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GENOTYPIC AND PHENOTYPIC DETERMINANTS OF JAPANESE ENCEPHALITIS VIRUS GEOGRAPHICAL EXPANSION

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GENOTYPIC AND PHENOTYPIC DETERMINANTS OF JAPANESE ENCEPHALITIS VIRUS GEOGRAPHICAL EXPANSION

by

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Dedication

For my Mom and Dad, June and Layton Schuh

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Japanese encephalitis virus (JEV) consists of at least four geographically and epidemiologically distinct genotypes (GI-IV). Genotypes II and IV have been isolated in tropical Asia only, and GI has recently replaced GIII as the dominant JEV genotype throughout Asia. Therefore, the objective of this dissertation was to elucidate genotypic and phenotypic determinants of JEV geographical expansion by relating differences in the molecular epidemiology of virus isolates to the phenotypic properties of the isolates through *in vitro* and *in vivo* investigations. Phylogeographic analysis revealed that GI consisted of two genetically and geographically distinct clusters, GI-a (tropical Asia only) and GI-b (primarily temperate Asia; emergent cluster). Further, it was demonstrated that both GIII and GI-b are temperate genotypes, suggesting that the spread and establishment of GI-b throughout Asia may have been due to its ability to efficiently overwinter in temperate Asia. Phylodynamic analysis indicated that a decade prior to the genotype displacement the relative genetic diversity of GI had surpassed that of GIII, thereby conferring a selective advantage to GI. No differences in viral multiplication were

observed among viral isolates representative of GI-IV of JEV in avian cells; however the GI-b isolate had significantly higher infectious titers in C6/36 *Aedes albopictus* cells (mosquito larvae derived cell line lacking a functional RNA interference response) from 24-48 hours post infection compared to the other viral isolates. In nature, an increased viral multiplication efficiency of GI-b viruses in mosquitoes may have resulted in a shortened extrinsic incubation period leading to an increased number of GI enzootic transmission cycles and the subsequent displacement of GIII. The blackbird model of JEV viremia revealed that 100.0% of blackbirds developed detectable viremias following inoculation with the GI-a and GI-b isolates, while only 66.7% of blackbirds developed viremias following inoculation with the GII and GIV isolates. These results suggest that GI-b may have emerged and established itself throughout Asia due to an increased multiplicative ability in avians, and genotypes II and IV may be limited to tropical Asia due to a decreased multiplicative ability in avians.

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List of Abbreviations

A	alanine
AES	acute encephalitis syndrome
AD	after death
ADD	average day of death
AI	association index
ANOVA	analysis of variance
AST	average survival time
ATCC	American Type Culture Collection
BF	Bayes factor
BGS	bovine growth serum
BSSVS	Bayesian stochastic search variable selection
C	Celcius
C	capsid
C	cysteine
C	carboxy
CNS	central nervous system
CO_2	Carbon dioxide
CP_{12}	codon positions one and two
CP_3	codon position three
CPE	cytopathic effect
CS1	cyclization sequence 1
CTMC	continuous-time Markov chain
Cx.	*Culex*
D	aspartic acid
DEAE	diethylaminoethyl
DEF	duck embryo fibroblast
DEPS	directional evolution in protein sequences
d_N	non-synonymous
d_N/d_S	ratio of non-synonymous to synonymous nucleotide substitutions
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPI	days post infection
d_S	synonymous
DTT	dithiothreitol
E	envelope
E	glutamic acid
EBF	empirical Bayes factor
EDTA	ethylenediaminetetraacetic acid
EIP	extrinsic incubation period

ELISA	enzyme linked immunosorbent assay
F	phenylalanine
FBS	fetal bovine serum
FEL	fixed effects likelihood
G	glycine
g	gram
GC	guanine-cytosine
GC_{12}	GC content at the first and second codon positions
GC_3	GC content at the synonymous third codon position
GI	genotype I
GII	genotype II
GIII	genotype III
GIV	genotype IV
GV	genotype V
H	histidine
HPD	highest posterior density
HPI	hours post infection
I	isoleucine
IACUC	Institutional Animal Care and Use Committee
IFEL	internal fixed effects likelihood
IFN	interferon
IFNAR1	interferon-α/β receptor 1
IFNAR2	interferon-α/β receptor 2
JAK	Janus kinase
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
K	lysine
kD	kilodalton
L	leucine
LD_{50}	50% lethal dose
M	membrane
M	methionine
MC	maximum exclusive single-state clade size
MCC	maximum clade credibility
MCMC	Markov chain Monte Carlo
MEM	minimum essential media
ML	maximum-likelihood
mL	milliliter
MOI	multiplicity of infection
MP	maximum-parsimony
MRCA	most recent common ancestor
MTase	methyltransferase
MVEV	Murray valley encephalitis virus

N	asparagine
N	amine terminal
N_C	codon usage index
NEAA	non-essential amino acids
NJ	neighbor-joining
nm	nanometer
NR	net relatedness
NS	non-structural
NS1	non-structural 1
NS2A	non-structural 2A
NS2B	non-structural 2B
NS3	non-structural 3
NS4A	non-structural 4A
NS4B	non-structural 4B
NS5	non-structural 5
NT	nearest taxa
NT	nearest taxa
NTPase	nucleoside triphosphatase
ORF	open reading frame
P	proline
PD	phylogenetic diversity
PFU	plaque forming units
PP	posterior probability
prM	precursor of the membrane
$PRNT_{80}$	80% plaque reduction neutralization test
PS	parsimony score
Q	glutamine
R	arginine
RdRp	RNA-dependent RNA-polymerase
REL	relaxed effects likelihood
RNA	ribonucleic acid
RNAi	RNA interference
RPM	revolutions per minute
RTPase	RNA triphosphase
S	serine
SAM	S-adenosyl-methionine
SD	standard deviation
SLAC	single-likelihood ancestor counting
STAT	signal transducer and activator of transcription
T	threonine
TAE	tris-acetate-EDTA
TPB	tryptose phosphate broth
UniFrac	unique fraction

USSR	Union of Soviet Socialist Republics
USUV	Usutu virus
UTMB	University of Texas Medical Branch
UTR	untranslated region
V	valine
WNV	West Nile virus
WRAIR	Walter Reed Army Institute of Research
WRCEVA	World Reference Center for Emerging Viruses and Arboviruses
Y	tyrosine
YFV	yellow fever virus
2K	2,000-molecular-weight

Chapter 1: Introduction

1.1 General overview

Japanese encephalitis virus (JEV) belongs to the JEV serocomplex within the genus *Flavivirus*, family *Flaviviridae*. The JEV serocomplex is comprised of eight antigenically related virus species and two subtype viruses, including Cacipacore virus, JEV, Koutango virus, Murray Valley encephalitis virus (MVEV) with subtype Alfuy virus, St. Louis encephalitis virus, Usutu virus (USUV), West Nile virus (WNV) with subtype Kunjin virus, and Yaounde virus (International Committee on Taxonomy of Viruses, 2011). The members of the JEV serocomplex are sylvatic, primarily circulating between Culicine mosquitoes and avians and/or rodents (Gaunt *et al*., 2001; Gould, 2002). However, several members of the JEV serocomplex infect humans, occasionally resulting in fatal encephalitis (Gould, 2002). In recent years, members of the JEV serocomplex have emerged and established in previously unoccupied geographic regions. In 1999, WNV was recognized for the first time in the Western Hemisphere when it caused an epidemic of encephalitis in New York City (Lanciotti *et al*., 1999). Since that time the virus has spread and established itself throughout most of the continental United States. Previously restricted to Sub-Saharan Africa, USUV was recognized for the first time in Austria in 2001 when it caused a massive blackbird die-off (Weissenbock *et al*., 2002) and the virus has since been detected in several European countries. Historically recognized only in Asia and Indonesia, JEV was isolated for the first time in Saipan in 1990 (Paul *et al*., 1993), Pakistan in 1992 (Igarashi *et al*., 1994), the Torres Strait of Australia in 1995 (Hanna *et al*., 1996). In terms of morbidity and mortality, JEV is considered the most important member of the JEV serocomplex, causing an estimated 68,000 human cases annually and a reported 10,000 deaths (Campbell *et al*., 2011). However, due to the lack of JEV surveillance, the availability of diagnostic testing,

misdiagnosis, and underreporting, the global incidence of JEV among humans is likely far higher.

1.2 HISTORICAL PERSPECTIVE

Recurrent epidemics of summer encephalitis suggestive of JE were recorded in Japan from 1871 onwards and major epidemics occurred in 1924 (6,000 cases, with a 60% case fatality rate), 1929, 1935 and 1937 (Lewis *et al*., 1947). The prototype Nakayama strain of JEV was isolated in mice from the brain of a male that died of summer encephalitis in Tokyo, Japan in 1935 (Lewis *et al*., 1947). Outbreaks of JE were first reported to occur in present day Beijing, China in 1935, 1938, and 1939 (Huang, 1982; Huang & Liu, 1940; Kuttner & Ts'un, 1936) (which was known as Peking at the time of the virus isolation), and in seacoast areas of the former Soviet Union in 1938 and 1939 (Grascenkov, 1964). The seasonal occurrence of epidemic encephalitis coupled with the abundance of culicine mosquitoes led to suggestions that JEV was transmitted by mosquito vectors, leading to the subsequent recovery of the virus by Japanese investigators from rice-paddy breeding *Culex tritaeniorhynchus* mosquitoes in 1938 (Mitamura *et al*., 1938).

During World War II, Major Albert Sabin developed an inactivated mouse brain-derived JEV vaccine using the prototype Nakayama strain, which was stockpiled for use only in an epidemic situation (Innis, 1995). Following an epidemic of JE among civilians in Okinawa, Japan, this stockpiled vaccine was given to over 60,000 American soldiers and was generally well tolerated (Innis, 1995). The mouse-brain derived JEV vaccine was replaced with a more immunogenic and efficacious chick embryo-derived JEV vaccine that was administered to all American soldiers in the Far East Command between 1948 and 1951 (Innis, 1995). However, the protective efficacy of this vaccine was only 80%

and the immunogenicity was variable (Innis, 1995). As a result, 201 cases of JE occurred among American soldiers stationed in Korea in 1950 (Lincoln & Sivertson, 1952).

Epidemiological observations made by the United States (US) Army during World War II and the Korean War prompted the US Army laboratory based in Tokyo, Japan to perform a series of ecological studies in the late 1950s that established herons and egrets as the maintenance host of JEV, domestic swine as the major amplifying host, and *Cx. tritaeniorhynchus* as the principal vector between these vertebrate hosts and the incidental, dead-end equine and human hosts (Buescher & Scherer, 1959; Buescher *et al*., 1959a; Buescher *et al*., 1959b; Buescher *et al*., 1959c; Buescher *et al*., 1959d; Hammon *et al*., 1951; Sabin *et al*., 1947; Scherer & Buescher, 1959; Scherer *et al*., 1959a; Scherer *et al*., 1959d).

1.3 Clinical epidemiology of JE

Following the inoculation of a human by the bite of an infective mosquito, five responses are possible: 1) no infection, 2) asymptomatic infection manifested by an immune response only, 3) mild febrile illness without signs of central nervous system (CNS) involvement, followed by recovery, 4) acute, self-limited meningomyeloencephalitis, followed by recovery with or without permanent neurological disorders, and 5) fatal meningomyeloencephalitis (Innis, 1995). The ratio of symptomatic to asymptomatic cases ranges from 1:1000 (estimated from children living in China) (Huang, 1982) to 1:25 (estimated from American soldiers serving in Korea) (Halstead & Grosz, 1962). Of symptomatic infections 20 to 30% are rapidly fatal, 30 to 50% suffer lingering neurologic and/or psychiatric sequelae, and only 25% fully recover (Campbell *et al*., 2011).

1.4 JAPANESE ENCEPHALITIS CASE DEFINITION

The World Health Organization-recommended case definition for human JE infection is reprinted below (Vaccine Assessment and Monitoring Team of the Department of Vaccines and Biologicals, 2003):

Recommended case definition

Clinical case definition

Clinically, a case of acute encephalitis syndrome (AES) is defined as a person of any age, at any time of year with the acute onset of fever and a change in mental status (including symptoms such as confusion, disorientation, coma, or inability to talk) and/or new onset of seizures (excluding simple febrile seizures). Other early clinical findings may include an increase in irritability, somnolence or abnormal behavior greater than that seen with usual febrile illness.

Case classification

Suspected case: A case that meets the clinical case definition for AES. Suspected cases should be classified in one of the following four ways: 1) Laboratory-confirmed JE: A suspected case that has been laboratory-confirmed as JE. 2) Probable JE: A suspected case that occurs in close geographic and temporal relationship to a laboratory-confirmed case of JE, in the context of an outbreak. 3) "Acute encephalitis syndrome" – other agent: A suspected case in which diagnostic testing is performed and an etiological agent other than JEV is identified. 4) "Acute encephalitis syndrome" – unknown: A suspected case in which no diagnostic testing is performed or in which testing was performed but no etiological agent was identified or in which the test results were indeterminate.

Laboratory criteria for confirmation

Clinical signs of JE are indistinguishable from other causes of AES. Laboratory confirmation is therefore essential for accurate diagnosis of JE. Laboratory confirmation of a JE virus infection includes: 1) presence of JEV-specific IgM antibody in a single sample of cerebrospinal fluid (CSF) or serum as detected by an IgM-capture enzyme linked immunosorbent assay (ELISA) specifically for JEV or any of the following: 2) detection of JEV antigens in tissue by immunohistochemistry; OR 3) detection of JEV genome in serum, plasma, blood, CSF, or tissue by reverse transcriptase polymerase chain reaction or an equally sensitive and specific nucleic acid amplification test; OR 4) isolation of JEV in serum, plasma, blood, CSF, or tissue; OR 5) detection of a four-fold or greater rise in JEV-specific antibody as measured by hemagglutination inhibition or

plaque reduction neutralization assay in serum collected during the acute and convalescent phase of illness. The two specimens for IgG should be collected at least 14 days apart. The IgG test should be performed in parallel with other confirmatory tests to eliminate the possibility of cross-reactivity.

1.5 GEOGRAPHICAL DISTRIBUTION OF JEV AND EPIDEMIOLOGY OF JE

Japanese encephalitis virus circulates throughout most of Asia, with the geographical boarders of virus activity extending north into maritime Siberia, east into Saipan (Paul *et al*., 1993), west into Pakistan (Igarashi *et al*., 1994) and south into the Torres Strait of Australia (Hanna *et al*., 1996) (Figure 1.1). Japanese encephalitis primarily occurs in rural areas, as the disease is associated with irrigated rice agriculture, domestic swine, and ardeids (waterbirds, including herons and egrets) (Innis, 1995). Transmission of the virus in temperate zones (e.g., Northern China and Japan) is epidemic with the majority of cases occurring in summer or monsoon season months, while transmission in tropical zones (e.g., Indonesia and Malaysia) is endemic and occurs year-round at lower rates (Innis, 1995).

1.6 ECOLOGY OF JEV

1.6.1 Transmission cycle

In nature, JEV is maintained in a transmission cycle primarily involving rice-paddy breeding culicine mosquitoes, ardeid maintenance hosts, and/or domestic swine amplifying hosts (Buescher & Scherer, 1959; Buescher *et al*., 1959a; Buescher *et al*., 1959b; Buescher *et al*., 1959c; Buescher *et al*., 1959d; Hammon *et al*., 1951; Sabin *et al*., 1947; Scherer & Buescher, 1959; Scherer *et al*., 1959a; Scherer *et al*., 1959d) (Figure 1.2).

1.6.2 Mosquito vectors

As mentioned previously, JEV was first isolated by Japanese investigators from rice-paddy breeding *Cx. tritaeniorhynchus* mosquitoes in 1938 (Mitamura *et al*., 1938). The virus since been isolated from over 30 mosquito species within the genera *Culex*, *Aedes*, *Anopheles*, *Armigeres*, and *Mansonia* (Mackenzie *et al*., 2006), as well as once from ixodid ticks of the species *Haemaphysalis japonica* and three times from biting midges (once from *Forcipomyia taiwana* and twice from the genus *Culicoides*) (Wang *et al*., 2007; Wu & Wu, 1957). However, rice-paddy breeding *Cx. tritaeniorhynchus* are considered the primary vectors of JEV throughout Asia (Innis, 1995). Other secondary or regional mosquito vectors listed in order of importance are *Cx. vishnui, Cx. gelidus*, *Cx. fuscocephala*, *Cx. pipiens*, and *Cx. quinquefasciatus* (Rosen, 1986). These mosquito vectors inhabit natural or man-made ground pools (immature stages), particularly irrigated rice paddies, are zoophilic, feed at night, prefer to feed outdoors, feed many times throughout their lifespan, and disperse widely following the ingestion of a blood meal (Innis, 1995). Mosquito infection rates exhibit geospatial variation, ranging from 1:369,146 in India (Dandawate *et al*., 1969) to 1:233 in Japan (Buescher *et al*., 1959c). In temperate zones, increases in mosquito infection rates are associated with the warmer summer months (Buescher *et al*., 1959c); while in tropical zones, increases in mosquito infection rates coincide with the onset of the monsoon season (Gajanana *et al*., 1997).

Laboratory experiments have confirmed that *Cx. tritaeniorhynchus* is a highly competent vector of JEV. The 50% infective dose for perorally blood fed *Cx. tritaeniorhynchus* was $10^{1.7}$ mouse brain 50% lethal dose (LD_{50}) of JEV/0.002 mL blood (estimated volume of each mosquito blood meal) (Hill, 1970; Soman *et al*., 1977; Takahashi, 1976). At blood meal virus concentrations of $10^{4.0}$ mouse brain LD_{50} of JEV per 0.002 mL/blood, almost 100% of *Cx. tritaeniorhynchus* became infected (Takahashi,

1976). Both the serum-agar method and the immunofluorescent staining technique demonstrated that *Cx. tritaeniorhynchus* perorally fed a blood meal containing $10^{3.5}$ mouse brain LD_{50} of JEV/ 0.002 mL blood first had virus in their salivary glands at six days post blood meal (Hill, 1970; Soman *et al*., 1977; Takahashi, 1976). In most cases, the virus titer for the salivary glands exceeded that of the midgut by 13 days post blood meal (Hill, 1970; Soman *et al*., 1977; Takahashi, 1976). Saliva collected from single JEV-infected *Cx. tritaeniorhynchus* forced to probe capillary tubes contained an average of $10^{3.0}$ mouse brain LD_{50} of virus, demonstrating that large amounts of virus can be transmitted by this mosquito (Takahashi, 1976).

1.6.3 Avian maintenance hosts

Japanese encephalitis virus has been isolated from the blood or antibodies to the virus have been detected in the serum of over 90 avian species (van den Hurk *et al*., 2009a). The first isolation of JEV from avians was made from the blood of sparrows (Family *Passeridae*; genus and species unknown) in Japan in 1937 (Mitamura, 1938). The virus was later isolated from 12 species of caged zoo birds in Japan during the summers of 1946 and 1947 (Kitaoka *et al*., 1953), neutralizing antibody to the virus was demonstrated in 13 species of caged zoo and farm birds during the summers of 1946 through 1948 (Kitaoka *et al*., 1953), and a serosurvey of wild birds in Japan conducted between 1950 and 1951 indicated that avian infection was widespread (Hammon *et al*., 1951). Experimental laboratory infections showed that susceptible young avians developed detectable viremias following subcutaneous inoculation of JEV (Buescher *et al*., 1959b) and another laboratory investigation found that, following subcutaneous inoculation with 1-320 mouse LD_{50} of JEV, 50.0% of the blue-crowned night herons (*Nycticorax nycticorax*), 33.3% of the little egrets (*Egretta garzetta*), and 75.0% of the

plumed egrets (*Egretta intermedia*) developed detectable viremias (log_{10}2.5-3.0 mouse LD_{50}/0.03 mL whole blood) that were obtained between two and four DPI and lasted at least two days (Buescher *et al*., 1959a). The coincidental infection of avians with JEV, as demonstrated by the recovery of virus or the appearance of antibody, and the isolation of JEV in mosquitoes during the summer months of 1952 through 1956 implicated ardeids as one of the natural hosts of JEV in Japan (Buescher *et al*., 1959d). A study in India showed that 100% of cattle egrets (*Bubulcus ibis*) and 100% of Indian pond herons (*Ardeola grayii*) developed detectable viremias by 2 DPI following inoculation of 2.7-3.5 dex LD_{50} of JEV and that the virus circulated in sufficiently high titers (at least 0.8 dex LD_{50}) for an adequate time period (at least 3 days) to infect *Cx. tritaeriorhynchus* suggesting an ardeid-mosquito-ardeid enzootic transmission cycle of JEV (Soman *et al*., 1977).

1.6.4 Swine amplifying hosts

Domestic swine are considered the major amplifying host of JEV. These animals have a high natural infection rate (JEV seroprevalence in swine in Japan during the 1950s ranged from 98-100%), maintain viremias of sufficient magnitude to infect mosquitoes for up to four days, are one of the preferred vector hosts, and have a high birth rate (provide an annual source of susceptible piglets) (Gresser *et al*., 1958; Scherer *et al*., 1959b; Scherer *et al*., 1959c). When used as sentinel animals to detect and monitor JEV circulation, 100% of swine become infected just prior to, or coinciding with, the commencement of human transmission and *Cx. tritaeniorhynchus* were observed feeding on the swine (Innis, 1995). Although clinical JE disease in domestic swine is rarely observed, pregnant sows can give birth to stillborn fetuses and aspermia may occur in boars (Burns, 1950; Takashima *et al*., 1988).

1.6.5 Overwintering mechanisms

The duration of viremia in avians and swine is too short for these vertebrates to maintain the virus throughout the winter in the temperate extremes of the JEV geographic range (e.g., Northern China and Japan). Therefore, it is likely that JEV is maintained in temperate climates year-to-year by hibernating mosquitoes, transovarial transmission in mosquitoes, poikilothermic vertebrates, and/or bats. Supporting this hypothesis, *Cx. tritaeniorhynchus* have been reported to overwinter as adults in Japan (Nabeshima *et al*., 2009), JEV has been isolated from overwintering *Cx. pipiens* in Korea (Rosen, 1986), and it has been demonstrated that *Cx. tritaeniorhynchus*, as well as other *Cx. sp*., can transmit JEV following experimental hibernation (Hurlbut, 1950). Transovarial transmission of JEV to the F1 adult stage was experimentally demonstrated in *Cx. tritaeniorhynchus*, *Cx. annulus*, *Cx. quinquefasciatus*, and *Armigeres subalbatus* (Rosen *et al*., 1980; Rosen *et al*., 1989). Interestingly, antibody to JEV (hemagglutination inhibition assay and virus neutralization test) has been demonstrated in several poikilothermic vertebrates found in temperate Asia, including lizards (*Takydromus tachydromoides*) (Doi *et al*., 1983), snakes (*Naja naja*, *Bungarus fasciatus*, *Ptyas korros* and *Elaphe rufodorsata*) (Shortridge *et al*., 1977; Shortridge *et al*., 1974), turtles (*Trionyx sinensis*) (Shortridge *et al*., 1975) and frogs (*Rana tigrina*) (Shortridge *et al*., 1977). Additionally, several lizard species (*Eumeces latiscutatus*, *Eumeces barbouri* and *Eumeces marginatus oshimensis*) have been experimentally infected with JEV (Oya *et al*., 1983), experimental hibernation studies have demonstrated that lizards (*Takydromus tachydromoides*) are able to maintain JEV throughout the winter (Doi *et al*., 1983), and experimental transmission of JEV has been shown from infected mosquitoes (*Cx. pipiens fatigans* and *Cx. pipiens pallens*) to uninfected lizards (*Takydromus tachydromoides* and *Eumeces latiscutatus*) and from infected lizards to mice through mosquitoes (Oya *et al*.,

1983). In temperate Asia, JEV has been isolated from various species of insectivorous and frugivorous bats, including *Rousettus leschenaultii* (Wang *et al*., 2009), *Murina aurata* (Wang *et al*., 2009), *Miniopterus schreibersii fuliginosus* (Sulkin *et al*., 1970), *Rhinolophus cornutus* (Sulkin *et al*., 1970). The virus was isolated throughout the winter months from *Miniopterus schreibersii fuliginosus* and *Rhinolophus cornutus* bats in Japan, demonstrating that bats are important reservoir hosts of JEV that are able to sustain infection year-round (Sulkin *et al*., 1970). Moreover, an experimental study found that *Eptesicus fuscus and Myotis lucifigus* bats infected with JEV and then subjected to temperatures experienced during hibernation were able to maintain viremias for up to 108 days (Sulkin & Allen, 1974).

