no.[b]
SD0810	2008	China, Shandong	*Culex tritaeniorhynchus*	JF706286*
SH03-103	2003	China, Shanghai	*Culex tritaeniorhynchus*	JN381847*
SH03-105	2003	China, Shanghai	*Culex tritaeniorhynchus*	JN381846*
SH04-10	2004	China, Shanghai	*Culex tritaeniorhynchus*	JN381856*
SH04-5	2004	China, Shanghai	*Culex tritaeniorhynchus*	JN381866*
SH17 M-07	2007	China	—	EU429297
SH-3	1987	China, Shanghai	CSF	JN381864*
SH-53	2001	China, Shanghai	*Culex tritaeniorhynchus*	JN381850*
SH-80	2001	China, Shanghai	*Culex tritaeniorhynchus*	JN381848*
T1P1	1997	Taiwan	*Armigeres subalbatus*	AF254453
TLA	1971	China, Liaoning	CSF	JN381868*
Vellore P20778	1958	India	Human brain	AF080251
XJ69	2007	China	*Culex pipiens pallens*	EU880214
XJP613	2007	China	*Culex tritaeniorhynchus*	EU693899
XZ0938	2009	China, Xizang	*Culex tritaeniorhynchus*	HQ652538*
YLG	1955	China, Fujian	CSF	JF706280*
YN	1954	China, Yunnan	CSF	JN381871*
YN05124	2005	China, Yunnan	*Culex tritaeniorhynchus*	JF706281*
YN05155	2005	China, Yunnan	*Culex tritaeniorhynchus*	JN381852*
YN0623	2006	China, Yunnan	*Culex tritaeniorhynchus*	JN381836*
YN0911	2009	China, Yunnan	*Culex tritaeniorhynchus*	JF706267*
YN0967	2009	China, Yunnan	*Culex tritaeniorhynchus*	JF706268*
YN79-Bao83	1979	China, Yunnan	*Culex tritaeniorhynchus*	JN381851*
YN82-BN8219	1982	China, Yunnan	Mosquito	JN381834*
YN83-Meng83-54	1983	China, Yunnan	*Lasiohelea taiwana* (Shiraki)	JF706282*
YN98-A151	2003	China, Yunnan	Mosquitoes	JN381861*
ZMT	1955	China, Fujian	CSF	JF706283*
ZSZ	1955	China, Fujian	CSF	JN381862*

[a] —, information not available.
[b] Asterisks indicate strains newly sequenced in this study.

mined using an ABI Prism 3730 sequence analyzer (Applied Biosystems, Foster City, CA). The SeqMan program in the DNAStar software package was used to splice, edit, and correct sequence fragments. A subset of the complete set of sequences that was representative of the sampled geographical regions, hosts, and vectors was submitted to GenBank (Table 1).

JEV genome sequence data set. Additional full-length genome sequences were downloaded from GenBank, combined with the new samples, and aligned using ClustalW, version 2.0 (29). Vaccine or derivative strains were excluded, and sequences sharing more than 98% similarity were removed from the data set by analyzing the alignment with the T-COFFEE software package (21), to leave a final data set containing 98 sequences. The complete sequence set contained samples isolated from a variety of hosts: mosquitoes and other insects ($n = 55$), with *Culex tritaeniorhynchus* as the major species ($n = 30$), midges ($n = 3$), bats ($n = 4$), a pig ($n = 1$), and humans ($n = 28$). The isolation dates ranged from 1935 to 2009, and samples were collected from the entire region in which JEV cases have been identified (latitude 15°S to latitude 45°N) (Fig. 1).

Bayesian Markov chain Monte Carlo (MCMC) analysis of JEV. The GTR+I+G substitution model was selected by MrModelTest (23), and the rate of nucleotide substitution, model and rate of population growth, and age of the most recent common ancestor (TMRCA) were estimated using the BEAST software package (2). Rates were estimated for the relaxed clock model, and the chain length was 1,000,000,000 generations with 10% burn-in. The demographics of genotypes I and III were compared by generating their respective Bayesian skyline plots with an uncorrelated log-normal relaxed molecular clock. There were insufficient data to allow the analysis of genotypes II and IV.