1.7 MOLECULAR VIROLOGY OF JEV

1.7.1 Virion structure

Flavivirus virions are approximately 50 nanometers in diameter and are icosahedral in shape (Murphy, 1980). The virions contain a nucleocapsid core, which is composed of a single molecule of viral genomic RNA in association with virus-derived capsid protein (Russell *et al*., 1980). The nucleocapsid core is surrounded by a host-derived lipid bilayer membrane embedded with virus-derived membrane and envelope proteins (Russell *et al*., 1980).

1.7.2 Virus life cycle

1.7.2.1 Receptor attachment and internalization

Following the attachment of flavivirus virions to host cell receptors, the virions are internalized via clathrin coated pits and transported to prelysosomal endocytic vesicles (Chu & Ng, 2004; Gollins & Porterfield, 1985, 1986). The acidic pH of the vesicles triggers fusion between the virus and host cell membranes, resulting in the

release of the virus nucleocapsid into the cytoplasm, followed by the disassociation of the viral capsid protein and the viral RNA, and the initiation of viral replication (Chu & Ng, 2004; Gollins & Porterfield, 1985, 1986).

1.7.2.2 RNA replication

The flavivirus replicase complex associates with cellular membranes and consists of the virus-encoded nonstructural proteins, viral RNA, and host factors (Lindenbach *et al*., 2007). Viral replication begins with transcription of the input positive-strand genomic RNA to synthesize complementary negative-strand genomic RNA, which is then used as a template for transcription of positive-strand genomic RNA (Lindenbach *et al*., 2007).

1.7.2.3 Translation

Flavivirus genomes possess a single-stranded, positive-sense RNA molecule that is approximately 11 kilobases in length and has a type I methylated 5' cap ($m^7GpppAmpN_2$), but lacks a 3' polyadenylated tail (Wengler & Gross, 1978) (Figure 1.3). Genomic RNA contains a 5' and 3' untranslated region (UTR), and a single open reading frame (ORF) encoding one polyprotein that is co- and post-translationally cleaved by viral (NS2B-NS3 protease) and host (host signal peptidase) proteases into the structural proteins: the capsid (C), the precursor of the membrane (prM)/membrane (M), and the envelope (E), followed by the non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, 2,000-moleuclar weight signal peptide [2K], NS4B, and NS5) (Chambers *et al*., 1990a) (Figure 1.3).

1.7.2.4 Virion assembly and release

Non-infectious, immature viral particles containing prM and E proteins, lipid membrane, and nucleocapsid, are formed in the lumen of the endoplasmic reticulum (Stadler *et al*., 1997). Generation of mature, infectious virions occurs in the trans-Golgi

network and coincides with the cleavage of prM by the protease furin (Stadler *et al*., 1997). Nascent virions are trafficked through the secretory pathway and are released from the host cell via exocytosis (Mackenzie & Westaway, 2001).

1.7.3 Important features of the virus untranslated regions

1.7.3.1 5' UTR

Secondary structures within the 5' UTR of flaviviruses play a key role in RNA translation and virus replication (Brinton & Dispoto, 1988; Thurner *et al*., 2004). Another major function of the 5' UTR is the complementary region for the negative-strand, which serves as the site of initiation for positive-strand synthesis during RNA replication (Cahour *et al*., 1995).

1.7.3.2 3' UTR

The 3' stem-loop secondary structure within the 3' UTR of flaviviruses has been found to enhance translation of reporter messenger RNAs containing flavivirus RNA (Cheney *et al*., 2002; Holden & Harris, 2004), interact with the viral replicase proteins NS3 and NS5 (Chen *et al*., 1997; Cui *et al*., 1998), and bind translation elongation factor 1A (Blackwell & Brinton, 1997; De Nova-Ocampo *et al*., 2002). Upstream of the 3' stem-loop structure is a 25-nucleotide region termed cyclization sequence 1 (CS1), which base pairs with a complementary sequence at the 5' end of the C gene termed 5'CS (Hahn *et al*., 1987). Complementarity between these cyclization sequences is necessary for selecting templates for RNA replication (Khromykh *et al*., 2001; Kofler *et al*., 2006).

1.7.4 Important features of the virus structural proteins

1.7.4.1 Capsid protein

The C protein is a highly basic protein with a molecular mass of approximately 11 kilodaltons (kD) (Lindenbach *et al*., 2007). The major role of the C protein is formation of the ribonucleoprotein complex with packaged genomic RNA (Lindenbach *et al*., 2007). The protein contains charged residues at the amine (N)- and carboxy (C)-termini separated by an internal hydrophobic region, which mediates membrane association (Ma *et al*., 2004). The nascent C protein possesses a C-terminal hydrophobic anchor that functions as a signal peptide for the translocation of prM protein from the endoplasmic reticulum (Lo *et al*., 1995).

1.7.4.2 Membrane/premembrane protein

The prM protein has a molecular mass of approximately 26 kD (Lindenbach *et al*., 2007), and as mentioned above, is translocated from the endoplasmic reticulum by the C-terminal hydrophobic anchor of nascent C protein (Lo *et al*., 1995). The prM protein folds rapidly and then supports in the correct folding of the E protein (Konishi & Mason, 1993; Lorenz *et al*., 2002). During the exit of virions through the secretory pathway, the prM protein is cleaved by furin to form the M protein (Stadler *et al*., 1997). Following cleavage, prM protein-E protein heterodimers dissociate, the prM protein fragments are released, and E homodimers form (Stiasny *et al*., 1996; Wengler, 1989).

1.7.4.3 Envelope protein

The E protein has a molecular mass of approximately 53-56 kD and represents the major constituent of the mature virion surface (Lindenbach *et al*., 2007). The protein folds into an elongated structure and forms head-to-tail homodimers that are positioned parallel to the virus envelope (Lindenbach *et al*., 2007). Each E protein monomer is

composed of three noncontiguous structurally distinct domains: domain I is centrally located and acts as a hinge between the other two domains, domain II is the dimerization domain and contains the fusion peptide at its distal end, and domain III is the putative receptor-binding domain (Rey *et al*., 1995). The stem-anchor region, located at the C-terminal of the E protein, plays a critical role in membrane fusion and virus assembly (Allison *et al*., 1999). The flavivirus E protein contains a variable number (0, 1, or 2) of N-linked glycosylation sites that may contribute to viral attachment and cell tropism (Fuchs *et al*., 2010). The E protein of JEV, as well as most other flaviviruses, can be glycosylated at positions 153 through 155 and almost all dengue virus isolates have a second N-linked glycosylation site a position 67 of the E protein (Mondotte *et al*., 2007). A previous study demonstrated that dengue-2 viruses lacking a N-linked glycan at position 67 were able to infect mammalian cells, translate and replicate the viral genome, however the production of nascent infectious virions was abolished (Mondotte *et al*., 2007). Dengue-2 viruses lacking the N-linked glycosylation site at position 153 of the E protein had reduced infectious in mammalian cells (Mondotte *et al*., 2007). However, dengue-2 viruses lacking one or both N-linked glycosylation sites were still able to replicate in mosquito cells (Mondotte *et al*., 2007).

1.7.5 Important features of the virus nonstructural proteins

1.7.5.1 Nonstructural 1 protein

The NS1 protein has a molecular mass of approximately 46 kD and exists in intracellular, cell-surface, and extracellular nonvirion forms (Lindenbach *et al*., 2007). The intracellular form of the NS1 protein plays an important role in RNA replication (Mackenzie *et al*., 1996; Westaway *et al*., 1997), and interaction between the NS1 and NS4A proteins is essential for replicase function (Lindenbach & Rice, 1999). The NS1

protein can localize to the cell surface where it is secreted, resulting in the elicitation of strong humoral immune responses and antibody dependent cellular cytotoxicity (Lindenbach & Rice, 2003). This protein also encodes complement fixing activity and has two potential N-linked glycosylation sites (Lindenbach & Rice, 2003).

1.7.5.2 Nonstructural 2A/2B proteins

The NS2A protein is a hydrophobic transmembrane protein of approximately 22 kD (Lindenbach *et al*., 2007). This protein is involved in RNA replication and virus assembly (Lindenbach & Rice, 2003; Liu *et al*., 2003), and functions as an interferon (IFN) antagonist by inhibiting IFN-α/β signaling (Liu *et al*., 2004; Liu *et al*., 2006; Munoz-Jordan *et al*., 2003). The NS2B protein is a membrane-associated protein with a molecular mass of approximately 14 kD (Lindenbach *et al*., 2007). The central region of this protein forms a complex with the N-terminal 180 amino acids of NS3 protein that encodes a serine protease. NS2B functions as an essential cofactor for the NS3 serine protease (Falgout *et al*., 1991).

1.7.5.3 Nonstructural 3 protein

The NS3 protein is a cytoplasmic, multifunctional protein with an approximate molecular mass of 70 kD (Lindenbach *et al*., 2007). The N-terminal third of the protein contains a serine protease, which in complex with NS2B cleaves at the junctions of the NS2A-NS2B, NS2B-NS3, NS3-NS4A, NS4A-2K and NS4B-NS5 proteins (Bazan & Fletterick, 1989; Chambers *et al*., 1990b; Gorbalenya *et al*., 1989a), creates the C-termini of the mature forms of the C (Amberg *et al*., 1994; Yamshchikov & Compans, 1994) and NS4A proteins (Lin *et al*., 1993), and cleaves internal sites within the NS2A and NS3 proteins (Chambers *et al*., 1990a). The C-terminal region of the NS3 protein encodes a supergroup 2 RNA helicase (Gorbalenya *et al*., 1989b), and experimental studies have

demonstrated that this region of the protein also possesses RNA-stimulated nucleoside triphosphatase (NTPase) and RNA unwinding activity (Warrener *et al*., 1993; Wengler, 1991), which play essential roles in RNA synthesis. The C-terminal of the NS3 protein also has RNA triphosphase (RTPase) activity, which is likely involved in the dephosphorylation of the 5' end of the genome in preparation for cap addition (Wengler, 1993).

1.7.5.4 Nonstructural 4A/4B proteins

The NS4A protein is a hydrophobic protein with a molecular mass of approximately of 16 kD (Lindenbach *et al*., 2007). Based on its subcellular distribution and association with the NS1 protein, the NS4A protein likely plays a role in RNA replication (Lindenbach & Rice, 1999). During viral replication, polyprotein cleavage generates a 2K peptide between NS4A and NS4B (Lindenbach *et al*., 2007). The NS4B protein is a hydrophobic, polytopic transmembrane protein with a molecular mass of approximately 27 kD (Lindenbach *et al*., 2007). The NS4B is likely important in RNA replication as it co-localizes with the NS3 protein and double-stranded RNA within endoplasmic reticulum-derived membrane structures (Miller *et al*., 2006; Westaway *et al*., 2002). The NS4A and NS4B proteins have also been shown to be involved with inhibition of IFN-α/β signaling (Munoz-Jordan *et al*., 2003).

1.7.5.5 Nonstructural 5 protein

The NS5 protein is a multifunctional protein with a molecular mass of 103 kD (Lindenbach *et al*., 2007). The N-terminus of the NS5 protein encodes a S-adenosyl-methionine (SAM)-dependent methyltransferase (MTase), and is therefore likely involved in methylation of the 5' cap structure (Koonin, 1993). The C-terminus of the NS5 protein is the RNA-dependent RNA-polymerase (RdRp), functioning to catalyze the

replication of RNA from an RNA template (Ackermann & Padmanabhan, 2001; Guyatt *et al*., 2001; Tan *et al*., 1996). There is also evidence that the JEV NS5 protein suppresses IFN-α/β-mediated Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling (Lin *et al*., 2006).

1.8 MOLECULAR EPIDEMIOLOGY OF JEV

1.8.1 Antigenic variation and genetic makeup

Early investigations examined antigenic variation among JEV isolates using polyclonal antisera (Ali & Igarashi, 1997; Ali *et al*., 1995; Banerjee, 1986; Hale & Lee, 1954; Huang, 1982; Okuno Y, 1968) and subsequently monoclonal antibodies (Hasegawa *et al*., 1994; Kedarnath *et al*., 1986; Kobayashi *et al*., 1985; Kobayashi *et al*., 1984). Prior to the determination of the first genomic sequence of JEV in 1987, T1 RNA oligonucleotide fingerprinting was utilized to examine the genetic variation of JEV isolates (Banerjee & Ranadive, 1989; Hori *et al*., 1986a; Hori *et al*., 1986b).

1.8.2 Phylogenetics

In 1987, the first nucleotide sequence of the full genome of JEV was reported based on the JaOArS982 isolate from Japan (Sumiyoshi *et al*., 1987). Unlike other flaviviruses, such as WNV and the dengue viruses, the nucleotide sequence of the ORF has been determined for only 15 wild-type JEV isolates (Solomon *et al*., 2003). Initially, genetic variation among JEV isolates was examined by determining the sequence of a 240-nucleotide region of the prM gene (Chen *et al*., 1990; Chen *et al*., 1992); however most current JEV phylogenetic studies utilize nucleotide sequence information derived from the entire E gene (1500 nucleotides in length). A previous review compared the phylogeny of JEV isolates generated from sequence information derived from a 240-nucleotide region of the prM gene with the phylogeny of JEV isolates generated from

sequence information derived from the complete E gene and found that only one of the virus isolates had been misclassified (Holbrook & Barrett, 2002). Genetic studies have divided JEV into at least four genotypes that exhibit distinct geographical ranges and epidemiological histories.

1.8.2.1 Genotype I

Genotype I (GI) includes isolates collected in northern Australia, northern Cambodia, China, Japan, Korea, Malaysia, Taiwan, Thailand, and Vietnam between 1967 and present. In recent years, multiple reports indicated that GI had displaced GIII as the most frequently isolated JEV genotype in a number of Asia countries including China (Wang *et al*., 2007), Thailand (Nitatpattana *et al*., 2008), South Korea (Nam *et al*., 1996), Japan (Ma *et al*., 2003), Malaysia (Tsuchie *et al*., 1997), Vietnam (Nga *et al*., 2004), India (Fulmali *et al*., 2011), and Taiwan (Chen *et al*., 2011). However, the reasons underlying this geographically expansive genotype displacement are unknown.

1.8.2.2 Genotype II

Genotype II (GII) includes isolates collected sporadically in northern Australia, Indonesia, Malaysia, Papua New Guinea, and southern Thailand between 1970 and 1999. Interestingly, GII has only been collected in tropical Asia (south of the Tropic of Capricorn [23.5°N].

1.8.2.3 Genotype III

Genotype III (GIII) includes isolates collected in China, India, Japan, Korea, Malaysia, Nepal, the Philippines, Sri Lanka, Taiwan, Thailand, and Vietnam between 1935 and present. Although a limited quantity of nucleotide sequence data are available for JEV isolates collected prior to the 1970s, GIII appears to have been the dominant JEV

genotype throughout Asia for at least 50 years (from the collection of the prototype Nakayama strain of JEV, a GIII virus, in 1935 until recently).

1.8.2.4 Genotype IV

Genotype IV (GIV) includes five isolates made from mosquitoes collected on three islands encompassing the Indonesian archipelago between 1980 and 1981 (Chen *et al*., 1992). Two of the isolates originated in Java (both isolated from mosquito pools), two originated in Bali (one isolated from *Cx. tritaeniorhynchus* and one isolated from a mosquito pool) and one originated in Flores (isolated from *Cx. tritaeniorhynchus*) (Chen *et al*., 1992). Based on JEV genomic data, GIV is the most divergent genotype, differing from GI-III by 17.4-19.6% at the nucleotide level and 4.8-6.5% at the amino acid level (Solomon *et al*., 2003). The reasons underlying the failure of GIV to spread beyond the Indonesian archipelago remain unknown. Further, the involvement of avians in the enzootic transmission cycle of GIV of the virus, or the ability of this genotype to cause viral encephalitis in non-avian vertebrates, remains to be determined.

1.8.2.5 Genotype V

A single viral isolate (Muar isolate) collected in Singapore from a patient living in Malaysia is thought to represent a fifth genotype (GV) of JEV on the basis of limited cross-neutralization studies using a set of monoclonal antibodies (Kobayashi *et al*., 1984) and phylogenetic studies (Solomon *et al*., 2003). Of note, the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch (UTMB) does not possess the Muar isolate.

1.8.2.6 Availability of genomic sequences prior to the initiation of this dissertation research

As stated above, prior to the initiation of this dissertation research, there were very few genomic sequences available for wild-type JEV isolates. Specifically, genomic sequences were available for one GI isolate, one GII isolate, 12 GIII isolates, and one GIV isolate. However, JEV E gene sequences were available for > 100 wild-type isolates.

1.8.3 Evolution

The geographical distribution of the genotypes of JEV was previously examined by categorizing all JEV isolates for which there was sequence data available into six groups based on their sampling location: 1) Indonesia-Malaysia, 2) Australia-Papua New Guinea, 3) Taiwan-Philippines, 4) Thailand-Cambodia-Vietnam, 5) Japan-Korea-China, and 6) India-Sri Lanka-Nepal (Solomon *et al*., 2003). Categorization of the viral isolates revealed that GI had been found in all regions with the exception of India-Sri Lanka-Nepal, GII had been found in all regions with the exception of India-Sri Lanka-Nepal and Japan-Korea-China, GIII had been found in all regions with the exception of Australia-Papua New Guinea (Solomon *et al*., 2003). Conversely, the most phylogenetically divergent genotypes, GIV and GV, had only been found in the Indonesia-Malaysia region (Solomon *et al*., 2003). Based on these findings, in addition to the fact that the Indonesia-Malaysia region is proximal to Australia, where MVEV circulates, (JEV's closest known relative), it was hypothesized that JEV originated in the Indonesia-Malaysia region from a virus common to JEV and MVEV (Solomon *et al*., 2003). From that ancestral virus, GIV-GV diverged (the most ancestral genotypes), followed by the divergence of GI-III (the most recent genotypes) (Solomon *et al*., 2003). Using a dataset of 18 JEV genomic sequences representative of GI-IV of JEV, it was estimated that GIV diverged approximately 350 years ago (± 150 years). However, the geographical location and time

of divergence of GI-III remain uncertain. Furthermore, only 13 Indonesian JEV isolates collected over a four-year time period have been phylogenetically characterized (Chen *et al*., 1990; Chen *et al*., 1992). Therefore, the extent of genetic variation among isolates of JEV may be greater than currently recognized.

1.9 PHENOTYPIC CHARACTERIZATION OF JEV

Phenotypic properties of JEV, as well as the closely related WNV, have been elucidated through *in vitro* (cell culture systems) and *in vivo* (mouse models of neurovirulence and neuroinvasiveness) investigations.

1.9.1 Cell culture systems

Various avian (Lee *et al*., 1965; Shapiro *et al*., 1971), mosquito (Bhatt *et al*., 2000; Hsu, 1971; Hsu *et al*., 1975; Shameem *et al*., 1988; Singh & Paul, 1968; Vrati *et al*., 1999), and mammalian (Inoue, 1964; Inoue & Nishibe, 1965; Lee *et al*., 1965; Shameem *et al*., 1988; Shapiro *et al*., 1971; Vrati *et al*., 1999) cell culture systems were found to be susceptible to infection with JEV. Growth curves have been performed to compare the multiplication and replication kinetics of wild-type versus vaccine (Bhatt *et al*., 2000), mutant (Inoue, 1964; Inoue & Nishibe, 1965), and/or naturally attenuated (Vrati *et al*., 1999) strains of JEV. However, no studies have utilized cell culture systems to investigate hypotheses regarding the distinct molecular epidemiologies and geographical distributions of GI-IV of JEV.

1.9.1.1 Duck embryo fibroblast cells

Duck embryo fibroblast (DEF) cells (avian IFN-α/β competent) (Staeheli *et al*., 2001)were found to be susceptible to infection with JEV (Lee *et al*., 1965), and DEF cell cultures have been previously used to investigate why the North American NY99 isolate of WNV is highly virulent in American crows, whereas the Kenyan KEN-3829 isolate of

WNV is considerably less virulent in American crows (Brault *et al*., 2004; Kinney *et al*., 2006). Temperature-sensitivity assays performed in DEF cell cultures at the high temperatures observed in the experimentally infected American crows demonstrated that virus derived from the NY99 infectious clone was able to efficiently multiply, while virus derived from the KEN-3829 infectious clone exhibited substantially decreased viral titers (Kinney *et al*., 2006). This study suggests that the ability of the NY99 isolate to multiply at the high temperatures observed in viremic birds may have been a factor in the emergence of WNV in North America in 1999 (Kinney *et al*., 2006).

1.9.1.2 Aedes albopictus C6/36 cells

Although the primary vector of JEV throughout Asia is *Cx. tritaeniorhynchus*, *Aedes albopictus* C6/36 cell cultures have been shown to be susceptible to infection with JEV and have been used to compare the viral multiplication kinetics of JEV isolates (Bhatt *et al*., 2000; Shameem *et al*., 1988; Vrati *et al*., 1999). However, C6/36 cells have never been utilized to investigate reasons underlying the emergence and establishment of GI as the dominant Asian JEV genotype, and the confinement of GIV to the Indonesian archipelago. It is plausible that GI may have emerged and established itself as the dominant Asian JEV genotype due to the enhanced multiplicative ability of this genotype in mosquitoes, while GIV may have remained confined to Indonesia due to the decreased multiplicative ability of this genotype in mosquitoes. The MRE16 strain of Sinbis virus (*Alphavirus*) was previously found to efficiently infect *Aedes aegypti* mosquitoes that ingested a viral blood meal (Myles *et al*., 2003). Conversely, the MRE16sp strain, a small plaque variant of the MRE16 strain containing a 90-nucleotide deletion in the envelope 2 protein, poorly infected *Ae. aegypti* following ingestion on an infectious blood meal (Myles *et al*., 2003). To investigate the role of this deletion the study, three infectious

clones were constructed: 1) pMRE16ic, 2) pMRE16icΔE200-Y229, and 3) pMRE16icΔE200-C220 (Myles *et al*., 2003). Viruses derived from both of the infectious clones containing deletions replicated 10-fold less efficiently compared to viruses derived from pMRE16ic (Myles *et al*., 2003). Further, both deletion mutants poorly infected *Ae. aegypti* and midgut infectivity and dissemination was drastically reduced (Myles *et al*., 2003). These findings suggest that C6/36 cells may represent a good model for mosquito infection *in vivo*.

1.9.1.3 Monkey kidney Vero cells

Monkey kidney Vero cells have been used extensively in virological studies to compare the multiplication kinetics of different virus strains and detect the temperature-sensitive phenotype implying that these cells can be used to detect phenotypic differences in isolates representative of GI-IV of JEV.

1.9.1.4 Adenocarcinomic human alveolar basal epithelial A549 cells

Unlike Vero cells, adenocarcinomic human alveolar basal epithelial A549 cells possess an intact IFN-α/β pathway. A previous investigation demonstrated that A549 cells were susceptible to infection with the neurovirulent RP-9 isolate of JEV due to the ability of the cells to suppress IFN signaling by inhibiting the IFN-α-stimulated tyrosine phosphorylation of STAT1 as early as 3 hours post infection (HPI) (Lin *et al*., 2004). A previous investigation demonstrated that the neurovirulent RP-9 isolate of JEV was able to effectively multiply in A549 cells due to the ability of this virus isolate to suppress IFN signaling by inhibiting the IFN-α-stimulated tyrosine phosphorylation of STAT1 as early as 3 hours post infection (HPI) (Lin *et al*., 2004) The virulent TX02 isolate of WNV lineage I was found to multiply to high infectious titers in A549 cells (IFN-α/β resistant), while the avirulent MAD78 isolate of WNV lineage II exhibited markedly decreased

infectious titers in A549 cells (IFN-α/β resistant) (Keller *et al*., 2006). The authors concluded that the emergence of WNV into the Western Hemisphere could have been due to the ability of particular WNV strains (namely WNV lineage I strains) to control the host IFN-α/β response to infection (Keller *et al*., 2006). Similarly, Genotype I-b of JEV may have emerged and established itself as the dominant JEV genotype due to the resistance of this genotype to the IFN-α/β produced by the reservoir host of the virus, while GIV of the virus may be confined to the Indonesian archipelago due to the sensitivity of this genotype to the IFN-α/β produced by the reservoir host of the virus.

1.9.2 Avian model of viremia

A previous study designed to assess the cross-protection between WNV and JEV in red-winged blackbirds (*Agelaius phoeniceus*) found that the eight WNV-seronegative blackbirds inoculated with the 826309 isolate of JEV (India, human, 1982, GIII) had viremias lasting an average of 3.3 days with an average peak viremia of 3.3 $\log_{10}$PFU/mL of serum, while blackbirds inoculated with the VN isolate of JEV (Vietnam, *Cx. tritaeniorhynchus*, 2003, GI-b) had viremias lasting an average of 3.8 days with an average peak viremia of 4.0 $\log_{10}$PFU/mL of serum (Nemeth *et al*., 2009). Furthermore, two weeks post inoculation all 16 of the blackbirds had developed JEV-specific antibodies, as determined by 80% plaque reduction neutralization test ($PRNT_{80}$) titers using the 826309 isolate of JEV (Nemeth *et al*., 2009). In terms of the magnitude and duration of viremia, the results of this study are in agreement with two older experimental studies involving ardeids (Buescher *et al*., 1959a; Soman *et al*., 1977). This suggests that blackbirds could be used to compare the magnitude and duration of viremias following inoculation with viruses representative of GI-IV of JEV. Unfortunately, the WRCEVA at UTMB does not possess any of the JEV isolates used in the experimental laboratory

infections of egrets and herons that were described in section 1.6.3. Therefore, the results of the older studies using herons and egrets cannot be directly compared to the recent studies using blackbirds. It is possible that GI-b may have emerged and established itself as the dominant Asian JEV genotype due to the increased multiplicative ability of this genotype in avians, while the failure of GIV to spread beyond the Indonesian archipelago may due to the decreased multiplicative ability of this genotype in avians (avians may not develop viremias of sufficient magnitude and/or duration for the virus to be transported to distant geographic regions). Although important in understanding the ecology and geographical distribution of JEV, none of the aforementioned studies include information regarding the temperature responses of birds to JEV infection.

1.9.3 Mouse models of neurovirulence and neuroinvasiveness

A previous study showed no large differences in neurovirulence (measured by 50% lethal dose [LD_{50}] and average survival time [AST]) among groups of 6-8 week old C57BL/6 mice intracranially inoculated with isolates representative of GI-IV of JEV (Beasley *et al*., 2004). However, no studies have described the mouse neuroinvasiveness of GI-IV of JEV. Several older studies have shown a correlation between age and resistance to intraperitoneal inoculation of viruses within the JEV serocomplex, namely JEV (Grossberg & Scherer, 1966), MVEV (Macdonald, 1952), and WNV (Eldadah *et al*., 1967). Specifically, mice were susceptible to a low intraperitoneal inoculum of only neuroinvasive JEV serocomplex virus strains until 3-4 weeks of age (Eldadah *et al*., 1967; Grossberg & Scherer, 1966; Macdonald, 1952). An older study was able to show differences in mortality in young A2G mice intraperitoneally inoculated with various yellow fever virus (YFV) isolates (Fitzgeorge & Bradish, 1980), suggesting that a young

mouse model of neuroinvasiveness could be used to show differences in mortality in young mice inoculated intraperitoneally with isolates representative of GI-IV of JEV.

1.10 SPECIFIC AIMS

The **overall objective** of this dissertation research is to utilize experimental approaches to better understand the genotypic and phenotypic determinants of JEV geographical expansion. The **central hypothesis** of this research is that GIV of JEV remained confined to Indonesia, whereas genotypes I and III spread throughout Asia due to viral molecular determinants that relate to differences in mosquito vector and/or avian host preference. This hypothesis was tested by relating differences in the molecular epidemiology of JEV isolates (specific aim 1) to the phenotypic properties of the isolates through *in vitro* (specific aim 2) and *in vivo* (specific aim 3) investigations. The overall hypothesis for specific aim 1 is that genetic characterization of isolates of JEV will identify the extent of genetic variation among and within the virus genotypes, thus providing an enhanced understanding of the phylogeography, phylodynamics, evolution and epidemiology of the virus. The overall hypothesis for specific aim 2 is that avian, mosquito, and mammalian cell cultures infected with GI-b will exhibit increased viral multiplication compared to cell cultures infected with representatives of the other virus genotypes, while cell cultures infected with GIV of JEV will exhibit decreased viral multiplication compared to cell cultures infected with GI-III of the virus. The overall hypothesis for specific aim **3** is that blackbirds inoculated with GI-b of JEV of will exhibit higher viremias compared to birds inoculated with representatives of the other virus genotypes and blackbirds inoculated with GIV of JEV will exhibit lower viremias compared to blackbirds inoculated with GI-III of the virus. Furthermore, young mice

inoculated intraperitoneally with representatives of GI-IV of JEV will exhibit different neuroinvasive phenotypes.

1.10.1 Specific aim 1a

Aim: Analyze the genetic variation among isolates of JEV by determining the nucleotide sequence of the NS5 gene/3'UTR and E gene of 26 isolates collected from the former Soviet Union, Japan and Korea during the 1930s through the 1990s.

Hypothesis: Genetic characterization of older isolates of JEV will identify the extent of genetic variation among and within the virus genotypes.

Rationale: There is a lack of genetic information on JEV isolates collected prior to the 1970s.

1.10.2 Specific aim 1b

Aim: Analyze the genetic variation, phylogenetic relationships, operation of selection, and codon usage using a dataset of ORF sequence information derived from isolates representative of GI-IV of JEV, including three newly sequenced GII isolates.

Hypothesis: Genotype II of JEV is most closely related to GI of the virus, and selection pressures and codon usage will vary according to viral genotype.

Rationale: The database of wild-type JEV isolates for which there is ORF nucleotide sequence information consists of 15 isolates, but only one of these isolates belong to GII of the virus (FU, Australia, 1995). The absence of sequence information on other GII isolates has prevented us from obtaining a firm understanding of the genetic variation and phylogenetic relationships among isolates of JEV; it also has precluded an investigation to determine whether selection and codon usage bias exist among isolates of the virus.

1.10.3 Specific aim 1c

Aim: Analyze the genetic variation and evolutionary adaptation among JEV isolates collected from Indonesia, the proposed geographic origin of JEV evolution, by determining the nucleotide sequence of 24 isolates collected between 1974 and 1987, and then performing a series of phylogenetic and evolutionary adaptation analyses.

Hypothesis: Genetic characterization of additional Indonesian JEV isolates collected over a 14-year time period will reveal that the extent of genetic diversity is greater than currently recognized.

Rationale: Only 13 Indonesian JEV isolates collected between 1978 and 1981 have been genetically characterized, consequently limiting our understanding of the genetic variation and evolution of the virus.

1.10.4 Specific aim 1d

Aim: Utilize a dataset consisting of 487 E-gene derived JEV sequences sampled from isolates collected from 12 countries over 75 years to determine when and where the virus and its genotypes originated, the geographical range of the genotypes, the existence of an association between genotype and climate of virus collection, and the amino acid substitutions involved in the phylogenetic divergence of the viral genotypes.

Hypothesis: Utilization of a comprehensive, spatiotemporally distributed JEV sequence dataset will reveal that the phylogeography, evolution, and epidemiology of the JEV genotypes are distinct.

Rationale: No studies have utilized a comprehensive dataset of viral sequences to examine the phylogeography, evolution, and epidemiology of the JEV genotypes.

1.10.5 Specific aim 1e

Aim: Utilize a dataset consisting of 453 GIII and GI E-gene derived JEV sequences sampled from 11 countries over 75 years to reconstruct the chronology and evolutionary dynamics of the genotype displacement on a spatiotemporal scale using Bayesian coalescent methods, and a series of adaptive evolutionary algorithms to elucidate genetic determinants associated with the emergence of GI.

Hypothesis: Utilization of a comprehensive, spatiotemporally distributed dataset consisting of E-gene derived GIII and GI JEV sequences will reveal the chronology, evolutionary dynamics, and genetic determinants underlying the geographically expansive genotype displacement.

Rationale: No studies have reconstructed the spatiotemporal chronology of the emergence and establishment of GI as the dominant JEV genotype throughout Asia, nor have any studies identified genetic determinants underlying the genotype displacement as it unfolded across Asia.

1.10.6 Specific aim 2a

Aim: Compare the *in vitro* multiplication kinetics of GI-IV of JEV in duck embryo fibroblast (DEF) cells incubated at 37, 41, and 44°C.

Hypothesis: Duck embryo fibroblast cell cultures infected with a GI-b isolate and incubated at 37°C will exhibit increased viral multiplication compared to cell cultures infected with isolates representative of the other genotypes, whereas DEF cell cultures infected with a GIV isolate and incubated at 37°C will exhibit decreased viral multiplication compared to cell cultures infected with isolates representative of the other genotypes. Additionally and/or alternatively, DEF cell cultures infected with a GI-b isolate and incubated at 41 and 44°C will exhibit increased viral multiplication compared to cell cultures infected with isolates representative of the other genotypes (GI-b will

exhibit a thermostable phenotype in DEF cells), whereas DEF cell cultures infected with a GIV isolate and incubated at 41 and 44°C will exhibit decreased viral multiplication compared to cell cultures infected with isolates representative of the other genotypes (GIV will exhibit a temperature-sensitive phenotype in DEF cells).

Rationale: An increased multiplicative efficiency of GI-b viruses in avians sustaining normal body temperatures and/or developing elevated body temperatures would have resulted in an increased number of JEV avian-mosquito-avian transmission cycles, in turn leading to the displacement of GIII by GI as the dominant Asian genotype of JEV. Conversely, a decreased multiplicative efficiency of GIV viruses in avians sustaining normal body temperatures and/or developing elevated body temperatures may have resulted in a decreased number of JEV avian-mosquito-avian transmission cycles, leading to the confinement of GIV to the Indonesian archipelago.

1.10.7 Specific aim 2b

Aim: Compare the *in vitro* multiplication kinetics of GI-IV of JEV in *Aedes albopictus* C6/36 cells incubated at 28°C.

Hypothesis: C6/36 cell cultures infected with a GI-b isolate will exhibit increased viral multiplication compared to cell cultures infected with isolates representative of the other genotypes, whereas C6/36 cell cultures infected with a GIV isolate will exhibit decreased viral multiplication compared to cell cultures infected with isolates representative of the other genotypes.

Rationale: An increased multiplicative efficiency of GI-b viruses in mosquitoes in nature would have resulted in an increased number of JEV mosquito-avian-mosquito transmission cycles, in turn leading to the displacement of GIII by GI as the dominant Asian genotype of JEV. Conversely, a decreased multiplicative efficiency of GIV viruses

in mosquitoes in nature may have resulted in a decreased number of JEV mosquito-avian-mosquito transmission cycles, leading to the confinement of GIV to the Indonesian archipelago.

1.10.8 Specific aim 2c

Aim: Compare the *in vitro* multiplication kinetics of GI-IV of JEV in Vero cells incubated at 37°C and the temperature-sensitivity of the genotypes in Vero cells by comparing the plaquing efficiency of cells incubated at 37 and 41°C.

Hypothesis: Vero cells infected with isolates representative of GI-IV of JEV will exhibit phenotypic differences as measured by differences in viral multiplication or temperature-sensitivity.

Rationale: Vero cells have been used extensively in virological studies to compare the multiplication kinetics of different virus strains and detect the temperature-sensitive phenotype implying that these cells can be used to detect phenotypic differences in isolates representative of GI-IV of JEV.

1.10.9 Specific aim 2d

Aim: Compare the *in vitro* multiplication kinetics of GI-IV of JEV in A549 cells (IFN-α/β-competent) incubated at 37°C.

Hypothesis: A549 cell cultures infected with a GI-b isolate will exhibit increased viral multiplication compared to cell cultures infected with isolates representative of the other genotypes, whereas A549 cell cultures infected with a GIV isolate will exhibit decreased viral multiplication compared to cell cultures infected with isolates representative of the other genotypes.

Rationale: Genotype I-b of JEV may have emerged and established itself as the dominant JEV genotype due to the resistance of this genotype to the IFN-α/β produced

by the reservoir host of the virus (resulting in an increased multiplicative efficiency) compared to the other viral genotypes, while GIV of the virus may be confined to the Indonesian archipelago due to the sensitivity of this genotype to the IFN-α/β produced by the reservoir host of the virus (resulting in a decreased multiplicative efficiency) compared to the other viral genotypes. Of note, most elements of the mammalian type I IFN system have been functionally conserved in avians (Staeheli *et al*., 2001). Comparative genomic analysis of chicken and human genomes has indicated two conserved genes, IFN-α/β receptor 1 [IFNAR1] and IFN-α/β receptor 2 [IFNAR2], which likely encode for the two subunits of the type I IFN receptor (Staeheli *et al*., 2001). Previous studies have also revealed that the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, which mediates IFN-α/β signal transduction in mammals, is also functional in avians (Staeheli *et al*., 2001).

1.10.10 Specific aim 3a

Aim: Compare the induction, magnitude, and duration of viremias in blackbirds inoculated subcutaneously with GI-IV of JEV.

Hypothesis: Blackbirds inoculated with GI-b of JEV will exhibit viremias of increased magnitude and/or duration compared to blackbirds inoculated with a GI-a virus or viruses representative of GII-IV of the virus, and blackbirds inoculated with GIV of JEV will exhibit viremias of decreased magnitude and/or duration compared to blackbirds inoculated with GI-III of the virus.

Rationale: Genotype I-b of JEV may have emerged and established itself as the dominant JEV genotype due to its enhanced multiplicative efficiency in avians compared to the other viral genotypes, while GIV of the virus may be confined to the Indonesian

archipelago due to its poor multiplicative efficiency in avians compared to the other viral genotypes.

1.10.11 Specific aim 3b

Aim: Compare the neuroinvasiveness in 8-day old mice inoculated intraperitoneally with isolates representative of GI-IV of JEV.

Hypothesis: Young mice inoculated with isolates representative of GI-IV of JEV will exhibit differences in neuroinvasiveness as measured by the LD_{50}, average survival time (AST) and average day of death (ADD).

Rationale: It has been demonstrated that young mice are susceptible to intraperitoneal inoculation of JEV implying that 8-day old mice can be used to show differences in the neuroinvasiveness among isolates representative of GI-IV of JEV.

Figure 1.1: Geographical range of JEV activity.

Figure 1.2: Transmission cycle of JEV.

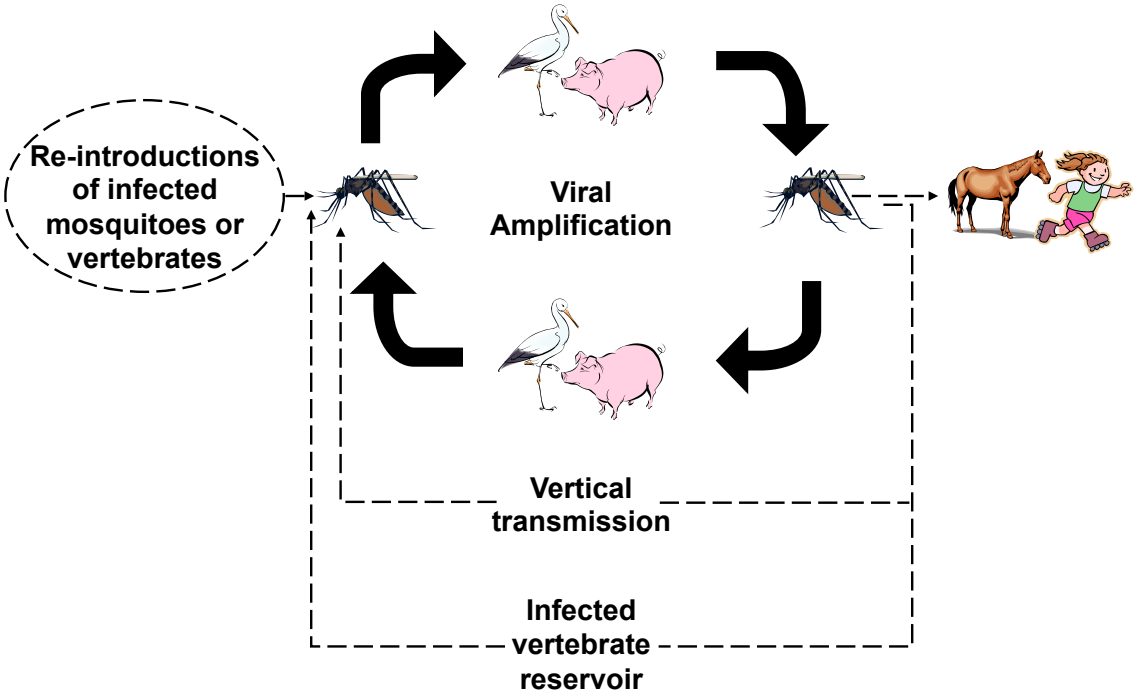
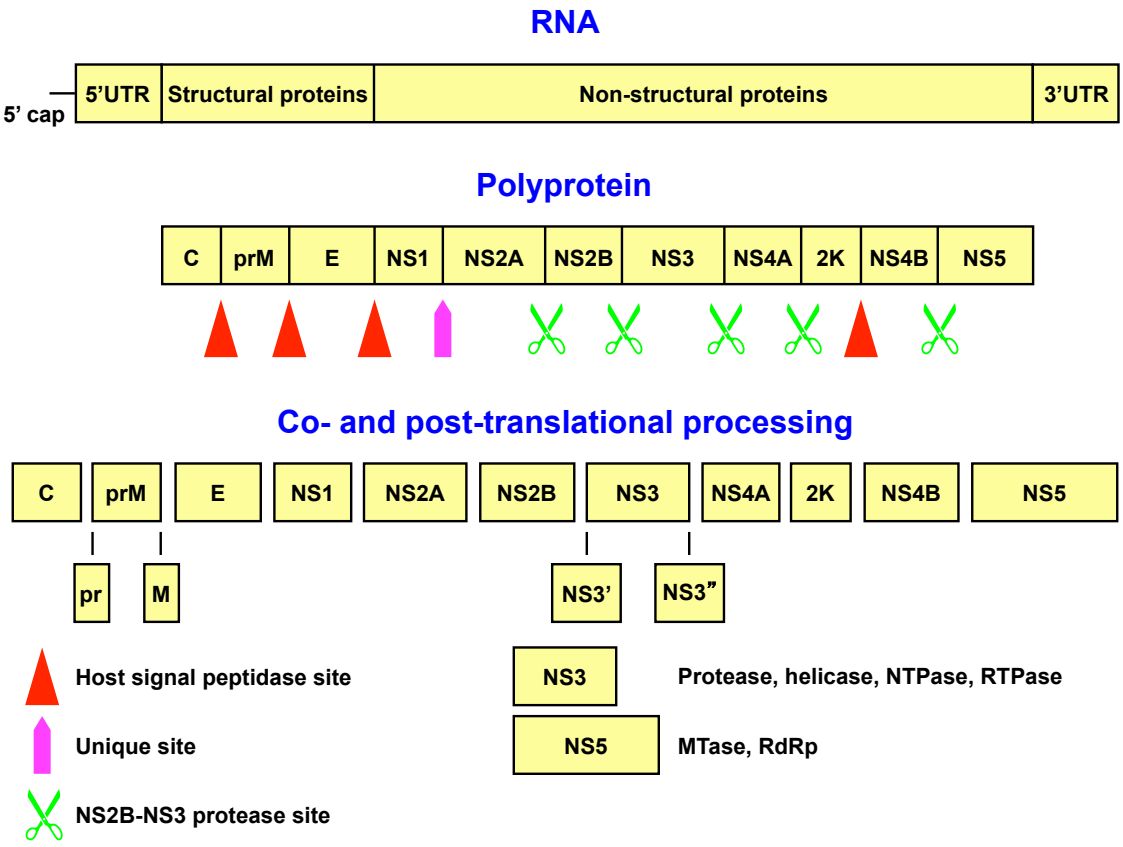


Figure 1.3: Schematic representation of the flavivirus RNA genome structure and polyprotein processing.



Chapter 2: Materials and methods

2.1 BUFFER AND CELL CULUTRE MEDIA RECIPES

2.1.1 2X Tryptose phosphate broth

29.5 g Tryptose phosphate broth (TPB) powder (Sigma-Aldrich, Saint Louis, MO)

500 mL distilled water

Autoclave for 30 minutes on liquid cycle

2.1.2 2% Diethylaminoethyl-dextran

2 g Diethylaminoethyl (DEAE)-dextran HCL salt (Sigma-Aldrich)

100 mL distilled water

Sterile filter

2.1.3 2% 7.5% sodium bicarbonate

7.5 g sodium bicarbonate powder (Sigma-Aldrich)

100 mL distilled water

Sterile filter

2.1.4 Monkey kidney Vero, adenocarcinomic human alveolar basal epithelial A549, and duck embryo fibroblast cell culture media

500 mL 1X minimum essential media (MEM) supplemented with Earle's salts and L-glutamine (Gibco, Carlsbad, CA)

50 mL (for culture) or 10 mL (for infection) bovine growth serum (BGS) (HyClone, Logan, UT)

5 mL penicillin-streptomycin liquid (5,000 units penicillin and 5,000 μg/mL streptomycin utilizing penicillin G and streptomycin sulfate in 0.85% saline) (Gibco)

5 mL 100X (10nM) non-essential amino acids (NEAA) (Gibco)

5 mL 100X (200 nM) L-glutamine (Gibco)

2.1.5 *Aedes albopictus* C6/36 cell culture media

500 mL 1X MEM with Earle's salts and L-glutamine (Gibco)

50 mL (for culture) or 10 mL (for infection) fetal bovine serum (FBS) (Hyclone)

25 mL TPB

5 mL penicillin-streptomycin liquid (5,000 units penicillin and 5,000 μg/mL streptomycin utilizing penicillin G and streptomycin sulfate in 0.85% saline) (Gibco)

5 mL 100X (10nM) NEAA (Gibco)

5 mL 100X (100 nM) sodium pyruvate (Gibco)

2.1.6 2X MEM

100 mL 10X MEM (Gibco)

35 mL 7.5% sodium bicarbonate

5 mL 100X (200 nM) L-glutamine (Gibco)

5 mL penicillin-streptomycin liquid (5,000 units penicillin and 5,000 μg/mL streptomycin utilizing penicillin G and streptomycin sulfate in 0.85% saline) (Gibco)

5 mL 100X (10nM) NEAA (Gibco)

20 mL FBS (HyClone)

5 mL 2% DEAE-Dextran solution

325 mL distilled water

2.2 CELL CULTURE

Cells were maintained in the appropriate media (see sections 2.1.4 and 2.1.5). Vero, DEF, and A549 cells were incubated at 37°C in the presence of 5% CO_2, while

C6/36 cells were incubated at 28°C in the absence of CO_2. Vero, DEF, and A549 cells were passaged by dissociating the cell monolayer from the flask through trypsin treatment (Gibco), while C6/36 cells were passaged by dislodging the cell monolayer from the flask by jarring. The American Type Culture Collection (ATCC) number, origin, and passage number(s) (for the viral multiplication kinetics experiments) for each of these cell lines is shown in Table 2.1.

2.3 ISOLATES OF JAPANESE ENCEPHALITIS VIRUS

Isolates of Japanese encephalitis virus (JEV) utilized for the studies described in this dissertation were obtained from the Barrett lab virus stocks, the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch (UTMB; Galveston, TX), and the Walter Reed Army Institute of Research (WRAIR; Silver Spring, Maryland). The details of the JEV isolates used in this dissertation research are provided in tables according to the specific study in which they were used (Chapters 3-8).

2.4 VIRUS GROWTH

Confluent 25 cm^2 flasks of either Vero or C6/36 cells were rinsed with 2 mL of phosphate buffered saline (PBS) (Gibco) (Chapters 3, 4, 5, 6, and 8). The monolayer was then inoculated with 100 μL of virus and 500 μL of PBS, and the flask was briefly rocked. The virus was allowed to adsorb for 30 minutes at room temperature and 7 mL of the appropriate cell culture medium was then added (see sections 2.1.4 and 2.1.5). Vero cells were incubated at 37°C in the presence of 5% CO_2, while C6/36 cells were incubated at 28°C. The cells were observed daily for cytopathic effect (CPE) or dislodgement. When approximately 50% of the cells exhibited CPE or had dislodged from the flask (typically occurred between 3 and 7 days post infection [DPI]), the cell

culture supernatant was transferred into a 15-mL conical tube and centrifuged at 2,000 revolutions per minute (RPM) for 5 minutes. The supernatant was then aliquoted into 14, 1.5-mL cryovials and stored at -80°C.