Construction of the JEV E gene data set. To obtain a simple estimate of the relative abundances of genotype I and III strains over time, we downloaded all JEV E gene sequences in GenBank as of January 2011. Derivative and genetically modified JEV strains were excluded, and only sequences with background information describing the place and time of isolation were retained, for a final data set of 537 elements.

Nucleotide sequence accession numbers. The virus sequences determined in this study have been deposited in GenBank under accession numbers JF706267 to JF706286 and JN381830 to JN381873.

RESULTS

Phylogenetic analysis. The maximum clade credibility (MCC) tree for the whole genomes of JEV is shown in Fig. 2. The tree contains four distinct clades corresponding to genotypes IV, III, II, and I. The most recent common ancestor for all genotypes is estimated to have occurred 1,695 years ago (95% highest posterior density [HPD], −548 to −3,153 years). The branching of the lineages occurred in the following order: genotype IV, genotype III at −973 years (95% HPD, −425 to −1,739 years), genotype II at −620 years (95% HPD, −266 to −1,141 years), and genotype I at −193 years (95% HPD, −104 to −308 years). The width of most of the 95% HPD intervals is due to the fact that for genotypes II and IV, only a single full-length sequence is available.

The JEV strains isolated from mosquitoes, midges, and JE patients were distributed throughout the evolutionary branches. Furthermore, no host adaptation was detected, and no specific branches were associated with isolation time, sample category, or geographical distribution, suggesting the absence of geographical or species barriers.

Rate of evolutionary change in the JEV genome. Based on the Bayesian MCMC approach and assuming an uncorrelated log-normal molecular clock, the mean nucleotide substitution rate for the entire sequence set was estimated as 1.11×10^{-4} substitution per site per year (95% HPD, 6.04×10^{-5} to 1.69×10^{-4}). For genotypes I and III, the estimated rates were 5.82×10^{-5} (95% HPD, 3.42×10^{-9} to 1.57×10^{-4}) and 7.91×10^{-5}

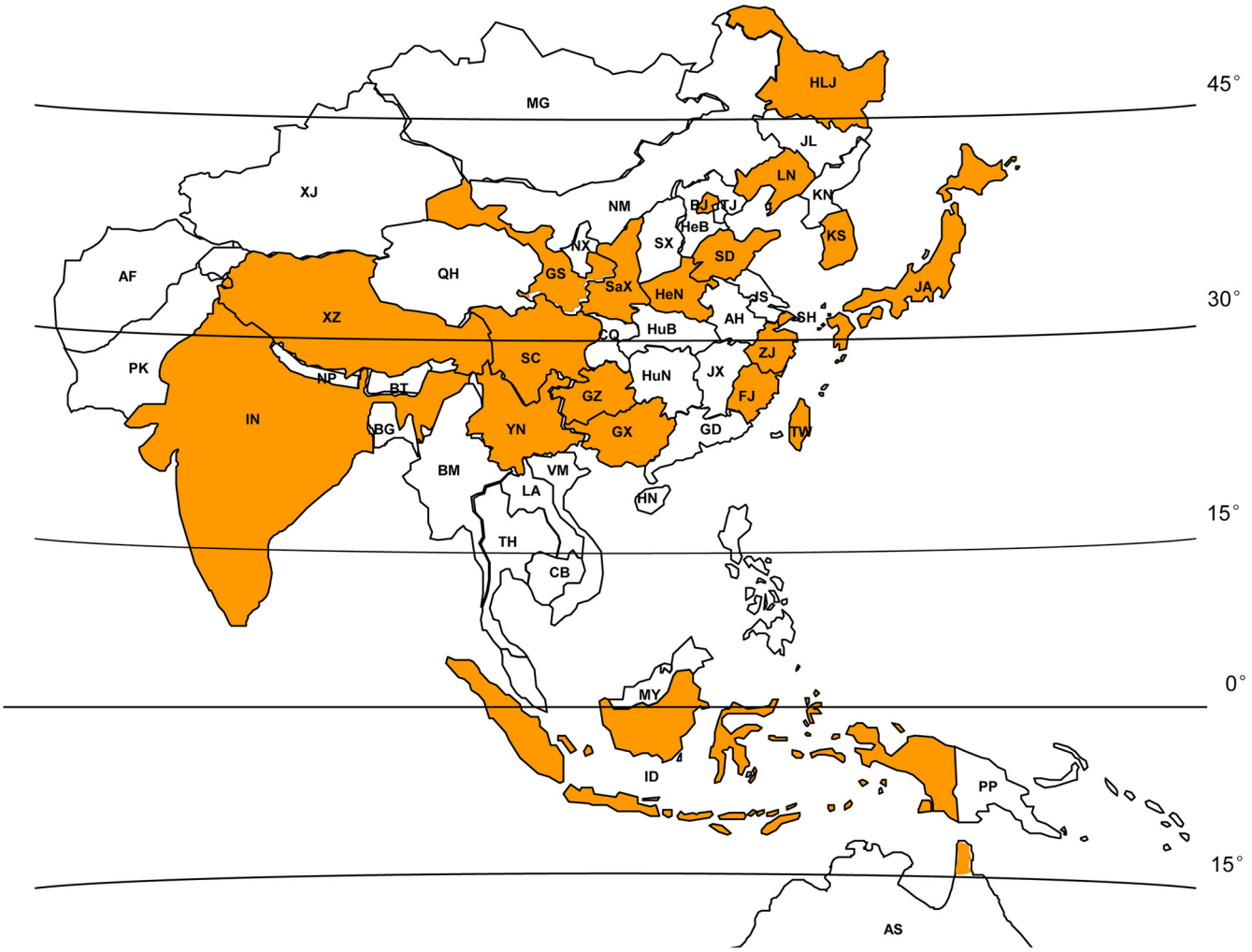

FIG. 1. Worldwide distribution of identified JEV cases. The provinces in China and the other countries from which JEV was isolated and used in this study are shaded. AF, Afghanistan; PK, Pakistan; IN, India; NP, Nepal; BT, Bhutan; BG, Bangladesh; BM, Burma; TH, Thailand; LA, Laos; VM, Vietnam; CB, Cambodia; MY, Malaysia; ID, Indonesia; PP, Papua New Guinea; AS, Australia; KN, North Korea; KS, South Korea; JA, Japan. Chinese provinces: HLJ, Heilongjiang Province; JL, Jilin Province; LN, Liaoning Province; NM, Neimenggu; XJ, Xinjiang; BJ, Beijing, TJ, Tianjin; HeB, Hebei Province; SX, Shanxi Province; SaX, Shaanxi Province; GS, Gansu Province; QH, Qinghai Province; NX, Ningxia; SD, Shandong Province; SH, Shanghai; JS, Jiangsu Province; AH, Anhui Province; HeN, Henan Province; XZ, Xizang; ZJ, Zhejiang Province; JX, Jiangxi Province; HuB, Hubei Province; CQ, Chongqing; SC, Sichuan Province; HuN, Hunan Province; GZ, Guizhou Province; YN, Yunnan Province; FJ, Fujian Province; GD, Guangdong Province; GX, Guangxi; HN, Hainan; TW, Taiwan; MG, Mongolia;.

(95% HPD, 4.28×10^{-5} to 1.18×10^{-4}) substitution per site per year, respectively.

Skyline plot and genetic diversity. The skyline plots for genotypes III and I are shown in Fig. 3a and b, respectively. For genotype III, there is an increase in genetic diversity for the first half of the plot, followed by a subsequent order of magnitude decrease. For the genotype I plot, the diversity appears to have remained relatively constant over the entire period. However, one notable feature of the plot for genotype I is the broad 95% HPD values; this uncertainty is also reflected in the 95% HPD intervals of the estimated average substitution rate for genotype I, given in the preceding section. This uncertainty is a consequence of the genome sequence set; genotype I has become prevalent only in recent years (the earliest isolate is from 1977), so a degree of extrapolation is involved in estimating quantities, and this is reflected in both of these 95% HPD intervals. Conversely, since genotype III was the dominant genotype in the latter half of the 20th century, and the earliest sampled sequence dates to 1935, the corresponding estimates are more robust. However, the uncertainty in these estimates can be seen to increase prior to this date.