2.5 PLAQUE ASSAY

Infectious titers of the aliquots were determined by plaque titration in Vero cells incubated at 37°C in the presence of 5% CO_2 (Chapters 7 and 8). Vero cells were grown to approximately 100% confluency in 6-well culture plates containing MEM in the presence of 10% BGS. Serial 10-fold dilutions of sample aliquots (100 µL) were inoculated onto Vero cells rinsed with PBS. The aliquots were adsorbed for 30 minutes at room temperature prior to overlaying the monolayer with 4 mL of a 1:1 solution of 2% agar:2X MEM. Four days following the first overlay, 2 mL of a 1:1 mixture of 2% agar:2X MEM containing 2% neutral red (Gibco) was applied to each well. Plaques were counted the following day and infectious titers expressed in plaque forming units (PFU)/mL were calculated.

2.6 VIRAL RNA EXTRACTION

Viral RNA was extracted from 140 µL of either infected Vero or C6/36 cell culture supernatants using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA) (Chapters 3, 4, 5, and 6).

2.7 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Either the non-structural (NS) 5 gene/3' untranslated region (UTR) (Chapter 3), envelope (E) gene (Chapters 3, 5, and 6), or open reading frame (ORF) (Chapter 4) of the viruses were amplified using the Titan One Tube RT-PCR System (Roche, Indianapolis, IN) (Table 2.2) and overlapping primer pairs (Table 2.2). The reverse transcription-

polymerase chain reaction (RT-PCR) reactions were performed in a thermocycler according to the parameters listed in Table 2.4.

2.8 DNA PURIFICATION

The resulting amplicons were separated by electrophoresis in ethidum bromide-stained 2% agarose/tris-acetate-ethylenediaminetetraacetic acid [EDTA] (TAE) buffer gels (Chapters 3, 4, 5, and 6). Amplicons were extracted from gels under ultraviolet light and purified using the QIAquick Gel Extraction Kit according to the manufacturer's instructions (Qiagen).

2.9 DNA SEQUENCING

Purified DNA was directly sequenced using the amplification primers (Table 2.2) (Chapters 3, 4, 5, and 6). Sequencing was performed using standard methods at the Molecular Genomics Core (Applied Biosystems ABI Prism 3100 Avant and 3130XL DNA sequencers) or the Protein Chemistry Core (Applied Biosytems 3100 Genetic Analyzers) at UTMB.

2.10 CONTIG ASSEMBLY, CONSTRUCTION OF NUCLEOTIDE AND AMINO ACID SEQUENCE ALIGNMENTS, AND CREATION OF DATASETS

The raw nucleotide sequence files were assembled using ContigExpress (Vector NTI, Invitrogen, Carlsbad, CA) and then aligned, using AlignX (Vector NTI, Invitrogen) with selected JEV sequences that were retrieved from GenBank (Chapters 3, 4, 5, 6, and 7). The nucleotide sequence alignment files were then transferred to BioEdit (Hall, 1999) to manually align gaps in the 3'UTR, to produce deduced amino acid alignment files, and to determine the percent nucleotide and amino acid sequence identity between among select JEV isolates.

The date, geographic origin, and host of viral isolate collection were recorded for each JEV sequence downloaded from GenBank. The nucleotide sequence alignment files used in the coalescent, phylogeographic, and phylodynamic analyses (Chapters 4, 5, 6, and 7) were pruned of sequences derived from non-wild type JEV isolates (e.g., vaccine strains, mouse-brain adapted strains, and plaque variants) and sequences absent of information regarding the date and country of collection of the viral isolate. For the phylogeographic analyses (Chapter 6), isolates collected north of the Tropic of Cancer (23.5°N) were classified as temperate, while isolates collected south of the Tropic of Cancer were classified as tropical. The climate of five Taiwanese isolates could not be discerned and therefore these isolates were not included in the climate phylogeographic analysis.

2.11 PHYLOGENETIC ANALYSES

Phylogenetic relationships were inferred using neighbor-joining (NJ), maximum-likelihood) ML (maximum-likelihood), MP (maximum-parsimony), and/or Bayesian methods available within SeaView v 4.2.12 (Gouy *et al*., 2010), the PHYLIP package (Felsenstein, 1989), the South of France bioinformatics platform (Guindon *et al*., 2010), and/or MrBayes v 3.1.2 (Ronquist & Huelsenbeck, 2003) (Chapters 3, 4, 5, 6, and 7). MODELTEST (Posada & Crandall, 1998) in conjunction with PAUP Beta 4.0 (Sinauer Associates, Sunderland, MA) was used to identify the best-fit nucleotide substitution model for use in the phylogenetic analyses. The robustness of the NJ and MP phylogenies were evaluated by bootstrap resampling with 1,000 replicates, while the robustness of the ML phylogenies were evaluated by bootstrap resampling with 100 replicates. The Bayesian analysis was executed twice for 1.6 million generations, model convergence was assessed using Tracer v 1.5 (Rambaut & Drummond, 2005), and the robustness of

the resulting phylogeny was assessed by determining posterior probability (PP) values for each node within the tree. The MVE-1-51 isolate of Murray Valley encephalitis virus (MVEV), a member of the Japanese encephalitis serocomplex, was used as the outgroup in all analyses. All trees were drawn using FigTree v 1.3.1 (Rambaut, 2008).

2.12 RECOMBINATION ANALYSES

The presence of recombination in a nucleotide sequence alignment can confound the results of selection and coalescent analyses. Therefore, the presence of recombination in the nucleotide sequence alignment files was analyzed using RDP (Martin & Rybicki, 2000), GENECONV (Padidam *et al*., 1999), Chimera (Posada & Crandall, 2001), MaxChi (Smith, 1992) and Bootscan (Martin *et al*., 2005b) methods implemented in RDP3 v Beta 41 (Martin *et al*., 2005a) (Chapters 4, 5, 6, and 7). Common program settings for all methods were to perceive sequences as linear, to require phylogenetic evidence, to refine breakpoints, and to check alignment consistency. The highest acceptable p-value was set at 0.05, after considering Bonferroni correction for multiple comparisons. All method-specific program settings remained at their default values. Only recombination events that were identified by at least two methods were considered as potential recombination events. The breakpoint positions and recombinant sequence inferred for every detected potential recombination event were manually verified using phylogenetic and recombination signal analysis features in RDP3.

2.13 TIME-SCALED PHYLOGENETIC ANALYSES

Time-scaled Bayesian phylogenies were inferred using a Bayesian Markov chain Monte Carlo (MCMC) method implemented in the BEAST package v 1.6.1 (Drummond & Rambaut, 2007) (Chapters 6 and 7). An SDR06 nucleotide substitution model, a relaxed-uncorrelated exponential molecular clock, and a piecewise constant Bayesian

skyline demographic model (20 coalescent-interval groups) were used in all analyses. The SDR06 nucleotide substitution model (links codon positions one and two [CP_{12}], but allows codon position three [CP_3] to vary in the rate of nucleotide substitution, the transition to transversion ratio, and the gamma-distributed rate heterogeneity) was selected as it has been shown to impose a reasonable balance of prior information to fit protein encoding nucleotide data (Shapiro *et al*., 2006). The relaxed-uncorrelated exponential molecular clock was found to best-fit the data when Bayes factor (BF) values were calculated (Tracer v 1.5.1) (Rambaut & Drummond, 2005) to evaluate the relative fit of strict and relaxed molecular clock models to the data by determining the natural logarithm of the ratio of the marginal likelihoods of the competing models (Suchard *et al*., 2001). The Bayesian skyline demographic model was utilized because it has been found to enforce the fewest demographic assumptions on the data (Drummond *et al*., 2005). Six (Chapter 7) to twelve (Chapter 6) independent runs of 50 million generations were executed, sampling every 1,000th state, using the Bioportal at the University of Oslo (Kumar *et al*., 2009). The log and tree files from the runs were combined and model convergence was ensured by confirming that the effective sample size for all parameters was ≥ 200 using Tracer v1.5 (Rambaut & Drummond, 2005). The log files were used to obtain mean overall rates of evolution, as well as evolutionary rates for CP_{12}, CP_3 and to compute the CP_{12}/CP_3 ratio (Chapter 7 only). Bayesian maximum clade credibility (MCC) phylogenies were generated from the posterior set of trees, and nodes were annotated with PP values and time of the most recent common ancestor (MRCA) with uncertainty in estimates reflected by 95% highest posterior density (HPD) values. Phylogenies were viewed in FigTree v 1.3.1 (Rambaut, 2008).

2.14 PHYLOGEOGRAPHIC ANALYSES

Viral dispersion patterns between geographical locations (country and climate) were inferred using a continuous-time Markov chain model (CTMC) implemented in BEAST (Lemey *et al*., 2009) (Chapters 6 and 7). The CTMC method estimates a reversible diffusion rate for each plausible migration pathway among the locations while simultaneously estimating coalescent parameters, thereby quantifying the uncertainty in ancestral state reconstructions. The Bayesian stochastic search variable selection (BSSVS) procedure was used to identify links between locations that explain the most likely virus dispersion pathways (Lemey *et al*., 2009). To assess the geographical location of the MRCA of phylogenetic nodes, state posterior probabilities for each plausible location were inferred for the corresponding nodes within the MCC phylogenies. Additionally, terminal branches of the MCC phylogenies were colored according to geographic location of the taxon at the tip, while internal braches were colored according to the most probable location of their parental node. Bayes factor tests were used to determine the statistical significance of the diffusion pathways between the countries by integrating over the posterior sample of phylogenies (Chapter 7 only). Pathways with a BF > 5 were considered statistically significant.

2.15 DISTRIBUTION OF INDONESIAN JEV ISOLATES ACCORDING TO YEAR OF COLLECTION AND GENOTYPE

To test the null hypothesis that there is no association between the year of collection of the Indonesian JEV isolates and their genotype, a list of all Indonesian JEV isolates for which there was nucleotide sequence information available was created (Chapter 5). The relationships between: 1) the year of collection of all of the Indonesian isolates and genotype and, 2) the year of collection of Java isolates only and genotype was evaluated by using the Pearson's chi-square test was performed at $\alpha = 0.05$ (IBM

SPSS Statistics v 19, Armonk, NY). Post-hoc analyses were then performed to determine which cell(s) in the table of year of collection versus genotype contributed most to the statistically significant Pearson's chi-square test. Residuals (the difference between the observed and the expected frequency) and standardized residuals (z-scores) were calculated and then the standardized residuals were compared against the critical z-value (± 1.96) for $\alpha = 0.05$ (IBM SPSS Statistics v 19).

2.16 DISTRIBUTION OF JEV ISOLATES ACCORDING TO GENOTYPE AND CLIMATE

Virus sequences derived from isolates collected north of the Tropic of Cancer (23.5°N) were classified as temperate, while isolates collected south of the Tropic of Cancer were classified as tropical. To test the null hypothesis of no association between climate and genotype, a Fisher's exact test was performed at $\alpha = 0.05$ (IBM SPSS Statistics v 20) (Chapter 7). Post-hoc analyses were then performed to determine which cell(s) in the table of genotype versus climate contributed the most to the statistically significant Fisher's exact test. Residuals and adjusted standardized residuals were calculated. The adjusted standardized residual values were then compared against the critical z-value (± 1.96) for $\alpha = 0.05$ (IBM SPSS Statistics v 20). Only GI-a, GI-b, GII and GIII were considered in these analyses, as GIV and GV are comprised of only three sequences each.

2.17 ANALYSES OF GEOGRAPHICAL STRUCTURE

To access the overall degree of geographical structure in the GIII and GI phylogenies (Chapter 7), the association index (AI), parsimony score (PS), unique fraction (UniFrac), nearest taxa (NT), net relatedness (NR) and phylogenetic diversity (PD) statistics were calculated for each genotype from the posterior set of trees generated by BEAST using Befi-BaTS v 0.1.1 (Parker *et al*., 2008). Additionally, a null distribution

was produced for each statistic. The reported p-value is the proportion of trees from the null distribution that are ≥ to the mean posterior estimate of the statistic generated from the posterior sample of trees. The degree of geographical structure was also accessed according to individual location using the maximum exclusive single-state clade size (MC) statistic.

2.18 PHYLODYNAMIC ANALYSES

Using the log and tree files produced from BEAST analyses, Bayesian skyline reconstructions were generated using Tracer v 1.5 (Rambaut & Drummond, 2005) to reveal the demographic histories of GIII and GI (Chapter 7). Demography was measured as the product of the effective population size and generation time (a measure of relative genetic diversity), as a function of time.

2.19 ESTIMATION OF THE GLOBAL RATIO OF NON-SYNONYMOUS TO SYNONYMOUS SUBSTITUTIONS

The single-likelihood ancestor counting (SLAC) method available on the Datamonkey webserver (Pond & Frost, 2005) was used to estimate the global number of synonymous nucleotide substitutions (d_S), non-synonymous nucleotide substitutions (d_N), and the d_N/d_S ratio (and the associated 95% confidence interval [CI]) (Chapters 4, 5, and 7).

2.20 DETECTION OF ADAPTIVE EVOLUTION

Evidence of adaptive evolution within the ORF, E gene, GIII, and GI alignments was evaluated using methods available within HyPhy v 2.0 or on the Datamonkey webserver (Pond & Frost, 2005; Pond *et al*., 2005) (Chapters 4, 5, and 7). The presence of positive selection ($d_N/d_S > 1$) was evaluated codon-by-codon across the GIII and GI nucleotide sequence alignments using the SLAC, random effects likelihood (REL;

Chapter 4 only), fixed effects likelihood (FEL) and internal FEL methods using NJ phylogenies and the reversible nucleotide substitution model (Kosakovsky Pond & Frost, 2005). Statistically significant evidence of positive selection was indicated by a p-value < 0.05. The SLAC methods compares the observed d_N/d_S ratio with the expected ratio assuming neutral evolution, the FEL method estimates d_N and d_S rates at every site (i.e., branch of the phylogeny) and the IFEL method considers only the internal branches of the phylogeny.

Evidence of directional selection within the E protein, GIII and GI amino acid alignments was assessed using the directional evolution in protein sequences (DEPS) method (Kosakovsky Pond *et al*., 2008) (Chapters 5 and 7). Neighbor-joining phylogenies and the amino acid substitution model that best-fit the data were used to identify statistically significant shifts in amino acid residue frequencies ($p < 0.05$) and/or significantly large number of substitutions towards a particular residue (empirical BF [EBF] > 100).

Identification of co-evolving sites within the E protein, GIII, and GI amino acid alignments was performed using the Spidermonkey method (Poon *et al*., 2007) (Chapters 5 and 7). The amino acid substitution model that best-fit the data was used to estimate substitutions over a NJ phylogeny. Reconstructed ancestral sequences were re-sampled, a two-parent directed network was used, and sites were filtered based on a minimum count of three substitutions across the phylogeny (threshold ≥ 3). Only sites with a PP ≥ 0.90 and a percent bootstrap replicate value ≥ 90 were reported.

2.21 CODON USAGE ANALYSES

The analysis of codon usage patterns was performed on a set of 44 nucleotide sequence alignment files, which were created by dividing the ORF dataset according to

genotype and gene, using the CodonW software package (Chapter 4). The GC content at the first and second codon positions (GC12), the GC content at the synonymous third codon positions (GC3), and the codon usage index (N_C) were calculated for each virus in all of the datasets and then averaged. The reported value of N_C is always between 20 (when only one codon is used for each amino acid) and 61 (when all codons are used equally). The relationship between GC3 content and GC12 content was examined to determine the relative effects of mutation pressure verses natural selection on codon composition. The Pearson's r correlation coefficient was used to measure the linear relationship between the two interval variables, GC12 and GC3 (IBM Statistics v 18). To examine the influence of GC content on codon usage, the relationship of N_C and GC3 content was plotted. This was compared with the N_C value that would result if GC content were solely responsible for the codon biases, calculated as $N_C = 2 + GC3 + (29/[(GC3)^2 + (1 - GC3)^2])$ (Wright & Bibb, 1992).

2.22 Mapping of important amino acid substitutions onto the E protein dimer structure of JEV

Important amino acid substitutions (genotype-defining amino acid substitutions, node-defining substitutions, substitutions involved in the adaptive evolution of JEV) within the E protein of the JEV isolates were mapped onto the E protein dimer structure of JEV (PBD ID: 3P54, MMDB ID: 87213) using MacPyMOL v 1.3 (Schrodinger, LLC, New York, NY) (Chapters 3, 5, 6, and 7).

2.23 Creation of geographical maps

Geographical maps were created using ArcView GIS v 9.1 (Environmental Research Systems Institute, Redlands, CA) from geographic boundary files downloaded from DIVA-GIS freely available at http://www.diva-gis.org/gData (Assessed on: August

15, 2012). Alternatively, maps were created using GIMP v 2.6.12 (Freely available at: http://www.gimp.org/; Accessed on: August 15, 2012) from a blank map of Asia downloaded from http://english.freemap.jp/ (Assessed on: August 15, 2012) (Chapters 5, 6, and 7).

2.24 VIRAL MULTIPLICATION KINETICS

The multiplication kinetics of isolates representative of genotypes I-IV (GI-IV) of JEV were compared in DEF, C6/36, Vero, and A549 cells (Chapters 7 and 8). The passage numbers of the cells used in the viral multiplication experiments are shown in Table 2.1. Cell cultures grown in 25 cm^2 flasks were rinsed with PBS and then infected in triplicate at a multiplicity of infection (MOI) of 0.1 with virus. Following adsorption for 30 minutes at room temperature, the monolayers were rinsed three times with PBS, 7 mL of the appropriate medium was added to each flask (see sections 2.1.4 and 2.1.5), and the cultures were incubated at the appropriate temperatures (DEF cells at 37, 41, and 44°C in the presence of 5% CO_2, C6/36 cells at 28°C, Vero cells at 37°C in the presence of 5% CO_2, and A549 cells at 37°C in the presence of 5% CO_2). Aliquots of culture medium were removed at 0 hours post infection (HPI) and thereafter at 12 to 24 hour intervals, and replaced with an equal volume of fresh medium. Aliquots were stored at -80°C until infectious titers were determined by plaque assay in Vero cells. One replicate for each experiment was initially plaque titrated. If there was a difference in virus titer between the isolates representative of GI-IV in the initial experimental replicate, the remaining two replicates were then plaque titrated (Chapter 7; only C6/36 cells). The mean and standard deviation (SD) of the infectious titers were plotted for each virus isolate according to HPI. The one-way analysis of variance (ANOVA) procedure was used to determine if there was an overall significant difference between the infectious titers

according to HPI (IBM SPSS Statistics v 18). To determine which of the infectious titers significantly differed from the others a post-hoc Tukey's test was performed (IBM SPSS Statistics v 18, Chicago, IL).

2.25 TEMPERATURE-SENSITIVITY ASSAYS

Virus isolates were plaque titrated in Vero cells at 37 and 41°C to screen for the temperature-sensitivity phenotype (Chapter 8). The temperature-sensitivity phenotype was defined as a ≥ 2.0 $\log_{10}$PFU/mL reduction in infectious titer at 41°C compared to 37°C.

2.26 BLACKBIRD VIREMIA PHENOTYPE

In collaboration with Dr. Richard Bowen of Colorado State University, red-winged blackbirds (*Agelaius phoeniceus*) with a known West Nile Virus (WNV) field serostatus were subcutaneously inoculated over the breast muscle with 3,000 PFU of isolates representative of GI-IV of JEV (5-6 blackbirds per virus isolate) (Chapter 8). All blackbirds were bled daily from 1 DPI to 5 DPI and then again at 7 DPI by jugular or ulnar venipuncture. Blood samples were mixed with bovine albumin-1 diluent following collection to allow for an approximate 1:10 serum dilution, allowed to clot for up to 30 minutes, centrifuged to obtain approximately 10% serum, and then stored at -80°C until plaque titrated in Vero cells. On 14 DPI the blackbirds were euthanized via sodium pentobarbital overdose.

2.27 YOUNG MOUSE NEUROINVASIVENESS PHENOTYPE

Litters (5-11 animals) of eight-day old Swiss Webster mice (Harlan, Houston, TX) were intraperitoneally inoculated with isolates representative of GI-IV of JEV, ranging in titer from 1 to 10,000 PFU (Chapter 8). One litter of mice was inoculated with PBS. Mice were observed until the onset of a moribund condition, and then sacrificed in

accordance with guidelines of the UTMB Institutional Animal Care and Use Committee (IACUC). Mice that were not moribund by 25 DPI were sacrificed. The 50% lethal dose (LD_{50}) was calculated for each virus isolate, and the mean (with the SD) average survival time (AST), and average day of death (ADD) were calculated according to dose for each virus isolate (IBM SPSS Statistics v 20, Armonk, NY). The AST estimate includes mice that were euthanized at 25 DPI (right-censored: Some subjects may still be alive at the end of the study period and therefore the exact survival times of these subjects are unknown). The ADD estimate excludes mice that survived until 25 DPI and were sacrificed. The ADD was estimated to provide a mean survival time for mice that died as a direct result of JEV infection. The Kaplan-Meir statistical method was used to generate time to progression and survival curves, the Mantel-Haenszel log-rank test was used to test the equality of the survival distributions, and the Tamhane's test was used to test the equality of the death distributions (IBM SPSS Statistics v 20).

Table 2.1: Details of the cell lines.

Cell line	ATCC number	Origin	Passage number(s)[1]
DEF	CCL-141	Duck (*Anas platyrhynchus domesticus*) embryo fibroblast cells	12, 13
C6/36	CRL-1660	Cells from the larvae of *Aedes albopictus*	11
Vero	CCL-81	Epithelial cells from the kidney of a normal adult African green monkey (*Cercopithecus aethiops*)	16, 17, 18
A549	CCL-185; Kindly provided by Terence Hill	Adenocarcinomic human alveolar basal epithelial cells initiated through explant culture of lung carcinomatous tissue from an adult Caucasian male	12

[1]Indicated in reference to the cell passage numbers used for the viral multiplication kinetics experiments.

Table 2.2: RT-PCR reaction mixture.

Reagent	Volume (µL)
Reaction buffer	10.00
Dithiothreitol (DTT), 100 mM	2.50
Deoxynucleotide triphosphate (dNTP), 10 mM	1.00
Enzyme mix	1.00
RNase inhibitor	0.25
Forward primer	1.00
Reverse primer	1.00
Viral RNA	5.00
High-performance liquid chromatography water	28.25

Table 2.3: Primers used to amplify and sequence the JEV isolates.

Primer	Sequence 5'-3'
11S	CTGTGTGAACTTCTTGGCTT
484A	GTCCGTATTGTTGACAGCCA
94S	CCATGACTAAAAAACCAGGAGGGCC
996A	CCATTCCCAGGCAATTGAAGCTGTAAGC
F879	GCTTTCCTGGCGGCGGTACTTG
R2570	CCTATCCACCCAGGCTTCCACGTCG
F1468	CGGCAAAGTTTACAGTAACACCCAATGC
R2088	GCACCTTTGAGTTGGCACTGGAAGTC
842S	GCTTTCCTGGCGGCGGTACTTG
1192A	GCCACCGTCGAGATGTCAGTG
940S	TTACTATCCTTCTGCTGTTGGTCGCTCCG
1720A	GTGGCGTGCGCCTCTTCAAA
1271S	GACACATGTGCTAAGTTCTC
1639A	TCTATTTCTCCATGCTGTGT
1598S	CTCGAAGTCATTCTTAGTCCACAGG
2171A	TTGTGCCAGTGATGATTGATCTGC
2115S	GATTCTTACATCGTGGTCGGAAGACG
2624A	CGCTTTGTGGACAATCTTTGCTAAGG
2091S	GAGGAGAAAAACAAATCAACC
2518A	CCACACCTCATCTCTTTTCTTG
2534S	CTTCGTACACAACGATGTGGAAGCTTGGG
3200A	AAGATCACTTTCCTCAACGCCATCTCCC
3053S	GGCGGTCCATAGTGACTTGTCG
3591A	GGCCAGAAACATCACCAGAAGG
3172S	GGGGAGATGGCGTTGAGGAAAGTGATC
3866A	TGCCCCTAGGACCAAAACCATGTTTTCT
3301S	TGGACTTTGATTATTGCCCA
4131A	CCAAGAGTACAGCTCCTTTC
3837S	CAAGAAAACATGGTTTTGGTCCTAGGGGC
4458A	CATCATCCAGCTTAACATCTAGCCTCCGG
3976S	TCCACCATCACCATGCCATT
4499A	CATGGAACACCGGGATCATC
4421S	GGGAAGCAGCCGGAGGCTAGATGTTAA
5122A	TCTTGACGGTCACCTTGCACAATAGCG
5072S	Unknown
5745A	CTGCTCTCTGGAGGCACATTGCTATCT
5603S	AATCCACGATTTGCAGGATGAGATCCCAG
5964A	CACTGGTTATGGGAGACGGATTTCCAAGA
5857S	AATTTTGGAGCGAGCAGGGT
6407A	CTTGCATCAAGCCATCTGGG
6352S	TAGTCACCCGGATGGGTGAGAGAAAGATC
6742A	AAAAAGGTGGCCAGCGTGAGCACTAAA
6619S	AGACCATCACACTCATCATTGC
7212A	CAAGAAAGACAAGGCCAACG
6652S	TGACAGGAGGATTCTTCCTGCTCATGATG
7376A	AACCATTCCGTCTACGACGGCATTCTT

Primer	Sequence 5'-3'
7097S	GGAATACGTCACCACATCATTAGC
7789A	GTGCGGTCCACCTCAATTATGG
7561S	TCTGGAATTCCACCACTGCCACAGGGC
8403A	GCCCTAACAGCACCTGGCTAGTCATGTTG
8194S	ACCGGGGACCTAGAGAGTTCTGC
8809A	TCGTTGAGCACTTCCTTGACTCC
8372S	CGGTGAACATGACCAGCCAG
9011A	AGGTGGTTTTCCCTCTCCAC
8714S	TGACCGACACAACCCCTTTTGG
9354A	CCAAAAGCTCCAGCACCTTGGC
9252S	AGGAAAGCAAGGAGGGAAAA
9588A	CACATGTTGTGGTCCAATAA
FU2	GCTGATGACACCGCCGGCTGGGACAC
CFD3	AGCATGTCTTCCGTGGTCATCCA
9751S	TCAGAAAAGACATCCAGGA
10347A	GTGAAGTCATGTAATCGACA
EMF1	TGGATGACSACKGARGAYATG
VD8	GGGTCTCCTCTAACCTCTAG
10201S	CATACGTGGGAAAGCGTGAGGACATCTGG
10944A	CCACCAGCTACATGTTTCGGCGCTC

Table 2.4: RT-PCR cycling parameters.

Temperature	Time	Number of cycles
50°C	30 min	1
94°C	2 min	
94°C	30 sec	35
65°C[1]	30 sec	
68°C	45 sec	
94°C	30 sec	2
65°C[1]	30 sec	
58°C	7 min	
4°C	∞	-

[1]Annealing temperatures were at least 5°C below the average melting temperatures of the primer pair used in the RT-PCR reaction.

Chapter 3: Genetic characterization of early isolates of Japanese encephalitis virus: Genotype II has been circulating since at least 1951[1]

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3.1 ABSTRACT

Although many recent isolates of Japanese encephalitis virus (JEV) have been genetically characterized, there is a lack of sequence data on isolates collected prior to the 1970s. In this study, phylogenetic characterization of 26 viral sequences derived from early isolates of JEV revealed that 24 of the isolates collected between 1935 and 1989 belonged to genotype III (GIII) and one of the Korean isolates collected in 1991 belonged to GI, thereby confirming that GIII was the predominant genotype of JEV in Japan and Korea from the 1930s to the 1990s. Sequencing of the Autumn 4 isolate (former Union of Soviet Socialist Republics [USSR], 1943) revealed that GIII existed as far north as the USSR. One of the Korean viral isolates (Bennett, circa 1951) belonged to genotype II (GII) demonstrating that this genotype circulated for at least 19 years longer than previously thought. Formerly, GII was associated with endemic disease only and this genotype had never been isolated in temperate Asia.

3.2 INTRODUCTION

Prior to the initiation of the work presented in this chapter, there was a lack of genetic information available on Japanese encephalitis virus (JEV) isolates collected prior to the 1970s and the vast majority of viruses that had been sequenced prior to this decade belonged to genotype III (GIII). Therefore, the extent of genetic variation between and within the genotypes of JEV was unclear. To fill this gap in the knowledge of JEV, the nucleotide sequences of 26 JEV isolates obtained from the Walter Reed Army Institute for Research (WRAIR) and the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) from specimens collected in Japan, Korea, and the former Union of Soviet Socialist Republics USSR between 1935 and 1991 were determined and phylogenetically characterized.

3.3 RESULTS

3.3.1 Phylogenetic analysis of the WRAIR isolates

The details of the 16 WRAIR JEV isolates examined in this chapter are shown in Table 3.1. Prior to their arrival at the University of Texas Medical Branch (UTMB), the WRAIR JEV isolates had been held at ambient temperature for over two years in the early 1990s when the freezer failed. Therefore, the non-structural 5 (NS5) gene-3' untranslated region (3'UTR) of the isolates was amplified using the universal mosquito-borne flavivirus primer pair EMF1/VD8 (Pierre *et al*., 1994). This primer pair was selected because it is able to amplify small amounts of complementary DNA, and because the 3'UTR of flaviviruses is hypervariable and is thus considered a good marker for predicting genetic relatedness (Shurtleff *et al*., 2001). However, EMF1/VD8 only amplifies a 562-nucleotide region of the virus genome, and therefore amplicons contain relatively few phylogenetically informative sites, which can result in low bootstrap values for some branches of the phylogeny. Hence, the envelope (E) gene of nine of the 16 WRAIR isolates was also amplified. The E gene of the remaining seven isolates (Autumn 4, JEV # 40783, Kalinina, Nakayama mb-1, V9-3182, V9-3702 and V9-4358) could not be amplified due to extremely low quantities of viral RNA. Phylogenetic trees produced from the NS5 gene/3'UTR and the E gene nucleotide sequence files identified four major clades (representing genotypes I-IV [GI-IV]) and revealed similar virus groupings (Figure 3.1A and B). Genotypes were defined as viruses that differed in nucleotide composition by ≥ 7.2% in NS5 gene/3'UTR and by > 9.2% in E gene.

Fifteen of the WRAIR JEV isolates belonged to GIII, which is composed of two major groups of viruses, group A and group B. Group A includes 10 isolates sequenced in this study (one isolate was collected in the USSR in 1943 and nine isolates were collected in Japan between 1935 and circa 1950) and group B includes five isolates

sequenced in this study (two isolates were collected in Korea between 1946 and circa 1950 and three isolates were collected in Japan between 1949 and before 1950) (Figure 3.1A and B). One of the WRAIR JEV isolates belonged to GII (Bennett, Korea, circa 1951) (Figure 3.1A and B), demonstrating that GII had circulated for at least 19 years longer than previously thought (prior to this study WTP-70-22 [Malaysia, 1970] was the oldest GII isolate (Chen *et al*., 1990). Additionally, the Bennett isolate represents the only isolate of GII that has ever been collected in Korea or in temperate Asia.

3.3.2 Phylogenetic analysis of the WRCEVA isolates

In an effort to determine whether GII circulated in Korea or the Bennett isolate represented a single imported case, the E gene sequences of 10 Korean JEV isolates obtained from the WRCEVA (Table 3.2) were determined and the E gene sequences of three previously sequenced Korean JEV isolates were retrieved from GenBank (Table 3.3 [also shows details of the other JEV sequences retrieved from GenBank]). Phylogenetic analyses revealed that of the 16 Korean JEV isolates examined, 12 belonged to GIII (GIII, group A: one isolate and GIII, group B: 11 isolates), three belonged to GI and the Bennett isolate represents the only isolate of a GII virus (Figure 3.1C).

3.3.3 Genetic variation between and within the genotypes of JEV

A WRAIR E protein amino acid sequence alignment was generated using sequence information derived from one GI isolate (Ishikawa), two GII isolates (Bennett and FU), four GIII isolates (Nakayama, Matsunaga, V9-3901 and "Korea Jap B" [representative of distinct nodes within the WRAIR E gene phylogeny]) and one GIV isolate (JKT6468) (Figure 3.2). The range of intergenotypic amino acid sequence divergence among the viruses included in Figure 2 is as follows: GI versus GII (2.2 to

3.4%), GI versus GIII (2.8 to 4.0%), GI versus GIV (6.6%), GII versus GIII (0.6 to 2.6%), GII versus GIV (4.4 to 5.6%) and GIII versus GIV (4.4 to 5.6%).

The Matsunaga, V9-3901 and "Korea Jap B" GIII isolates (GIII, group I) exhibited six amino acid substitutions when compared to the prototype Nakayama isolate (GIII, group A; V51S, K83E, T176I, P223S, N276S and R290K) (Figure 3.3A). In addition to these six amino substitutions, the "Korea Jap B" isolate (GIII, group B) possessed three unique amino acid mutations when compared to the Nakayama isolate and the other GIII, group A isolates (S64L, T146A and K336R) (Figure 3.3A).

The GII isolate FU (Australia, 1995) possessed six amino acid substitutions within the E protein that differentiated it from the Bennett isolate (Korea, circa 1951) (F108S, S208P, K307N, F308S, A311R and A366S). The locations of these six amino acid substitutions were mapped onto the E protein dimer structure of JEV (Figure 3.3B). Four of the amino acid substitutions (K307N, F308S, A311R and A366S) mapped to domain III of the E protein; while two of the substitutions mapped to domain II of the E protein (F108S and S208P), with F108S specifically mapping to the fusion loop of domain II. Five of these amino acid substitutions (S208P, K307N, F308S, A311R and A366S) are unique to the FU isolate and three other GII isolates (M15, NO and M40) that were also collected in Australia in 1995. One of the amino acid substitutions (F108S) is also found in the Australian M15, NO and M40 isolates that were collected in 1995, as well as in the GII Indonesian JKT5441 isolate that was collected in 1981.

3.4 DISCUSSION

Prior to the initiation of the work presented in this chapter, there was a lack of genetic information available on JEV isolates collected prior to the 1970s. Therefore, 26

JEV isolates collected from Japan, Korea and the "USSR" between 1935 and 1991 were sequenced and genetically characterized.

3.4.1 Two genetically distinct strains of GIII co-circulated in Japan in 1949

In Japan, the first outbreak suggestive of JE was recorded in 1871 and major epidemics occurred in 1924, 1935 and 1948 (Innis, 1995). The majority of the Japanese isolates sequenced in this study belonged to GIII-group A, which differs from GIII-group II by three amino acids within the E protein (GIII-group A to group B: S64L, T146A and K336R). As expected, two isolates (Nakayama mb-1 and Kalinina) collected from humans in the 1935 epidemic in Tokyo grouped closely (Kudo *et al*., 1937). The Matsunaga isolate was collected from a human in Japan in 1939 and groups with Equine, which was collected from a horse in Japan in 1947. The Taira isolate was collected during the 1948 epidemic in Japan and shares a common ancestor with four isolates (V9-3702, V9-3901, V9-3902 and V9-4399) collected between 1949 and circa 1950 in Japan. Interestingly, two isolates (V9-3182 and V9-4358) collected during the same time period in Japan were located within the second group of GIII isolates and differed by 19 nucleotides within the amplified NS5 gene-3'UTR (the E gene sequence of these two viruses isolates could not be amplified), suggesting that two genetically distinct strains of JEV were co-circulating.

3.4.2 Northern border of GIII prevalence extended to the USSR

A JE epidemic was reported for the first time in restricted seacoast areas of the USSR in 1938, reappeared in epidemic form in 1939, and occurred sporadically in subsequent years (Grascenkov, 1964). The Autumn 4 isolate, a GIII virus, was collected from a human in the USSR in 1943 and represents the first genetically characterized isolate of JEV from this geographic region at that time.

3.4.3 GII circulated at least 19 years longer than previously recognized

Major outbreaks of JE were recorded in Korea among American soldiers in 1946, in the civilian population in 1949, and among American military personnel in 1950 (Lincoln & Sivertson, 1952; Sabin *et al*., 1947). Japanese encephalitis had been suggested to occur in Korea as early as 1926, but was never proven (Sabin *et al*., 1947). For this reason the American forces stationed in Korea were not given JE vaccine during 1946, as were those stationed in Japan (Sabin *et al*., 1947). Consequently, three cases of severe encephalitis, resulting in one fatality, occurred among 1,500 American soldiers stationed in Kunsan, Korea in 1946 (Sabin *et al*., 1947). JEV was isolated from the fatal case and it is likely that this is the Roum isolate (Sabin *et al*., 1947). The Roum isolate and the "Korea Jap B" isolate, which were collected in Korea circa 1950, group together. The collection of the "Korea Jap B" isolate coincides with an epidemic of JE resulting in 17 deaths that occurred among a camp of 300 American soldiers stationed to the 1949-1950 defense "perimeter" about Pusan, Korea (Lincoln & Sivertson, 1952). Interestingly, the Bennett isolate was collected in Korea during the same time period (circa 1951) as the "Korea Jap B" isolate, indicating two genotypes were in Korea concurrently. The Bennett isolate was collected at least 19 years prior to the oldest published GII isolate, WTP-70-22, which was collected in Malaysia in 1970 (Chen *et al*., 1990). Furthermore, the Bennett isolate represents the only isolate of GII that has been made in Korea or in temperate Asia. The FU isolate (Australia, 1995), as well as three other GII JEV isolates collected in Australia in 1995 possesses six amino acid substitutions in the E protein that differentiate them from the Bennett isolate (F108S, S208P, K307N, F308S, A311R and A366S), while the JKT5441 isolate collected in Indonesia in 1981 possesses only one of these substitutions (F108S). This evidence supports the hypothesis that the E protein

amino acid substitutions unique to the four virus isolates collected in Australia in 1995 evolved recently.

Since there is no sequence information available on Korean isolates collected between 1951 and 1971, it remains unknown whether the collection of the Bennett isolate coincided with a GII epidemic focus that quickly died off, or GII circulated in Korea for a period of time prior to its extinction, or the Bennett isolate represented a single imported case from another region. The latter is improbable given that almost all United States ground forces arrived to the Korean War zone by sea after a journey devoid of culicine mosquito exposure for days, and there was little or no rest and recuperation (troops were withdrawn from the line and sent to Japan for a five day break), which occurred in Japan, for United States ground forces assigned to the Korean War zone (Hastings, 1987). Thus, the possibility of importation is unlikely, and if importation did occur the origin of infection was nearby Japan. The Bennett isolate was most likely collected from an United States soldier bitten by an infective mosquito in Korea, as minimum infection rates of mosquitoes even during intense JEV outbreaks among susceptible humans are low (Gingrich *et al*., 1992). Therefore, it is likely that there was at least transient transmission occurring locally among amplifying hosts to generate the infective mosquito that transmitted the Bennett isolate.

3.4.4 Conclusions

Overall, the results of this chapter confirmed that GIII was the predominant genotype of JEV in Japan and Korea between 1935 (isolation of the prototype isolate, a GIII virus) and 1991 (collection of the GI JE-91 isolate of JEV in Korea). The reasons underlying the initial disproportionate expansion and establishment of GIII as the most widely distributed genotype of JEV throughout East and Southeast Asia is uncertain.

Sequencing of the Autumn 4 isolate (USSR, 1943) revealed that GIII existed as far north as the USSR. Genetic characterization of the Bennett isolate (Korea, circa 1951) demonstrated that GII has been circulating for at least 19 years longer than previously thought. Prior to this study, GII was associated with endemic disease and this genotype had never been isolated in temperate Asia. The data presented in this chapter have contributed to a better understanding of the epidemiology and evolution of JEV.

Table 3.1: Details of the WRAIR JEV isolates sequenced in this study.

Isolate	Origin	Year	Host	Genotype	Passage number	GenBank accession number(s)
Bennett	Korea	Circa 1951	Human	GII	5	FJ515927, FJ872376
Kalinina	Japan	1935	Human	GIII, group I	5	FJ515928
Nakayama mb-1	Japan	1935	Human	GIII, group I	3	FJ515931
Matsunaga	Japan	1939	Human	GIII, group I	3	FJ515930, FJ872381
Autumn 4	USSR	1943	Human	GIII, group I	4	FJ515932
Equine	Japan	1947	Equid	GIII, group I	27	FJ515929, FJ872378
Taira	Japan	1948	Human	GIII, group I	5	FJ515933, FJ872384
V9-3702	Japan	1949	Human	GIII, group I	9	FJ515934
V9-3901	Japan	Circa 1950	Human	GIII, group I	8	FJ515935, FJ872382
V9-3902	Japan	Circa 1950	Human	GIII, group I	8	FJ515936, FJ872383
V9-4399	Japan	Circa 1950	Human	GIII, group I	7	FJ515937, FJ872380
Roum	Korea	1946	Human	GIII, group II	5	FJ515922, FJ872377
V9-3182	Japan	1949	Human	GIII, group II	5	FJ515924
V9-4358	Japan	Circa 1950	Human	GIII, group II	6	FJ515925
“Korea Jap B”	Korea	Circa 1950	Unknown	GIII, group II	7	FJ515926, FJ872379
JEV # 40783	Korea	Circa 1971	Human	GIII, group II	8	FJ515923

Table 3.2: Details of the WRCEVA JEV isolates sequenced in this study.

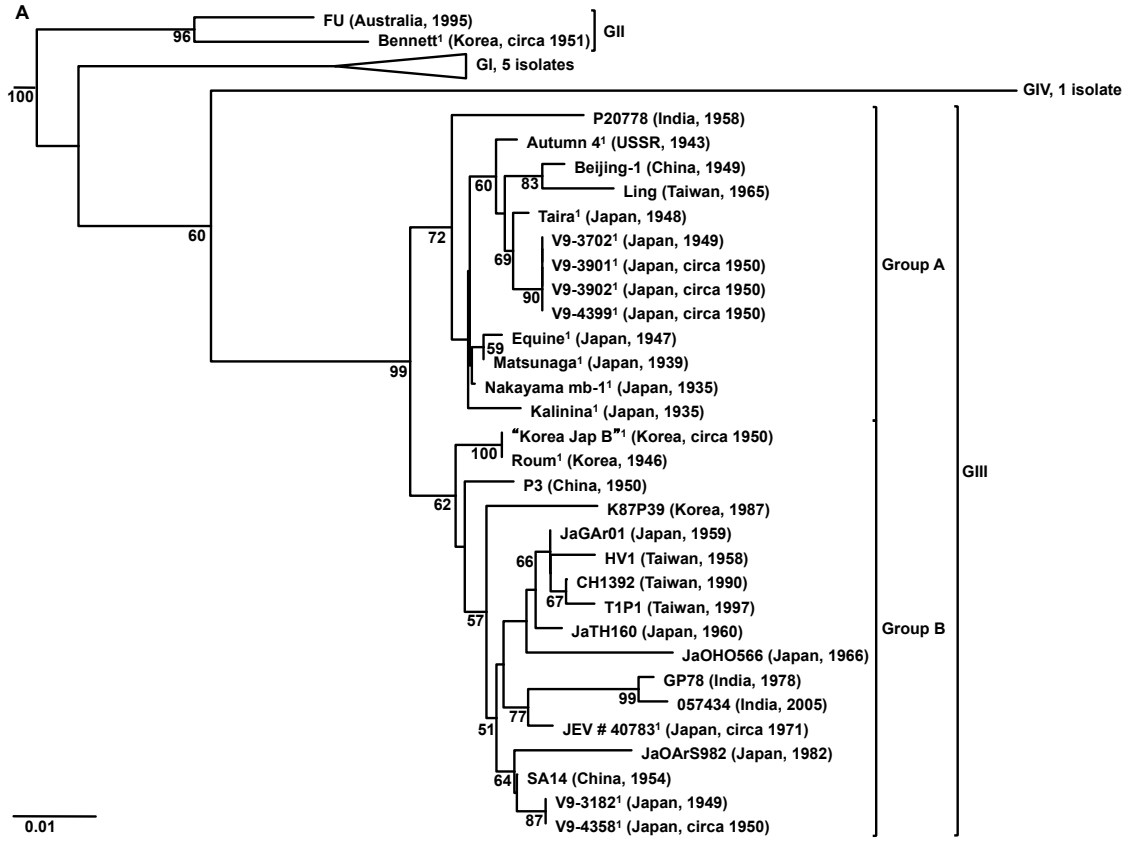
Isolate	Origin	Year	Host	Genotype	GenBank accession number(s)
JE-91	Korea	1991	Mosquito	GI	GQ415355
K-29	Korea	1949	Human	GIII, group I	GQ415356
JE-82	Korea	1982	Mosquito	GIII, group II	GQ415347
JE-83	Korea	1983	Mosquito	GIII, group II	GQ415348
JE-84	Korea	1984	Mosquito	GIII, group II	GQ415349
JE-85	Korea	1985	Mosquito	GIII, group II	GQ415350
JE-86	Korea	1986	Mosquito	GIII, group II	GQ415351
JE-87	Korea	1987	Mosquito	GIII, group II	GQ415352
JE-88	Korea	1988	Mosquito	GIII, group II	GQ415353
JE-89	Korea	1989	Mosquito	GIII, group II	GQ415354

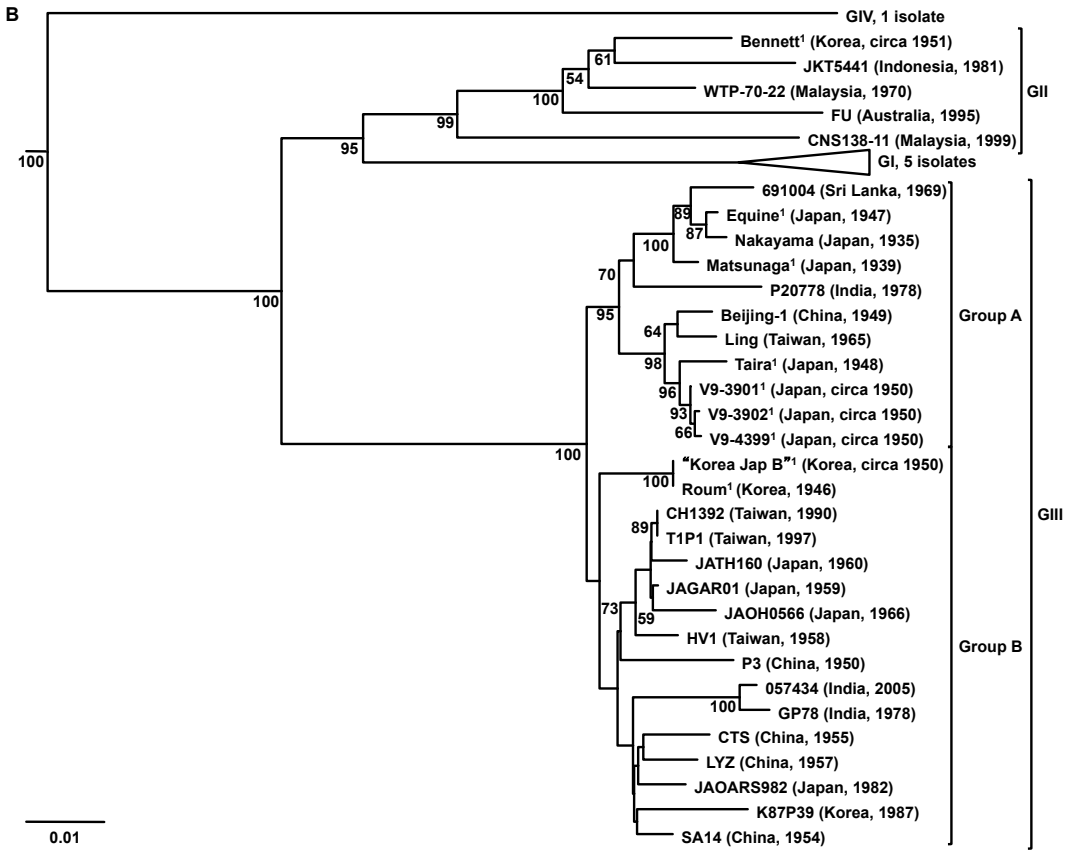
Table 3.3: Details of the JEV isolates retrieved from GenBank for use in this study.