Figure 3c shows a plot of the median genetic diversity values of both genotypes on the same scale and the ratio of dated genotype I to genotype III isolates submitted to GenBank over time. The genotypes show a marked difference in their variation in genetic diversity over time. Since the first genotype III sample was isolated in 1935 (Fig. 3c, blue arrow), the diversity of this genotype has decreased by an order of magnitude, while the diversity of genotype I (first isolated in 1977 [Fig. 3c, red arrow]) has remained almost constant. Figure 3c also shows a plot of the ratio of genotype I to genotype III E gene isolates

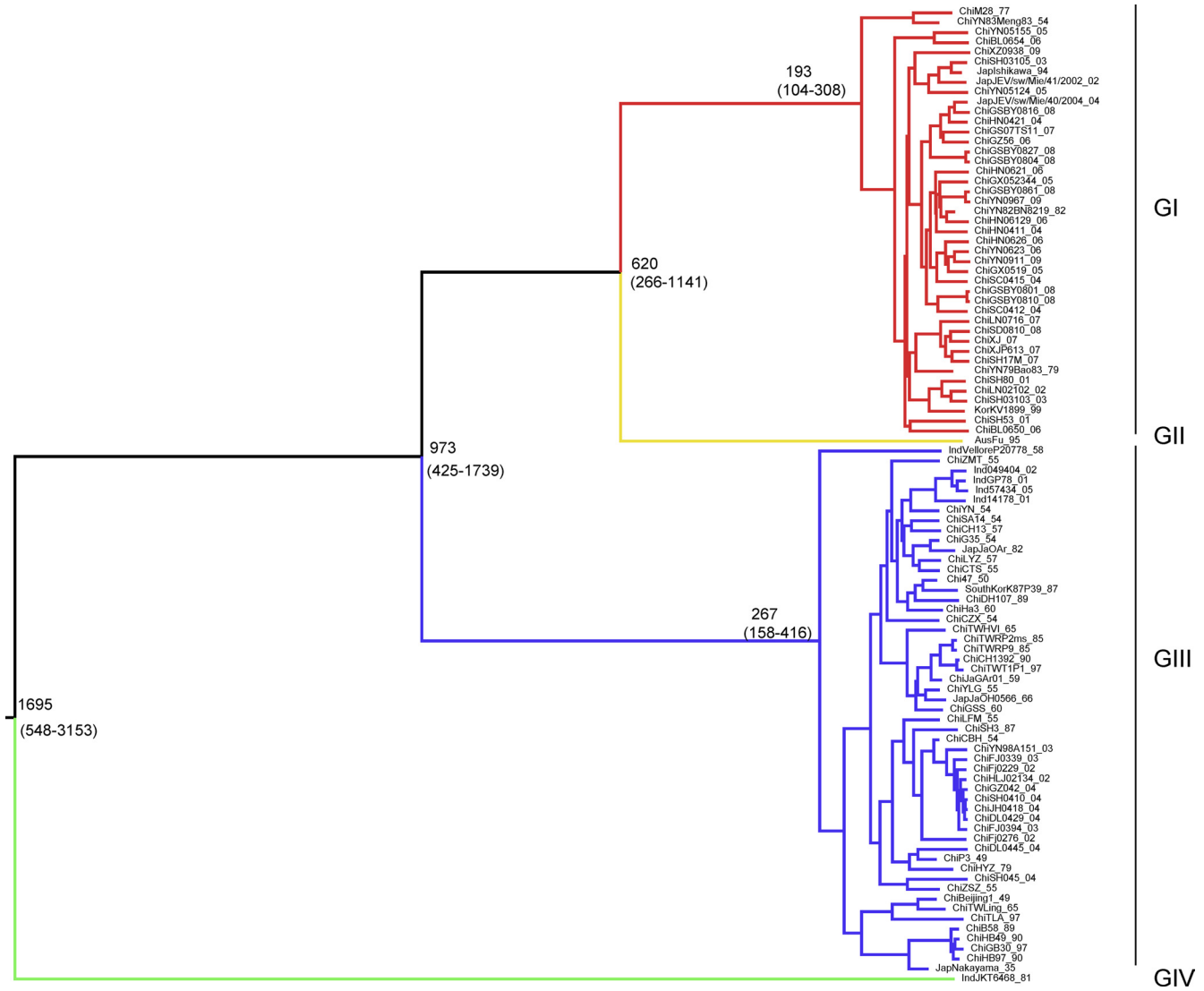

FIG. 2. Maximum clade credibility tree for 98 whole-genome sequences of JEV. Consistent with previous studies, the tree identifies four distinct lineages: genotype I (GI) (red), genotype II (yellow), genotype III (blue), and genotype IV (green). Estimated TMRCAs of these lineages (with their 95% HPD values in parentheses) are shown.

based on the E gene data set described in Materials and Methods. Although this is a simple estimate of the relevant abundances of the two genotypes, the features of the plot are consistent with the estimates of genetic diversity for both genotypes. In particular, the most rapid decrease in the genetic diversity of genotype III precedes the most rapid increase in the number of genotype I samples collected.