Isolate	Origin	Year	Host	Genotype	GenBank accession no(s).
K94P05	Korea	1994	Mosquito	GI	AF045551
Ishikawa	Japan	1999	Mosquito	GI	ABO51292
KV1899	Korea	1999	Swine	GI	AY316157
JEV/sw/Mie/40/2004	Japan	2004	Swine	GI	AB241118
XJ69	China	2007	Mosquito	GI	EU880214
WTP-70-22	Malaysia	1970	Mosquito	GII	U70421
JKT5441	Indonesia	1981	Mosquito	GII	U70406
FU	Australia	1995	Human	GII	AF217620
CNS138-11	Malaysia	1999	Human	GII	AY184213
Nakayama	Japan	1935	Human	GIII, group A	EF571853
Beijing-1	China	1949	Mosquito	GIII, group A	L48961
P20778	India	1958	Human	GIII, group A	AF0802561
Ling	Taiwan	1965	Human	GIII, group A	L78128
691004	Sri Lanka	1969	Human	GIII, group A	Z34097
P3	China	1950	Mosquito	GIII, group B	U47032
SA14	China	1954	Mosquito	GIII, group B	JEU14163
CTS	China	1955	Human	GIII, group B	AY243830
LYZ	China	1957	Human	GIII, group B	AY243834
HV1	Taiwan	1958	Human	GIII, group B	AF098735
JaGAr01	Japan	1959	Mosquito	GIII, group B	AF069076
JaTH160	Japan	1960	Human	GIII, group B	AB269326
JaOH0566	Japan	1966	Human	GIII, group B	AY508813
GP78	India	1978	Human	GIII, group B	AF075723
JaOArS982	Japan	1982	Mosquito	GIII, group B	NC_001437
K87P39	Korea	1987	Mosquito	GIII, group B	AY585242
CH1392	Taiwan	1990	Mosquito	GIII, group B	AF254452
T1P1	Taiwan	1997	Mosquito	GIII, group B	AF254453
05734	India	2005	Human	GIII, group B	EF623988
JKT6468	Indonesia	1981	Mosquito	GIV	AY184212
MVE-1-51[1]	Australia	1951	Human	MVEV	AF161266

[1]All strains are JEV except for MVE-1-51, which is an isolate of Murray Valley encephalitis virus (MVEV).

Figure 3.1: Neighbor-joining phylogenies based on sequence information derived from the: A) NS5 gene/3'UTR of the WRAIR isolates, B) E gene of the WRAIR isolates, and C) E gene of the WRAIR and WRCEVA isolates. The trees were rooted using an isolate of MVEV (MVE-1-51), which is a member of the Japanese encephalitis serocomplex, but has been removed to allow for better visualization of branch lengths. Horizontal branch lengths are proportional to the genetic distance between isolates and the scale at the lower-left of each tree indicates the number of nucleotide substitutions per site. GI–IV are represented to the right of each tree. Numbers to the left of the nodes represent percent bootstrap values based on 1000 replicates. Only bootstrap values > 50% are indicated. WRAIR isolates sequenced in this study are indicated by '1' and WRCEVA isolates sequenced in this study are indicated by '2'.

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GIV, 1 isolate
Bennett[1] (Korea, circa 1951)
JKT5441 (Indonesia, 1981)
WTP-70-22 (Malaysia, 1970)
FU (Australia, 1995)
CNS138-11 (Malaysia, 1999)
GI, 5 isolates
GII
691004 (Sri Lanka, 1969)
Equine[1] (Japan, 1947)
Nakayama (Japan, 1935)
Matsunaga[1] (Japan, 1939)
P20778 (India, 1978)
Beijing-1 (China, 1949)
Ling (Taiwan, 1965)
Taira[1] (Japan, 1948)
V9-3901[1] (Japan, circa 1950)
V9-3902[1] (Japan, circa 1950)
V9-4399[1] (Japan, circa 1950)
Group A
"Korea Jap B"[1] (Korea, circa 1950)
Roum[1] (Korea, 1946)
CH1392 (Taiwan, 1990)
T1P1 (Taiwan, 1997)
JATH160 (Japan, 1960)
JAGAR01 (Japan, 1959)
JAOH0566 (Japan, 1966)
HV1 (Taiwan, 1958)
P3 (China, 1950)
057434 (India, 2005)
GP78 (India, 1978)
CTS (China, 1955)
LYZ (China, 1957)
JAOARS982 (Japan, 1982)
K87P39 (Korea, 1987)
SA14 (China, 1954)
Group B
GIII
100
61
54
100
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96
93
66
100
100
89
73
59
100
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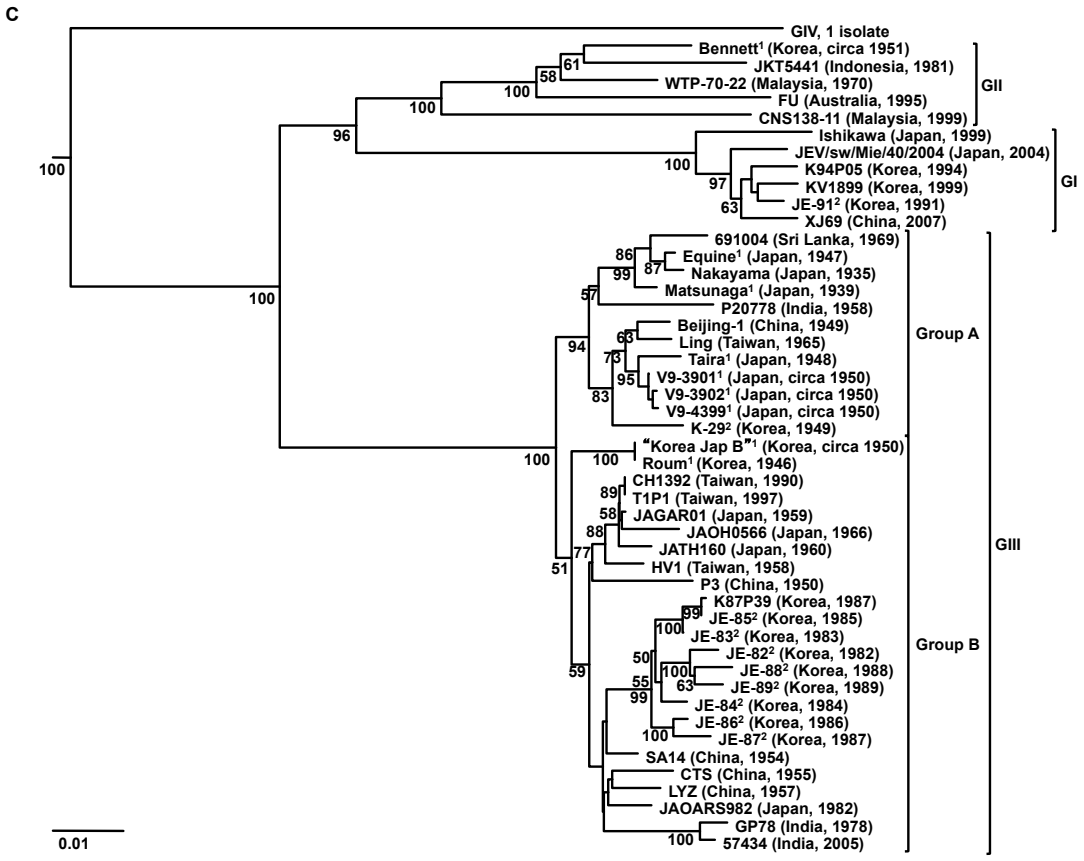
C
GIV, 1 isolate
Bennett[1] (Korea, circa 1951)
JKT5441 (Indonesia, 1981)
WTP-70-22 (Malaysia, 1970)
FU (Australia, 1995)
CNS138-11 (Malaysia, 1999)
GII
Ishikawa (Japan, 1999)
JEV/sw/Mie/40/2004 (Japan, 2004)
K94P05 (Korea, 1994)
KV1899 (Korea, 1999)
JE-91[2] (Korea, 1991)
XJ69 (China, 2007)
GI
691004 (Sri Lanka, 1969)
Equine[1] (Japan, 1947)
Nakayama (Japan, 1935)
Matsunaga[1] (Japan, 1939)
P20778 (India, 1958)
Beijing-1 (China, 1949)
Ling (Taiwan, 1965)
Taira[1] (Japan, 1948)
V9-3901[1] (Japan, circa 1950)
V9-3902[1] (Japan, circa 1950)
V9-4399[1] (Japan, circa 1950)
K-29[2] (Korea, 1949)
Group A
"Korea Jap B"[1] (Korea, circa 1950)
Roum[1] (Korea, 1946)
CH1392 (Taiwan, 1990)
T1P1 (Taiwan, 1997)
JAGAR01 (Japan, 1959)
JAOH0566 (Japan, 1966)
JATH160 (Japan, 1960)
HV1 (Taiwan, 1958)
P3 (China, 1950)
K87P39 (Korea, 1987)
JE-85[2] (Korea, 1985)
JE-83[2] (Korea, 1983)
JE-82[2] (Korea, 1982)
JE-88[2] (Korea, 1988)
JE-89[2] (Korea, 1989)
JE-84[2] (Korea, 1984)
JE-86[2] (Korea, 1986)
JE-87[2] (Korea, 1987)
SA14 (China, 1954)
CTS (China, 1955)
LYZ (China, 1957)
JAOARS982 (Japan, 1982)
GP78 (India, 1978)
57434 (India, 2005)
Group B
GIII
0.01

Figure 3.2: Alignment of JEV GI–IV E protein amino acid sequences relative to the prototype Nakayama isolate. Dots indicate consensus. The letter X represents amino acids that are unidentified in the Nakayama isolate retrieved from GenBank.

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                    10        20        30        40        50        60        70        80        90       100
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Nakayama            FNCLGMGNRDFIEGASGATWVDLVLEGDSCLTIMANDKPTXDVRMINIEAVQLAEVRSYCYHASVTDISTVARCPTTGEAHNKKRADSSYVCKQGFXDRG
V9-3901             ........................................L.........S.............................E.............T...
Equine              ........................................L.....................................................T...
"Korea Jap B"       ........................................L.........S............L..................E.............T...
Ishikawa            .T......................................L.........S........Y......................E.............T...
Bennett             ........................................L.........S..............................E.............T...
FU                  ........................................L.........S..............................E.............T...
JKT6468             ..............V.......................R..L.........S..............................E.............T...

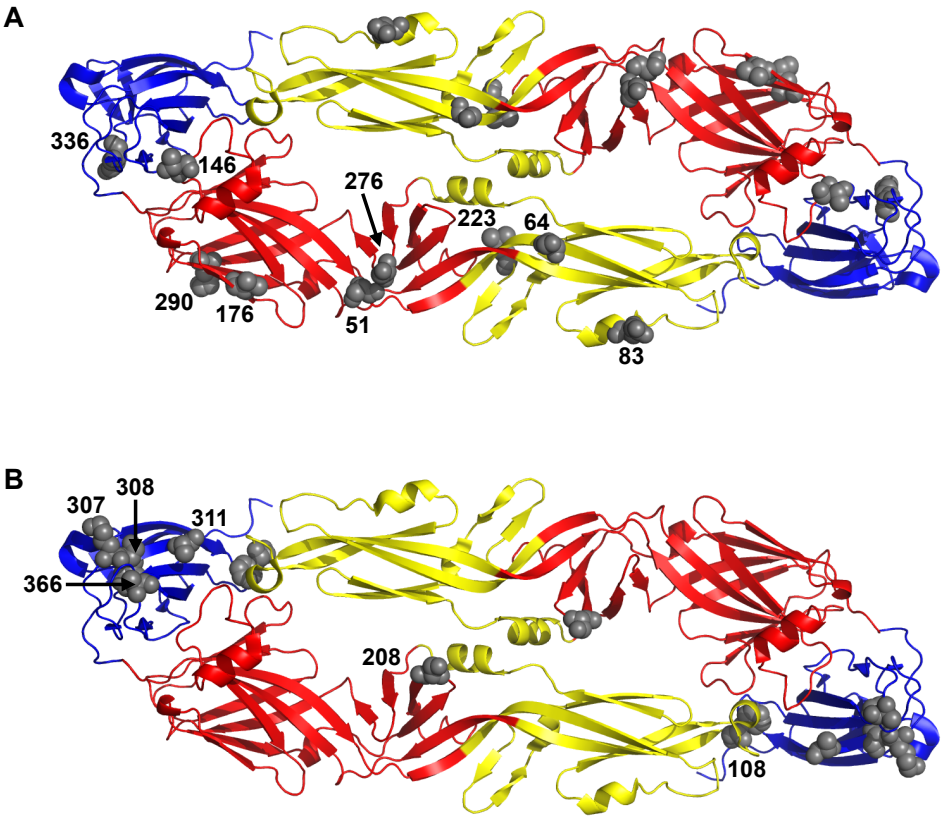
                   110       120       130       140       150       160       170       180       190       200
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Nakayama            WGNGCGLFGKGSIDTCAKFSCTSKAIGRTIQPENIKYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVTPNAPSTTLKLGDYGEVTLDCEPRSGLNTEA
V9-3901             ..................................................................................I.....................
Equine              ..................................................................................I.....................
"Korea Jap B"       ..........................................A.......................................I.....................
Ishikawa            ..K.........................M.....................................................I......F.........K.......
Bennett             ..................................................................................I.....................
FU                  .......S..........................................................................I.....................
JKT6468             ........................T.K.............................T..I.........I......I.....................

                   210       220       230       240       250       260       270       280       290       300
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Nakayama            FYVMTVGSXSFLVHREWFHDLALPWTPPSSTAWRNRELLMEFEEAHATKQSVVALGSQEGGLHQALAGAIVVEYSNSVKLTSGHLKCRLRMDKLALKGTT
V9-3901             ........K.................S.............................................S.............K..........
Equine              ........K.....................................................................................
"Korea Jap B"       ........K.................S.............................................S.............K..........
Ishikawa            ........K............S....S.............................................S.............K..........
Bennett             ........K............S....S.............................................S.............K..........
FU                  .......PK............S....S.............................................S.............K..........
JKT6468             ........K.................SS.N..............................RA.............S.............K..........

                   310       320       330       340       350       360       370       380       390       400
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Nakayama            YGMCTEKFSFAKNPADTGHGTVVIELSYSGSDGPCKIPIVSVASLNDMTPVGRLVTVNPFVATSSANSKVLVEMEPPFGDSYIVVGRGDKQINHHWHKAG
V9-3901             ..................................................................................................
Equine              ..................................................................................................
"Korea Jap B"       ..............................R...................................................................
Ishikawa            ........................T.....................................S.....L.I.............K.......R....
Bennett             ........................T.....................................S..................................
FU                  ......NS..R..............T.....................................................................
JKT6468             ........................L.....................................S..Q...................E.........P.