DISCUSSION

The earliest observations of JEV were recorded in the late 19th century in Japan, and subsequent reports trace its gradual spread into neighboring regions in Southeast Asia (4, 16, 31). Compared to those of other viruses, the JEV genome is highly conserved, and previous phylogenetic studies have been based on the more variable prM or E gene sequences. Many of these studies have focused on the classification of isolates by genotype and have highlighted the gradual displacement of genotype III by genotype I (14, 19, 22, 32, 35). The earliest comprehensive attempt to investigate the origin of JEV found that the virus probably originated from Indonesia/Malaysia (26). With the development of more-sophisticated phylogenetic analysis techniques (2), some recent studies have reinvestigated the relationships between the different genotypes in an effort to understand the origin of JEV. These studies also found that tropical Southeast Asia plays an important role in the introduction of new strains and indicated that birds and windblown mosquitoes may be responsible for importing these new strains from mainland China into Taiwan and Japan (10, 18). All of these studies were based on prM and E gene sequences. In this work we report the first detailed phylogenetic analysis of the epidemiology of JEV based on full-length genome sequences. Unlike previous investigators, in addition to examining the origin of the virus, we also considered the roles

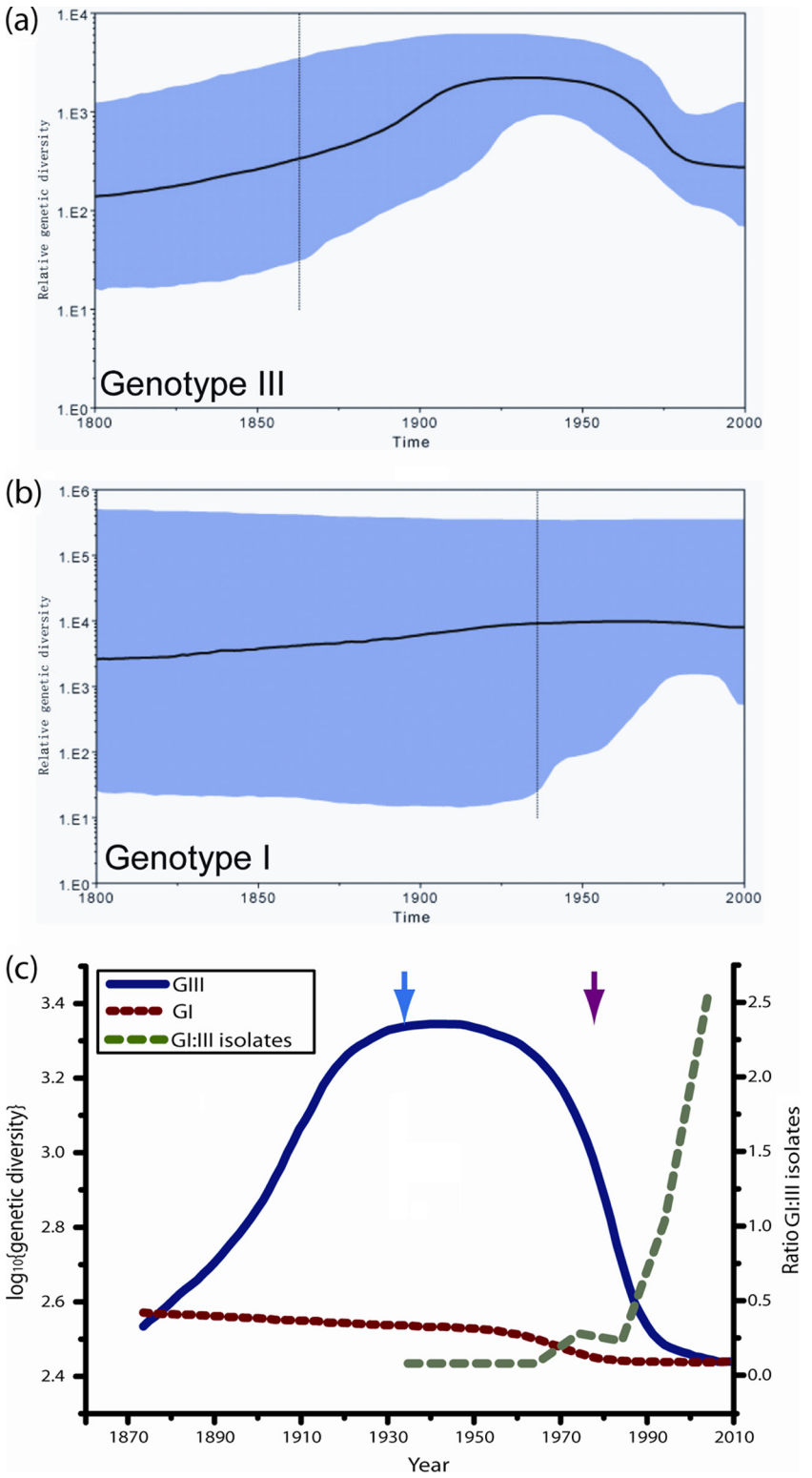

FIG. 3. (a and b) Bayesian skyline plots for genotype III (a) and genotype I (b). Highlighted areas correspond to 95% HPD intervals. The broad 95% HPD for genotype I is a consequence of the sequence set used in the estimation. (c) Medians of the skyline plots for both genotypes drawn on the same scale. Dotted red line, genotype I; solid blue line, genotype III; dashed green line, ratio of the number of genotype I isolates to the number of genotype III isolates deposited in GenBank by year. The blue arrow marks the earliest isolate of genotype III; the red arrow marks the earliest isolate of genotype I. The order of magnitude drop in the estimated genetic diversity of genotype I is matched by a corresponding order of magnitude increase in the ratio of genotype I to genotype III isolates. See the text for full details.

of genotypes I and III in the spread of the virus and the displacement of genotype III strains by genotype I.

Our results lead to an estimate that the most recent common ancestor of JEV occurred 1,695 years ago, with the divergence of the four JEV genotypes occurring in the following order: genotype IV (1,695 years ago), genotype III (973 years ago), genotype II (620 years ago), and genotype I (193 years ago). Our prediction that genotype I is the youngest genotype is supported by growing evidence that genotype I is replacing genotype III in several regions. Acute encephalitis syndrome (AES) induced by genotype I JEV has been reported in Japan (11), India (5), Yunnan Province in southwest China (35, 36), and Gansu (35) and Shanxi (34) Provinces in northwest China, covering a region spanned by latitude 24°37′ to 42°57′N and longitude 92°13′ to 111°15′E in China. Vietnam and Thailand (20) first reported the isolation of genotype I JEV in the 1980s. In Japan, all samples isolated after 1994 belonged to genotype I (14), and in Thailand, all samples isolated from insects and pigs after 2000 belonged to genotype I (20). National JEV surveillance data for China revealed that from 2001 to 2005, genotype I samples accounted for 71% of all isolates (32). The region in which genotype I samples have been isolated now spans latitudes 10°N (Thailand) to 35°N (Gansu, China).

Our work also provides insight into the relationship between genotype I and genotype III, the two major JEV genotypes in Asia. Our results indicate that genotype I, as the youngest genotype, began to replace genotype III to become the dominant genotype approximately 20 years ago. Genotype I appeared to be a minor strain until the early 1970s, at which point it seemed to reach some critical size and expanded rapidly through the region, accompanied by a rapid drop in the number of genotype III isolates and a corresponding drop in the genetic diversity of genotype III. It is also interesting that by the time the first genotype III isolate was collected, the genetic diversity of this genotype had already reached a plateau (Fig. 3c); i.e., although surveillance data suggest that genotype III was dominant during this period, it seems it already had a limited selective advantage.

Japanese encephalitis is a vaccine-preventable disease, and vaccines demonstrate cross-protection between genotypes (1, 3). Nevertheless, the currently available live attenuated and inactivated vaccines were derived when genotype III was dominant and cases of genotype I JE were relatively rare. Since genotype III has now been displaced, and given a recent report of isolation of a JEV genotype I strain from the cerebrospinal fluid (CSF) of a vaccinated JE patient in Yunnan Province (36), it would be prudent to monitor the protective effect of current vaccines closely.

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