                   410       420       430       440       450       460       470       480       490       500
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Nakayama            STLGKAFSTTLKGAQRLAALGDTAWDFGSIGGVFNSIGKAVHQVFGGAFRTLFGGMSWITQGLMGALLLWMGVNARDRSIALAFLATGGVLVFLATNVHA
V9-3901             ..................................................................................................
Equine              ................V.................................................................................
"Korea Jap B"       ..................................................................................................
Ishikawa            ............................R.....................................................................
Bennett             ..................................................................................................
FU                  ..................................................................................................
JKT6468             ..........................................................V......I........M...V...T.L........
```

Figure 3.3: Important amino acid substitutions indicated on the E gene protein dimer structure of JEV: A) GIII and B) GII. Domain I is indicated in red, domain II in yellow and domain III in blue. The substitutions are indicated in gray and are only numbered on the lower monomer.



Chapter 4: Genetic characterization of Japanese encephalitis virus genotype II strains isolated from 1951 to 1978[2]

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4.1 ABSTRACT

Five genotypes (GI-V) of Japanese encephalitis virus (JEV) have been identified; however, to date, the nucleotide sequence of the ORF of only one GII virus has been determined (FU isolate, Australia, 1995). Therefore in this study, the nucleotide sequence of the open reading frame (ORF) of three additional GII isolates collected between 1951 and 1978 in Korea, Malaysia and Indonesia were determined and then compared with the FU isolate, as well as with virus isolates representative of the other three genotypes. Based on nucleotide and amino acid composition, GII isolates were the most similar to GI isolates; however, these two genotypes are epidemiologically distinct. Selection analyses revealed that all genotypes and genes of JEV are predominantly under purifying selection and evidence of positive selection was detected at amino acid 24 of the non-structural 4B gene-protein, a protein which functions as an interferon-α/β signaling inhibitor.

4.2 INTRODUCTION

Genotype II (GII) of Japanese encephalitis virus (JEV) includes isolates that have been collected sporadically between 1951 and 1999 in Northern Australia, Indonesia, Korea, Malaysia, Papua New Guinea and southern Thailand. Historically, the southeastern limit of JEV activity was considered to lie west of the Wallace line (Williams *et al*., 2000). However, in 1995 an outbreak due to GII of the virus that consisted of three human cases, two of which were fatal, occurred on Badu Island in Australia's Torres Strait (Hanna *et al*., 1995). Prior to the initiation of the work presented in this chapter, the database of wild-type JEV isolates for which there was open reading frame (ORF) nucleotide sequence information consisted of 29 isolates, and only one of these isolates belonged to GII of the virus (FU, Australia, 1995). As such, the

phylogenetic and evolutionary relationships among GII isolates, and between GII and the other virus genotypes were poorly understood. In this study, the nucleotide sequence of the ORF of three JEV GII isolates collected in Korea, Malaysia, and Indonesia between 1951 and 1978 were determined, and then compared to sequence data derived from the GII isolate collected in Australia in 1995, as well as sequence data derived from 28 JEV isolates representative of genotype I (GI), genotype III (GIII), and genotype IV (GIV) that were collected throughout Asia between 1935 and 2007. Additionally, a series of selection and codon usage analyses were performed according to gene and genotype.

4.3 RESULTS

4.3.1 Phylogenetic relationships between the four genotypes of JEV and among GII isolates of the virus

The nucleotide sequences of the ORF of three isolates of GII of JEV were determined (Bennett [Korea, circa 1951], WTP-70-22 [Malaysia, 1970], and JKT654 [Indonesia, 1978]). These were compared with 29 wild-type homologous JEV sequences and one sequence of Murray Valley encephalitis virus (MVEV) that were retrieved from GenBank for a final data set comprising 33 virus isolates, each of which is 10,308 nucleotides (3,436 amino acids) in length (Table 4.1). All three methods of phylogenetic inference (Bayesian, neighbor-joining [NJ], and maximum-likelihood [ML]) confirmed previous studies and identified four major clades (representing GI-IV), as well as revealed identical tree topologies and virus groupings (Figure 4.1). Genotypes of JEV in this study were defined as isolates that differed in nucleotide composition by > 9.1% over the entire ORF.

Nine of the 32 JEV isolates belong to GI, four isolates belong to GII (including the three isolates sequenced in this study), 18 isolates belong to GIII, and one isolate

belongs to GIV. Genotype IV of JEV diverged from MVEV first, followed by GIII, GII and lastly GI.

Of the GII isolates, the Bennett isolate (Korea, circa 1951) diverged first, followed by the divergence of the JKT654 isolate (Indonesia, 1978), and finally the WTP-70-22 (Malaysia, 1970) and FU (Australia, 1995) isolates, which form a monophyly.

4.3.2 Genetic divergence among the four genotypes of JEV

Pairwise comparisons of both nucleotide and amino acid sequences were used to determine the genetic relatedness of GII isolates to the other three genotypes of JEV (Table 4.2). Based on nucleotide sequence divergence, GII isolates were the most similar to GI isolates (9.1% [Bennett|JEV/Sw/Mie/40/2004] to 10.7% [FU|K94P05, KV1899]), followed by GIII isolates (10.2% [Bennett|JaGAr01] to 11.7% [FU|GP78]), and lastly the GIV isolate (16.2% [Bennett|JKT6468] to 16.6% [FU|JKT6468]. This same pattern held true when the amino acid sequence divergence was examined; GII isolates were the most closely related to GI isolates (1.5% [Bennett|JEV/Sw/Mie/40/2004, HEN0701] to 3.3% [FU|K94P05]), followed by GIII isolates (1.7% [Bennett|JaGAr01, K87P39] to 3.0% [FU|TC]), and finally the GIV isolate (5.0% [WTP-20-22|JKT6468] to 5.4% [FU|JKT6468]).

A number of amino acid substitutions were specific for individual genotypes (genotype-specific amino acid substitutions), and some define individual nodes within the phylogeny (node-defining amino acid substitutions) (Table 4.3). Genotype IV possessed 144 genotype-specific amino acid substitutions (20 in the capsid [C] protein, 15 in the premembrane [prM] protein, 22 in the envelope [E] protein, 17 in the non-structural 1 [NS1] protein, 11 in the non-structural 2 [NS2A] protein, 15 in the non-structural 3 [NS3]

protein, 7 in the non-structural 4A [NS4A] protein, 8 in the non-structural 4B [NS4B] protein, and 29 in the non-structural 5 [NS5] protein); GIII had 17 genotype-specific substitutions (three in the C protein, two in the E protein, two in the NS1 protein, three in the NS2A protein, three in the NS3 protein, one in the NS4B protein, and three in the NS5 protein); GI + GII had 27 node-defining substitutions (three in the C protein, one in the prM protein, three in the E protein, three in the NS1 protein, four in the NS2A protein, one in the non-structural 2B [NS2B] protein, four in the NS3 protein, three in the NS4B protein, and five in the NS5 protein); GII had 23 genotype-specific substitutions (one in the C protein, one in the prM protein, four in the NS1 protein, six in the NS2A protein, one in the NS2B protein, two in the NS3 protein, one in the NS4A protein and seven in the NS5 protein); GI had 21 genotype-specific substitutions (one in the C protein, three in the prM protein, one in the E protein, four in the NS1 protein, one in the NS2A protein, two in the NS2B protein, three in the NS3 protein, one in the NS4A protein and five in the NS5 protein).

4.3.3 Genetic diversity within GII of JEV

The intra-genotypic nucleotide and amino acid divergence was calculated to determine the extent of genetic variation within each genotype (Table 4.2 [only the intra-genotypic divergence within GII is shown]). The intra-genotypic nucleotide sequence divergence ranged from 0.7% (XJP613|SH17M-07) to 3.2% (K94P05|KV1899) among GI isolates, from 2.5% (Bennett|WTP-70-22) to 4.3% (JKT654|FU) among GII isolates, and from 0.1% (CH1392|T1P1) to 4.0% (Beijing-1|GP78) among GIII isolates. While, the amino acid sequence divergence ranged from 0.3% (JEV/Sw/Mie/40/2004|SH17M-07, HEN0701| JEV/Sw/Mie/41/2002) to 2.8% (K94P05|KV1899) among GI isolates, 0.5% (Bennett|WTP-70-22, JKT654, WTP-70-22|JKT654) to 0.9% (JKT654/FU) among

GII isolates, and 0.3% (04940-4|57434, JaGAr01|CH1392, JaOArS982, JaTH160) to 2.1% (TC/GP78) among GIII isolates.

Table 4.4 shows the amino acid substitutions within the ORF that are specific to the four isolates of GII of JEV. Compared to the Bennett isolate, the WTP-70-22 isolate exhibited 255 nucleotide differences resulting in 17 amino acid substitutions, the JKT654 isolate exhibited 309 nucleotide differences resulting in 15 amino acid substitutions, and the FU isolate exhibited 402 nucleotide differences resulting in 26 amino acid substitutions. Interestingly, the NS2B protein is conserved among the four GII isolates of JEV.

4.3.4 Recombination and selection analyses

Given that recombination in a nucleotide sequence alignment can adversely affect the results of selection analyses, recombination analyses were performed on the JEV nucleotide sequence alignment prior to selection analyses. Preliminary analyses of the nucleotide sequences yielded 10 statistically significant ($p < 0.05$) potential recombination events that were detected by at least two of the five detection methods used. However, after manual verification of the potential recombination events, using phylogenetic and recombination signal analysis features in the RDP3 program, only four of the 10 potential recombination events were confirmed (Table 4.5). All confirmed recombination events identified K94P05 (Korea, 1994, mosquito, GI) as the recombinant and JaOArS982 (Japan, 1982, mosquito, GIII) as the minor parent, while the major parent was identified as a different GI sequence in each of the four recombination events. Since the K94P05 isolate is not available in the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) to re-sequence, it cannot be determined whether the sequence of this isolate represents a true recombination event that occurred in nature or is

the result of a laboratory sequence assembly error. Therefore, the K94P05 sequence was removed from the dataset prior to all subsequent analyses.

Selection analyses were performed according to gene and genotype (44 datasets) using the single-likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), internal FEL (IFEL), and relaxed effects likelihood (REL) methods. (Table 4.6). The ratio of non-synonymous to synonymous nucleotide substitutions (d_N/d_S) over the first 9,999 nucleotides of the ORF for all virus isolates was estimated to be 0.035, which suggests predominantly purifying selection. Estimates for separate genes for all isolates ranged from 0.014 for the NS2B protein to 0.147 for the C protein. When the ORF was examined, estimates of the d_N/d_S ratio ranged from 0.020 for GII to 0.067 for GI. This pattern persisted when the ORF dataset was parsed into individual genes; GII consistently had the lowest d_N/d_S ratio when compared to the other genotypes in seven of the 10 individual protein genes examined, while genotypes I and III consistently had higher d_N/d_S ratios when compared to GII in all of the individual protein genes examined. These data suggests that GI and GIII viruses are under stronger purifying selection compared to GII viruses.

The maximum number of codons that exhibited patterns of negative selection when all the ORF isolates were examined was 1,432, using REL method, and the maximum number of codons that exhibited patterns of positive selection was 10, using the REL method. However, only one codon, site 2,296 of the ORF (amino acid 24 of the NS4B protein), was identified as being under strong positive selection using three of the four selection detection methods. When the selection analysis was performed according to protein for all of the isolates, site 24 of the NS4B protein was again identified as being under strong positive selection using two of the four selection detection methods: FEL ($p = 0.03$) and IFEL ($p = 0.04$). Site 24 of the NS4B protein is characterized by a non-

conservative serine to proline/leucine substitution that occurs on some of the internal and external braches of the phylogeny (Figure 4.2). This substitution is present in four of the eight GI isolates, all four of the GII isolates, and two of the 16 GIII isolates.

4.3.5 Codon usage analyses

To determine if codon usage differs among JEV isolates according to genotype and individual gene, the GC content at the first and second codon positions (GC12), the GC content at the synonymous third codon position (GC3), and the codon usage index (N_C) values were calculated over the ORF for all genotypes, as well as according to genotype and gene (Table 4.7). The mean GC12 value was 0.51 and ranged from 0.47 (GII, GIII, GIV|prM gene) to 0.54 (GIII|NS4B gene and GI, GIV|NS2B gene). The GC3 values ranged from 0.46 (GII|NS2B gene) to 0.59 (GII, GIV|NS2A gene), with a mean value of 0.52. The mean N_C value was 55.81 and ranged 45.07 (GII|NS4A gene) to 61.00 (GI|NS2B gene; GIV|prM gene). Therefore, the extent of codon usage bias is small, although some variation is present.

To determine if the observed codon usage bias is controlled by mutational pressure or natural selection, the observed codon usage bias was compared with the bias that would be expected under the null hypothesis that mutation pressure is the sole determinant. First, GC3 was plotted against GC12 for all genotypes and genes (overall) (Figure 4.3A), as well as according to genotype (Figure 4.3B) and gene (Figure 4.3C). A marginally statistically significant negative correlation between GC3 and GC12 was observed overall ($r = -0.33$, $p = 0.04$), suggesting that patterns of base composition may be the result of mutational pressure. However, no statistically significant correlations between GC3 and GC12 were observed according to genotype ($r = 0.76$, $p = 0.24$) or gene ($r = -0.43$, $p = 0.22$), indicating that patterns of base composition are most likely not

the result of mutational pressure since the effects are not present at all codon positions. Secondly, GC3 was plotted against N_C for all genotypes and genes (Figure 4.3D), as well as according to genotype (Figure 4.3E) and gene (Figure 4.3F). For each plot a curve was drawn and represents the expected codon usage if GC compositional constraints alone were responsible for the codon usage bias. In the plot of GC3 versus N_C over all genotypes and genes, the vast majority of points lie under the curve, while a few points lie on the curve and above the curve (Figure 4.3D). When GC3 was plotted against N_C according to genotype, all points were positioned slightly below the curve suggesting that the actual codon usage indices are close to the values expected from their GC base composition, and furthermore that no difference exists among the four genotypes of JEV (Figure 4.3E). However, in the plot of GC3 verses N_C according to gene, some points overlap the curve (NS2B and prM genes), while others lie farther away (NS2A gene), suggesting that base composition as well as other factors may contribute to the observed codon usage bias (Figure 4.3F).

4.4 DISCUSSION

Until recently, the known geographical range of JEV activity was limited to East and Southeast Asia and Indonesia. Yet, in recent years the range of virus activity has been detected as far west into Pakistan (Igarashi *et al*., 1994), as far east as Saipan (Paul *et al*., 1993), and as far south as Australia (Hanna *et al*., 1996). In April of 1995, an outbreak of three human cases resulting in two fatalities occurred on Badu Island, which is located in the Torres Strait between mainland Queensland, Australia and Papua New Guinea (Hanna *et al*., 1995). Subsequent investigations revealed that the outbreak was due to GII of the virus (Hanna *et al*., 1996) and was most likely introduced from Papua New Guinea by wind-blown mosquitoes or migratory birds (see chapter 6) (Johansen *et*

al., 2000). Genotype II isolates continued to be collected from islands located in Australia's Torres Strait until 1998 (Pyke *et al*., 2001), and a single GII isolate was collected in Malaysia in 1999 (CNS138-11, Malaysia, 1999) (Solomon *et al*., 2003). However, in January 2000 a new genotype of JEV (GI) emerged in the Torres Strait (Pyke *et al*., 2001). As GII of JEV has not been isolated since 1999, it remains unknown whether this genotype is still circulating or it has become extinct. It is important to note that following the collection of the initial isolate of GI of JEV in 1967 (M859/Cambodia/1967/Mosquito) another GI isolate was not collected until 1979, and this genotype has recently replaced GIII as the most frequently isolated genotype throughout a number of Asian countries (Nga *et al*., 2004; Nitatpattana *et al*., 2008). To better understand the genetic variation, phylogenetic relationships and evolution among isolates of JEV, the nucleotide sequence of the ORF of three GII isolates collected between 1951 and 1978 were determined and compared to sequence information derived from an isolate collected during the 1995 JEV outbreak in Australia (FU, Australia, 1995), as well as to isolates representing GI, GIII and GIV of the virus.

4.4.1 GII and GI are closely related

Nucleotide and amino acid pairwise comparisons over the ORF, as well as phylogenetic analyses revealed that GII is the most similar to GI. Interestingly, GI has been isolated in East Asia, Southeast Asia, Indonesia and Northern Australia, whereas GII has been isolated on two occasions only in geographic areas where JE is considered to be epidemic, i.e., Korea (Bennett isolate, circa 1951) and Australia (FU isolate, 1995). Japanese encephalitis virus utilizes a variety of vector species and hosts; geographical differences in vector or host availability may explain why the geographical range of GII of JEV is/was limited to tropical regions where JEV is endemic. Furthermore, it remains

unclear if GI has replaced GII in Australia and Papua New Guinea or if both virus genotypes are circulating, as no JEV sequence has been published from either of these countries recently.

Correlation of the topology of the phylogeny with the JEV amino acid alignment further illustrated the close evolutionary relationship between genotypes II and I; GII + GI shared 27 node-defining amino acid substitutions, while GII possessed 23 genotype-specific substitutions and GI possessed 21 genotype-specific substitutions. The low magnitude of genetic variation among the four geospatiotemporally distributed GII isolates is consistent with that observed for genotypes III and I, and indicates that the overall genomic tolerance for mutation is minimal. The NS2B protein was conserved among all of the GII isolates. The central region of the NS2B protein of flaviviruses is a cofactor of the serine protease of the NS3 protein and is thus necessary for the activation of this protease (Mastrangelo *et al*., 2007).

4.4.2 Evidence of positive selection at site 24 of the NS4B protein

Selection analyses revealed that JEV isolates are under predominantly purifying selection, which results in virus-encoded proteins being conserved over time due to selective pressure against deleterious variants. Evidence of strong positive selection was detected at amino acid 24 of the NS4B protein in the GI-IV, ORF nucleotide sequence file by three of the four detection methods and in the GI-IV, NS4B protein nucleotide sequence file by two of the four detection methods. This signifies that the non-conservative, serine to proline/leucine substitution that occurred at this site in four of the eight GI isolates, all four of the GII isolates, and two of the 18 GIII isolates tended to be beneficial, rather than neutral or deleterious. The NS4B protein of flaviviruses has been found to inhibit the interferon (IFN)-α/β signaling cascade at the level of signal

transducer and activator of transcription (STAT) phosphorylation, suggesting that amino acid mutations at site 24 of this protein may be important in the antagonization of the host IFN response to the virus (Munoz-Jordan *et al*., 2003; Munoz-Jordan *et al*., 2005). Amino acid residues 22 and 24 of the West Nile virus (WNV) NS4B protein, which are analogous to JEV residues 22 and 24, respectively, have been shown to control IFN-α/β resistance in HeLa cells expressing subgenomic replicons lacking the structural genes, although no effect was shown on the expression of full-length infectious genomes (Evans & Seeger, 2007). Although potentially interesting, it should be noted that the amino acids at residues 22 and 24 of the NS4B protein are different for WNV and JEV so extrapolation must be made with care.

4.4.3 Individual JEV genes exhibit differences in codon usage

The results of the overall codon usage analysis revealed that patterns of base composition may be the result of mutational pressure. However, when the data were parsed according to genotype and gene, the patterns of base composition are most likely not the result of mutational pressure since the effects were not present at all codon positions. Also, little variance in the extent of codon usage bias among the genotypes of JEV was found, however individual genes of the virus varied to a small degree in their extent of codon usage bias.

4.4.4 Conclusions

Overall, this chapter elucidated the genetic variation, phylogenetic relationships, the operation of selection and the patterns of codon usage among isolates of JEV, including three newly sequenced isolates of GII that were temporally isolated in geographically distinct regions. Compared to other flaviviruses such as YFV, the nucleotide and amino acid divergence among and within the four genotypes of the virus

is low (von Lindern *et al*., 2006). However, the four genotypes of JEV exhibit distinct epidemiological histories and geographical ranges of virus activity. At the time this chapter was written, no phenotypic differences among the virus genotypes had been described. Once phenotypic properties of the genotypes have been delineated, the genotype-specific amino acid substitutions and the GII/GI-specific, positively selected site within the NS4B protein identified in this chapter may correlate with these properties. Identification of genotype-defining molecular determinants and their associated phenotypic properties is vital to understanding the evolution and epidemiology of JEV, and may have an impact on future vaccine development strategies.

Table 4.1: Details of the JEV isolates used in this study.

Isolate	Origin	Year	Host	Genotype	GenBank accession number
K94P05	Korea: Wando Island, Chollanam-Do	1994	Mosquito	GI	AF045551
Ishikawa	Japan: Ishikawa	1998	Mosquito	GI	AB051292
KV1899	Korea: Gyeonggi	1999	Swine	GI	AY316157
JEV/Sw/Mie/41/2002	Japan: Mie	2002	Swine	GI	AB241119
JEV/Sw/Mie/40/2004	Japan: Mie	2004	Swine	GI	AB241118
XJP613	China	2007	Mosquito	GI	EU693899
HEN0701	China	2007	Swine	GI	FJ495189
SH17M-07	China	2007	Unknown	GI	EU429297
XJ69	China: Zhejiang	2007	Mosquito	GI	EU880214
Bennett[1]	Korea	Circa 1951	Human	GII	HQ223285
WTP-70-22[1]	Malaysia: Kuala Lumpur	1970	Mosquito	GII	HQ223286
JKT654[1]	Indonesia: Java, Kapuk	1978	Mosquito	GII	HQ223287
FU	Australia: Badu Island	1995	Human	GII	AF217620
Nakayama	Japan: Nakayama	1935	Human	GIII	EF571853
Beijing-1	China: Beijing	1949	Mosquito	GIII	L48961
SA14	China: Xian	1954	Mosquito	GIII	U14163
HV1	Taiwan	1958	Human	GIII	AF098735
JaGAr01	Japan: Gunma	1959	Mosquito	GIII	AF069076
JaTH160	Japan: Tokyo	1960	Human	GIII	AB269326
Ling	Taiwan	1965	Human	GIII	L78128
TC	Taiwan	1965	Human	GIII	AF098736
TL	Taiwan	1965	Human	GIII	AF098737
JaOH0566	Japan: Osaka	1966	Human	GIII	AY508813
GP78	India: Gorakhpur	1978	Human	GIII	AF075723
JaOArS982	Japan: Osaka	1982	Mosquito	GIII	M18370
K87P39	Korea: Wando Island, Chollanam-Do	1987	Mosquito	GIII	U34927
CH1392	Taiwan: Changhua City	1990	Mosquito	GIII	AF254452
T1P1	Taiwan: Liu-Chiu Islet	1997	Mosquito	GIII	AF254453
14178	India: Lakhimpur	2001	Human	GIII	EF623987
04940-4	India: Maharashtra	2002	Mosquito	GIII	EF623989
057434	India: Gorakhpur	2005	Human	GIII	EF623988

Isolate	Origin	Year	Host	Genotype	GenBank accession number
JKT6468	Indonesia: Flores, Golock	1981	Mosquito	GIV	AY184212
MVE-1-51[2]	Australia	1951	Human	MVEV	AF161266

[1]Isolates sequenced in this study. [2]All strains are JEV except for MVE-1-51, which is an isolate of MVEV.

Table 4.2: Percent nucleotide and amino acid sequence divergence among JEV isolates.

	GI (9 isolates)	GII (Bennett)	GII (WTP-70-22)	GII (JKT654)	GII (FU)	GIII (18 isolates)	GIV (JKT6468)
GI (9 isolates)		1.5-2.9	1.7-3.0	1.7-3.1	1.9-3.3	1.6-3.8	5.0-6.2
GII (Bennett)	9.1-9.7		0.5	0.5	0.8	1.7 - 2.6	5.1
GII (WTP-70-22)	9.5-10.1	2.5		0.5	0.8	1.9-2.8	5
GII (JKT654)	9.8-10.4	3.1	2.8		0.9	1.9-2.8	5.1
GII (FU)	10.1-10.7	4	3.3	4.3		2.0-3.0	5.4
GIII (18 isolates)	10.8-11.9	10.2-10.9	10.5-11.3	10.7-11.5	10.9-11.7		4.6-5.5
GIV (JKT6468)	16.6-17.2	16.2	16.4	16.6	16.6	15.4-16.1	

Nucleotide divergence is in the lower diagonal and amino acid divergence is in the upper diagonal.

Table 4.3: Genotype-specific and node-defining amino acids within the ORF of JEV isolates.

Protein	Protein length	Site	Consensus	Substitution			
				GI	GII	GIII	GIV
C	127	6	G				R
		10	C				I
		15	N				Y
		35	M				I
		41	R				I
		44	V				L
		51	I				V
		52	T				S
		60	A				S
		70	R			K	
		72	V				L
		100	R	K	K		
		109	E				G
		110	G	S	S		
		111	S				T
		112	I				T
		113	M				L
		115	L				F
		116	A				M
		120	V	I	I	V	I
		121	V				A
		122	I	T	M	I	A
		123	A				V
		125	A				V
prM	167	1	M				L
		13	T				A
		14	I				V
		57	T	A			
		58	M	V			P
		63	E				Q
		71	N				H
		76	V		I		
		85	R				S
		88	K				Q
		89	R				T
		97	Q				H
		113	D				N
		122	M				V
		129	I				V
		140	V	A	A		
		149	N	S			S
		151	Q				P
E	500	15	A				V

Protein	Protein length	Site	Consensus	Substitution			
				GI	GII	GIII	GIV
		38	K				R
		126	I				T
		128	R				K
		129	T	M			
		156	S				T
		159	V				I
		171	V				I
		222	A	S	S		
		228	P				S
		230	S				N
		260	G				R
		261	G				A
		327	S	T	T	S	L
		366	A	S	S/A	A	S
		369	K				Q
		389	D				E
		399	A				P
		466	A				V
		473	V				I
		482	L				M
		486	A				V
		490	V				T
		492	V				L
NS1	352	41	S				A
		50	H		Y/H		
		51	K	Q/K			M
		54	V				I
		57	V		I		
		70	A	S	S		
		147	H	R/H	R	H	H
		175	S	N/T	S	S	N
		182	A				T
		188	V				I
		205	R				H
		206	Y	L/Y	F	Y	L
		220	V				I
		242	I		V		
		251	K	R	R		
		271	N				D
		298	V	I/L			
		317	S				T
		326	E				G
		327	N				S
		335	I				V
		339	R				K
		343	T				A
		350	V				A
NS2A	227	2	N				S

Protein	Protein length	Site	Consensus	Substitution			
				GI	GII	GIII	GIV
		6	V	I/V	I	V	I
		34	I		V		V
		70	S	S	N	S/N	N
		92	M				A
		97	T	A	A		
		98	R		K		
		105	V		M		
		119	V				A
		128	I				M
		139	V				I
		140	R				K
		149	S	T	T	S	T
		150	V				I
		151	T	A			
		154	V				L
		159	T				A
		176	V				I
		179	I				V
		187	K	R	R	K/E	R
		188	K				R
NS2B	131	55	E	D			
		65	D	E			
		97	V		I		
		99	V	L	L		
NS3	619	14	S	L/S	L	S/L	A
		44	N				S
		62	E		G/E		
		78	A	S	S		
		105	A	P			
		107	V				T
		117	R				C
		175	V				I
		177	E	D			
		180	T				N
		182	N	S	S	N	S
		185	F	K	K	F	K
		210	K				R
		294	S				G
		340	I				V
		354	E	D			
		356	A		S		
		374	M				V
		495	M				L
		586	M				T
		591	I				V
NS4A	149	3	V				I
		17	M				A
		35	K				R

Protein	Protein length	Site	Consensus	Substitution			
				GI	GII	GIII	GIV
		58	V		I		
		61	T				A
		72	M				K
		88	T				V
		100	P				S
		110	I	V/I			
NS4B	255	20	K	R/K			
		21	T				A
		22	Q				P
		23	A				V
		24	S	P/S	P		
		26	L				M
		31	S				G
		59	L				I
		73	N	S	S		
		115	M				V
		118	V	V	V	A/V	T
NS5	905	15	K				R
		22	E	D	D		
		49	I				K
		78	I				V
		101	R	K/R	K		
		135	K				R
		144	F				L
		253	K		R		
		275	N		D		D
		277	E				G
		280	K	R			
		287	K	R/K			
		296	K	R			
		298	P		S/P		
		370	A				P
		372	A	V	V	A	V
		381	H				Y
		429	D	G/S/D	G/S/D	D/N	G
		432	R	L/R			
		438	D	N/D			
		453	I		V		V
		455	N				H
		503	E				R
		526	G				R
		528	Q				E
		546	R				K
		576	R				K
		586	A				S
		587	A				T
		588	E	G	G	E	D
		637	L				V

Protein	Protein length	Site	Consensus	Substitution			
				GI	GII	GIII	GIV
		644	N		T		
		661	T				S
		684	H				Y
		754	K				R
		787	A				T
		830	M		T		E
		860	S				R
		878	V	I/V	I/M		
		883	N				T
		889	T		L		

Table 4.4: Amino acid substitutions within the ORF of GII isolates of JEV.

Protein	Protein length	Site	Bennett	WTP-70-22	JKT654	FU
C	127	44	V	A	A	A
		90	T	T	T	I
		123	A	V	G	A
prM	167	58	P	S	S	T
E	500	108	F	F	F	S
		126	I	T	I	I
		208	S	S	S	P
		307	K	K	K	N
		308	F	F	F	S
		311	A	A	A	R
		366	S	S	S	A
NS1	352	50	H	Y	Y	Y
		79	L	F	L	L
		214	R	R	K	R
		240	E	E	E	D
		284	K	K	K	T
		326	E	E	E	D
		338	V	V	V	A
NS2A	227	61	T	A	A	A
		134	I	I	T	I
		188	K	K	R	K
		220	I	V	V	A
NS2B	131					
NS3	619	62	E	G	G	G
		117	R	C	R	R
NS4A	149	96	A	A	T	A
NS4B	255	8	K	K	K	R
		31	S	I	S	S
		74	S	S	A	S
NS5	905	3	P	P	A	P
		135	K	R	R	K
		155	E	E	E	D
		298	S	S	S	P
		398	R	K	K	K
		429	G	S	G	D
		5887	A	A	A	S
		682	A	A	A	S
		835	I	V	I	I
		878	V	I	V	I
		897	A	V	A	V

Table 4.5: Confirmed recombination events.

Event	Gene	Nucleotide breakpoint positions	Recombinant	Minor parent	Major parent	Algorithm	p-value
1	NS5	8838-9063	K94P05	JaOArS982	JEV/Sw/Mie/40/2004	RDP	2.63×10^{-27}
						GENECONV	8.27×10^{-26}
						Bootscan	1.20×10^{-24}
						Maxchi	2.40×10^{-8}
						Chimera	9.23×10^{-5}
2	NS5	7883-8072	K94P05	JaOArS982	XJ69	RDP	4.68×10^{-19}
						GENECONV	4.28×10^{-18}
						Bootscan	3.75×10^{-19}
						Maxchi	9.44×10^{-5}
						Chimera	9.53×10^{-5}
3	NS4A-NS4B	6697-6880	K94P05	JaOArS982	SH17M-07	RDP	2.05×10^{-12}
						GENECONV	4.25×10^{-12}
						Bootscan	1.73×10^{-12}
						Maxchi	0.02
						Chimera	1.05×10^{-2}
4	NS3	5903-6009	K94P05	JaOArS982	JEV/Sw/Mie/41/2002	RDP	8.83×10^{-10}
						GENECONV	9.28×10^{-9}
						Bootscan	7.34×10^{-10}
						Maxchi	> 0.05
						Chimera	> 0.05

Table 4.6: Evidence for positive and negative selection using four detection methods.

			SLAC		FEL		REL		IFEL	
Protein	**Genotype**	**Overall d_N/d_S**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**
ORF	GI-IV	0.035	0	807	1	1432	10	1204	1	855
	GI	0.067	0	19	0	143	0	0	0	13
	GII	0.02	0	12	0	232	0	43	0	0
	GIII	0.057	0	126	0	383	1	29	0	112
C	GI-IV	0.147	0	14	0	26	0	54	0	16
	GI	0.111	0	0	0	5	0	13	0	0
	GII	0.133	0	0	0	4	3	0	0	0
	GIII	0.166	0	1	0	5	0	0	0	2
prM	GI-IV	0.048	0	33	0	78	0	0	0	41
	GI	0.061	0	0	0	8	1	0	0	0
	GII	0.015	0	0	0	20	1	35	0	0
	GIII	0.083	0	6	0	19	0	0	0	6
E	GI-IV	0.033	0	135	0	225	0	0	0	141
	GI	0.069	0	4	0	23	0	77	0	4
	GII	0.023	0	3	0	42	0	0	0	0
	GIII	0.065	0	21	0	58	0	129	0	19
NS1	GI-IV	0.043	0	81	0	145	0	0	1	83
	GI	0.041	0	13	0	49	0	24	0	10
	GII	0.031	0	3	0	28	0	55	0	0
	GIII	0.061	0	14	0	52	0	6	0	12
NS2A	GI-IV	0.052	0	46	0	81	0	0	0	46
	GI	0.047	0	3	0	13	0	24	0	2
	GII	0.05	0	1	0	11	1	1	0	0
	GIII	0.069	0	6	0	19	0	55	0	6
NS2B	GI-IV	0.014	0	31	0	65	0	0	0	32
	GI	0.012	0	0	0	5	0	0	0	0
	GII	5.310 x 10-16	0	0	0	7	0	0	0	0
	GIII	0.038	0	6	0	14	0	0	0	4
NS3	GI-IV	0.025	0	167	0	285	0	3	0	186
	GI	0.058	0	2	0	25	0	0	0	0
	GII	0.006	0	0	0	46	0	0	0	0
	GIII	0.054	0	25	0	72	0	172	0	29
NS4A	GI-IV	0.045	0	31	0	57	1	90	0	23
	GI	0.08	0	0	0	7	0	0	0	1
	GII	0.012	0	0	0	8	0	0	0	0
	GIII	0.073	0	4	0	14	3	16	0	4
NS4B	GI-IV	0.025	0	61	1	130	0	0	1	64
	GI	0.056	0	1	0	11	0	0	0	2
	GII	0.019	0	2	0	14	0	0	0	0
	GIII	0.035	0	9	0	37	2	21	0	8
NS5	GI-IV	0.033	0	210	0	373	1	47	0	243
	GI	0.081	0	3	0	35	0	109	0	1
	GII	0.025	0	1	0	46	0	144	1	0
	GIII	0.014	0	5	0	21	0	48	0	5

Table 4.7: GC12, GC3, and N_C values according to genotype and gene.

		Genotype														
		All			GI			GII			GIII			GIV		
		GC12	GC3	N_C	GC12	GC3	N_C	GC12	GC3	N_C	GC12	GC3	N_C	GC12	GC3	N_C
Gene	ORF	0.51	0.53	55.55	0.51	0.54	55.13	0.51	0.52	55.85	0.51	0.53	55.68	0.51	0.51	55.73
	C	0.51	0.49	55.52	0.51	0.51	47.23	0.52	0.51	60.06	0.51	0.48	58.79	0.50	0.50	53.19
	prM	0.47	0.57	59.45	0.48	0.58	57.98	0.47	0.52	59.73	0.47	0.58	60.03	0.47	0.52	61.00
	E	0.51	0.51	56.56	0.51	0.48	57.26	0.51	0.48	55.82	0.51	0.53	56.40	0.50	0.49	56.11
	NS1	0.50	0.52	56.08	0.50	0.53	56.44	0.50	0.51	56.71	0.50	0.52	55.82	0.50	0.49	55.3
	NS2A	0.50	0.57	54.18	0.50	0.58	53.47	0.49	0.59	52.44	0.50	0.56	54.81	0.49	0.59	55.43
	NS2B	0.53	0.48	60.54	0.54	0.52	61.00	0.53	0.46	58.16	0.53	0.47	60.98	0.54	0.50	58.11
	NS3	0.51	0.51	55.04	0.51	0.53	53.44	0.51	0.51	54.86	0.51	0.51	55.84	0.52	0.49	55.75
	NS4A	0.52	0.53	53.80	0.52	0.57	50.58	0.51	0.53	45.07	0.52	0.51	56.78	0.52	0.52	61.00
	NS4B	0.54	0.55	56.20	0.53	0.58	54.89	0.53	0.51	55.85	0.54	0.56	57.13	0.53	0.50	51.22
	NS5	0.51	0.55	54.17	0.51	0.56	54.31	0.51	0.55	53.86	0.51	0.54	54.09	0.50	0.53	55.5

Figure 4.1: Bayesian phylogeny based on nucleotide sequence information derived from the ORF of the JEV isolates. The trees were rooted using an isolate of MVEV (MVE-1-51), which is a member of the Japanese encephalitis serocomplex, but has been removed to allow for better visualization of branch lengths. Horizontal branch lengths are proportional to the genetic distance between isolates and the scale at the lower-left of the tree indicates the number of nucleotide substitutions per site. GI-IV are represented to the right of the tree. All nodes within the phylogeny are supported by a posterior probability of 1.0 unless otherwise indicated to the right of the node. The isolates sequenced in this study are indicated by "1".

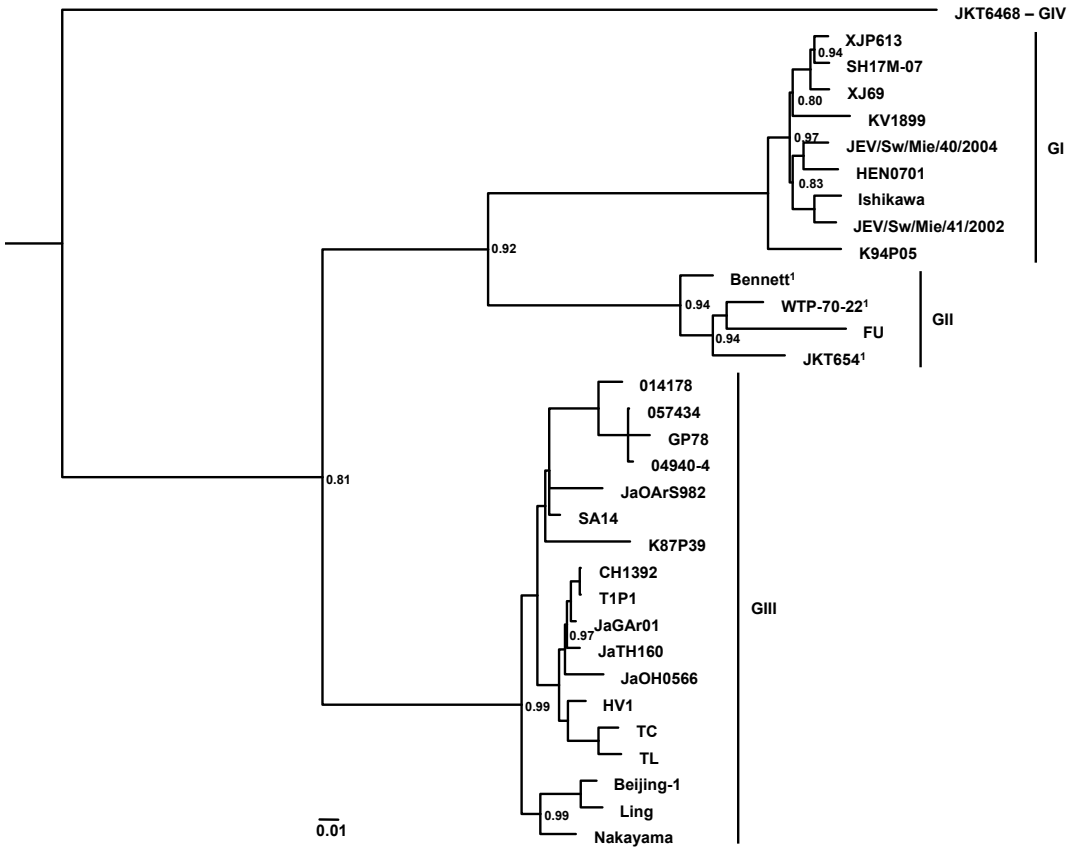


Figure 4.2: JEV Bayesian phylogeny with branches colored according to the amino acid residue present at site 24 of the NS4B protein. The key to the branch coloring scheme is located to the lower-left of the phylogeny.

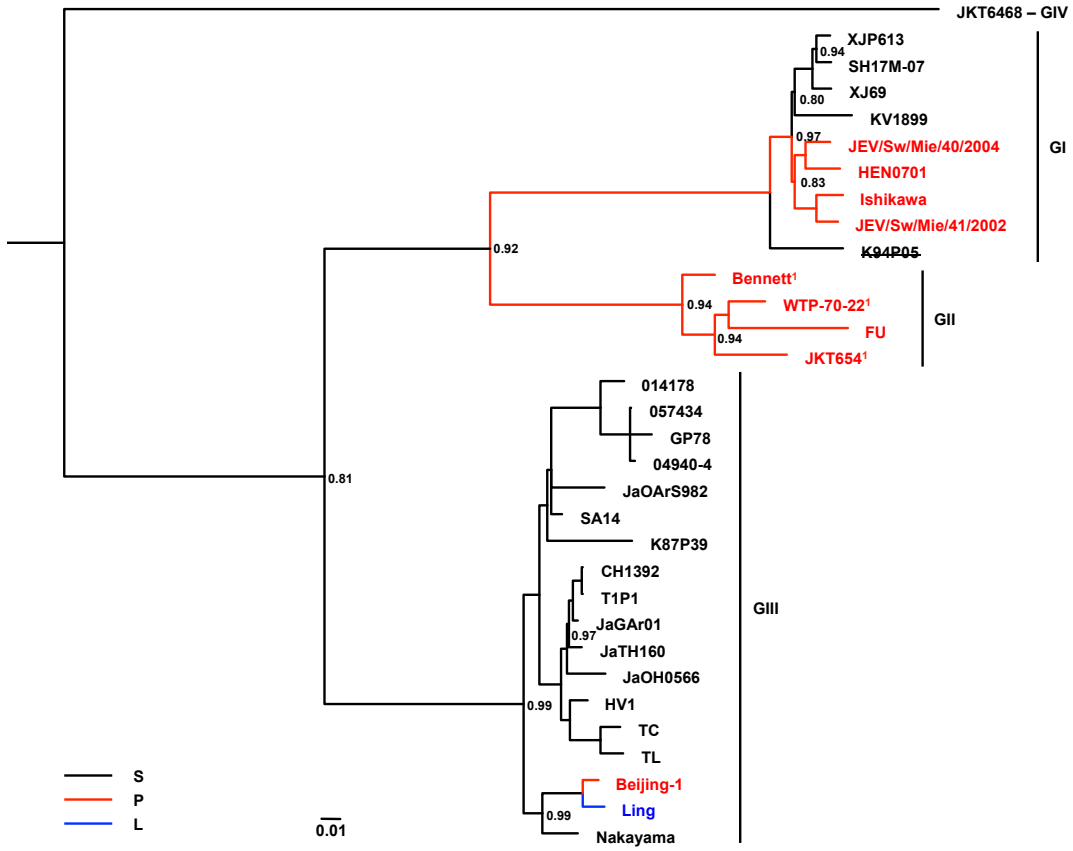
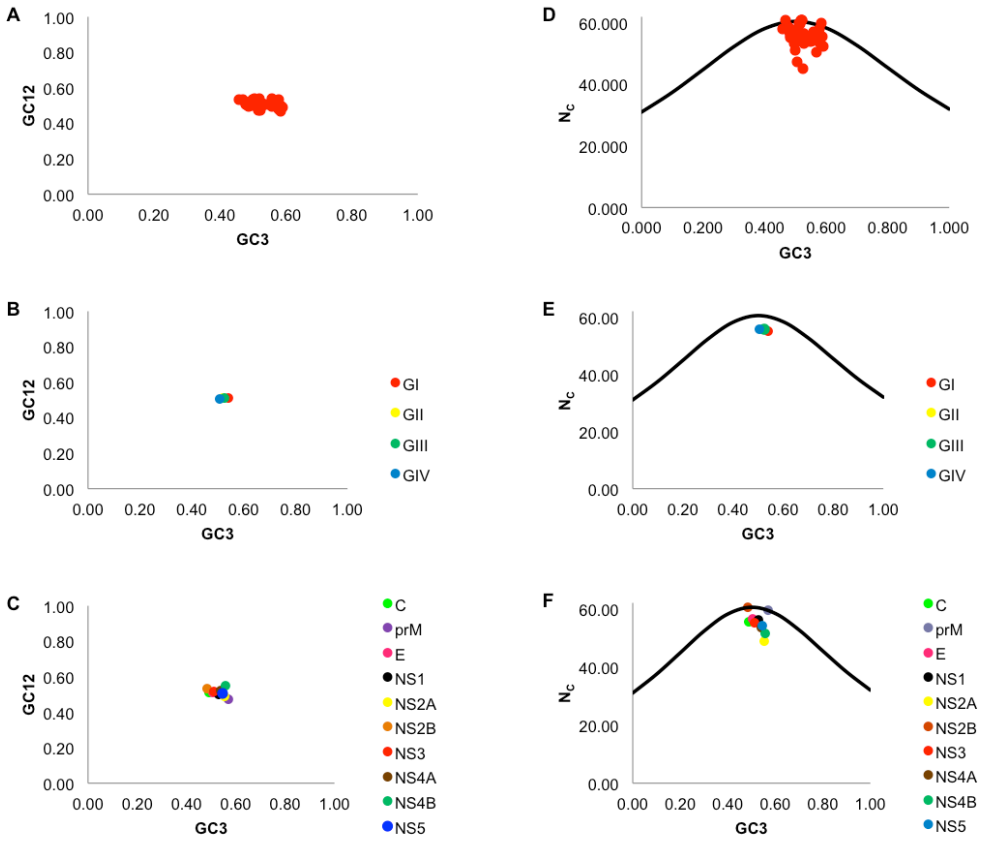


Figure 4.3: Correlation between GC12 and GC3 among all genotypes and genes (A), as well as according to genotype (B) and gene (C). Distribution of GC3 and NC among all genotypes and genes (D), as well as according to genotype (E) and gene (F). The curve indicates the expected codon usage if GC compositional constraints alone account for codon usage bias.



Chapter 5: Genetic diversity of Japanese encephalitis virus isolates obtained from the Indonesian archipelago between 1974 and 1987[3]

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5.1 ABSTRACT

It has been previously proposed that Japanese encephalitis virus (JEV) originated in the Indonesia-Malaysia region; however, only 13 Indonesian JEV isolates collected within a four-year time period have been genetically characterized. In this study, the nucleotide sequences of the envelope genes of 24 JEV isolates that were made from mosquitoes collected throughout the Indonesian archipelago from 1974 to 1987 were determined, and a series of phylogenetic and evolutionary adaptation analyses were performed. Phylogenetic analysis indicated that over a 14-year time span three genotypes of JEV circulated throughout Indonesia and a statistically significant association between the year of virus collection and genotype was revealed: isolates collected between 1974 and 1980 belonged to genotype II (GII), isolates collected between 1980 and 1981 belonged to genotype IV (GIV), and isolates collected in 1987 belonged to genotype III (GIII). Interestingly, three of the GII Indonesian isolates grouped with an isolate that was collected during the JE outbreak that occurred in Australia in 1995, two of the GIII Indonesian isolates were closely related to a Japanese isolate collected 40 years previously, and two Javanese GIV isolates possessed six amino acid substitutions within the E protein when compared to a previously sequenced GIV isolate collected in Flores. Several amino acids within the E protein of the Indonesian isolates were found to be under directional evolution and/or co-evolution. Conceivably, the tropical climate of the Indonesia/Malaysia region, together with its plethora of distinct fauna and flora, may have driven the emergence and evolution of JEV. This is consistent with the extensive genetic diversity among JEV isolates observed in this study, and further substantiates the hypothesis that JEV originated in the Indonesia-Malaysia region.

5.2 INTRODUCTION

A previous study proposed that Japanese encephalitis virus (JEV) originated in the Indonesia-Malaysia region from an ancestral virus common to JEV and Murray Valley encephalitis virus (MVEV). The Indonesia-Malaysia region is the only area where all five viral genotypes (GI-V) are represented and until recently was the only area where the most divergent genotypes (GV and GIV) had been found (Solomon *et al*., 2003). Further, the Indonesia-Malaysia region is geographical proximal to Australia, where JEV's closest relative, MVEV, circulates (Solomon *et al*., 2003). However, prior to the initiation of the work presented in this chapter, only 13 Indonesian JEV isolates collected between 1978 and 1981 had been genetically characterized (Chen *et al*., 1992), consequently limiting our understanding of the genetic variation and evolution of the virus. In this study, the nucleotide sequence of the envelope (E) gene of 24 JEV isolates that were made from mosquitoes collected throughout the Indonesian archipelago from 1974 to 1987 were determined, and a series of phylogenetic and evolutionary adaptation analyses were performed.

5.3 RESULTS

5.3.1 Geographical distribution of the Indonesian JEV isolates

Figure 5.1 depicts the geographical distribution of the 24 Indonesian JEV isolates sequenced in this study, as well as 10 others that were retrieved from GenBank (Table 5.1).

5.3.2 Genetic relationships among the Indonesian JEV isolates

Phylogenies generated using all three methods of phylogenetic inference sequence (neighbor-joining [NJ], maximum-parsimony [MP], and maximum-likelihood [ML]) identified four major clades (representing GI–IV) (Figure 5.2). Twenty of the 24

Indonesian JEV isolates sequenced in this study belong to GII (collected between 1974 and 1979 in Jakarta, Kapuk and West Java, and Lombok), two isolates belong to GIII (collected in 1987 in Central Java), and two isolates belong to GIV (collected in 1981 in Bantul and Central Java).

The topology of the phylogeny was compared to the deduced amino acid alignment to identify coding substitutions that could be involved in phylogenetic divergence and potentially result in phenotypic changes. Node-defining amino acid substitutions within the E protein that were specific to the Indonesian viruses sequenced in this study were determined and modeled onto the E protein dimer structure of JEV (Figure 5.3A).

Genotype II of the phylogeny is composed of two major groups of viruses. The first group is comprised of three Indonesian isolates (JKT2212, JKT2380 and JKT2362 [Kapuk, Java, October/November-1979]) that were sequenced in this study and share the A146T node-defining amino acid substitution, as well as the FU isolate (Australia, 1995). The second group is comprised of the remaining seventeen GII Indonesian isolates sequenced in this study; WTP-70-22 (Malaysia, 1970) diverged first, followed by the Bennett isolate (Korea, circa 1951) and then the DjAr703 isolate (West Java, 1974). The DjAr703 isolate is the oldest virus sequenced in this study and is ancestral to the other 16 GII isolates that were all collected in 1979 in Java, Lombok or undefined locations within Indonesia. Four of these viruses (JKT4312, JKT4331, JKT4332 and JKT5441) share the S230N node-defining amino acid substitution.

Two of the viruses sequenced in this study grouped together within GIII (JKT27-085 and JKT27-087 [Central Java, January-1987]) and share the A157T node-defining amino acid substitution. These two viruses grouped closely with the Equine isolate

(Japan, 1947) and these three viruses are defined by two node-defining amino acid substitutions (A51V and S276N).

The JKT7089 (Bantul, Java, June-1981) and JKT7180 (Central Java, July-1981) isolates sequenced here belonged to GIV along with the JKT6468 isolate (Flores, 1981). When the JKT7089 and JKT7180 isolates were compared with the JKT6468 isolate six amino acid substitutions were noted: T126I, I159V, R260G, Q369K, E389D and T490I.

5.3.3 Re-examination of three previously sequenced Indonesian JEV isolates

The phylogeny revealed that three previously sequenced viruses (JKT1749 [U70405], JKT1724 [U70404] and JKT5441 [U70406]) were not phylogenetically distributed according to the pattern observed in this study (year of collection versus genotype) (Figure 5.2). Given these discrepancies, the E gene of these three viruses was re-sequenced and it was found that the JKT1724, JKT1749, and JKT5441 isolates belonged to GII (Figure 5.2).

5.3.4 Distribution of Indonesian JEV isolates according to year of collection and genotype

Indonesian isolates of JEV that were sequenced in this study and isolates for which nucleotide sequence information was retrieved from GenBank were categorized according to their year of collection and genotype (Table 5.2).

A Pearson's chi-square test was used to test the null hypothesis that there is no association between the year of virus collection and genotype. Based on $\alpha = 0.05$, the null hypothesis was rejected and it was concluded that there is a relationship between the year of virus collection and genotype (Pearson's chi-square = 64.891, exact p-value = 0.000). Post-hoc analysis revealed that among virus isolates collected in 1979 and 1981, there were fewer GIV (standardized residual = -2.1) and GII (standardized residual = -2.1)

isolates than expected, respectively. Furthermore, among virus isolates collected in 1981 and 1987, there were more GIV (standardized residual = 4.3) and GIII (standardized residual = 5.5) isolates than expected, respectively.

Given that the majority of the Indonesian JEV isolates were collected in Java the hypothesis was re-tested using Java isolates only. Again, the null hypothesis was rejected and it was concluded that there is a relationship between the year of virus collection and genotype in Java (Pearson's chi square = 50.000, exact p-value = 0.000). Post-hoc analysis indicated that among virus isolates collected in 1981 and 1987 in Java there were more GIV (standardized residual = 4.2) and GIII (standardized residual = 4.6) isolates than expected, respectively.

5.3.5 Recombination and molecular adaptation analyses

No evidence of recombination was obtained.

The global ratio of non-synonymous to synonymous nucleotide substitutions (d_N/d_S) across the E gene alignment was 0.055 (95 % confidence interval [CI]: 0.045, 0.066), which suggested the occurrence of predominantly purifying selection (virus-encoded proteins are conserved over time due to the selective pressure against deleterious variants). No sites were identified to be under positive selection using the single-likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), internal FEL (IFEL) and relaxed effects likelihood (REL) methods.

The directional evolution in protein sequences (DEPS) analysis revealed elevated amino acid substitution rates towards seven residues: A, I, M, N, P, T and V (Table 5.3). Of these seven residues, directional evolution towards P was subjected to the strongest bias (83.26), but affected the smallest proportion of sites (1.35%), whereas evolution towards T was subjected to a weak bias (4.31), but affected the largest proportion of sites

(16.36%) (Table 5.3). Fifteen sites were found to be involved in this directional evolution: 51, 76, 123, 129, 146, 169, 222, 227, 230, 367, 375, 400, 474, 483, 484 (Table 5.4). The locations of the 15 sites are indicated on the E protein dimer structure of JEV (Figure 5.3B).

The Spidermonkey method detected three sets of sites that exhibited evidence of co-evolution: 1) S51V and S276N, 2) S222A and T327S, and 3) T363A and K398R (Table 5.5). These sites are indicated on the E protein dimer structure of JEV (Figure 5.3C).

5.4 DISCUSSION

Although it has been suggested that the Indonesia-Malaysia region is the origin of JEV evolution, prior to this study only 13 isolates collected within a four-year time span from Indonesia had been studied genetically. Therefore, the objective of the current study was to determine the extent of genetic variation among Indonesian JEV isolates by expanding the phylogenetic analysis of Chen *et al*., (1992) through the incorporation of 24 newly sequenced Indonesian isolates collected between 1974 and 1987. This study revealed a statistically significant association between the year of virus collection and genotype: isolates collected between 1974 and 1980 belonged to GII, isolates collected between 1980 and 1981 belonged to GIV, and isolates collected in 1987 belonged to GIII. Interestingly, only GIII and GI of JEV circulated throughout all of the other countries encompassing the geographical range of JEV activity between 1974 and 1987. This not only indicates that the genetic diversity of JEV in Indonesia is extensive, but provides additional support for the hypothesis that JEV originated from its ancestral virus in the Indonesia-Malaysia region (Solomon *et al*., 2003).

5.4.1 Two previously sequenced Indonesian isolates belong to GII, not GIII

Three previously sequenced Indonesian isolates (JKT1749 [U70405], JKT1724 [U70404] and JKT5441 [U70406]) were re-sequenced here because they were not phylogenetically distributed according to the pattern observed in this study (year of collection versus genotype), and a previous study revealed that the JKT1749 [U70405] and JKT1724 [U70404] isolates belonged to GIII in an E gene phylogeny, but belonged to GII in a prM gene phylogeny (Williams *et al*., 2000). Re-sequencing of the E gene of the JKT1749 and JKT1724 isolates revealed that the original sequences contained errors, and in agreement with Williams and colleagues it was found that the isolates do indeed belong to GII.

5.4.2 GII of JEV in Indonesia

Twenty of the 24 Indonesian JEV isolates sequenced in this study belonged to GII; three of which (JKT2212, JKT2380 and JKT2362 [all from Kapuk, Java, October/November-1979]) group together and share the A146T node-defining amino acid substitution. Site 146 was also found to be under directional evolution. The JKT2212, JKT2380 and JKT2362 isolates share a most recent common ancestor with the FU isolate, from an asymptomatic human during the 1995 JE outbreak that occurred on Badu Island, which is located in the Torres Strait between Queensland, Australia and Papua New Guinea (Hanna *et al*., 1996). A subsequent study found that the JEV isolates from Papua New Guinea were > 99% identical to virus isolates collected during the 1995 outbreak on Badu Island, and therefore concluded that JEV may have been introduced from Papua New Guinea to Australia (Johansen *et al*., 2000). The close phylogenetic grouping between the three Indonesian isolates collected in 1979 and the FU isolate suggest that JEV may have been introduced to the Papua New Guinea-Australia region from Indonesia sometime between 1979 and 1995. A previous hypothesis suggests that

JEV gradually moved eastwards along the Indonesian archipelago, through natural mosquito-bird and mosquito-pig transmission cycles, until it arrived undetected in Papua New Guinea (Mackenzie *et al*., 2002). In fact, strong serological evidence suggests that JEV arrived in Papua New Guinea in the late 1980s (Mackenzie *et al*., 2002). Although flaviviral serological results are difficult to interpret due to heterotypic cross-reactivity, it is noteworthy that JEV seropositive swine were observed in Timor and Irian Jaya between 1989 and 1991 (Mackenzie *et al*., 2002). It is also plausible that JEV may have been introduced into Papua New Guinea and subsequently into Australia through wind-blown mosquitoes (Ritchie & Rochester, 2001), black flying foxes (*Pteropus* sp.) (van den Hurk *et al*., 2009b), and/or wind-blown biting midges (*Culicoides* sp.) (Mackenzie *et al*., 2002).

The remaining 17 GII Indonesian isolates sequenced here (collected in Java, Lombok and unknown locations in Indonesia) were descendants of the WTP-70-22 (Malaysia, 1970) and Bennett (Korea, circa 1951) isolates and four of these viruses (JKT4312, JKT4331, JKT4332 and JKT5441) share the S230N node-defining amino acid substitution. Site 230 was also found to be under directional evolution. Between 1972 and 1974, the time period coinciding with the collection of the DjAr703 isolate (West Java, 1974), the United States Naval Medical Research Unit No. 2 and the Indonesian Ministry of Health collected mosquitoes from Kapuk, Java ultimately resulting in 12 isolates of JEV (van Peenen *et al*., 1975). During the study period, Kapuk was an area of Jakarta reserved for the raising and slaughter of swine and 11 of the 12 JEV isolates that were made came from pools of *Cx. tritaeniorhynchus*, thereby implicating domestic swine and *Cx. tritaeniorhynchus* in the enzootic transmission cycle of JEV (van Peenen *et al*., 1975). An additional nineteen isolates of JEV were made from mosquitoes (*Cx. tritaeniorhynchus, geldius, vishnui, and fuscocephala*) captured between October 1978

and April 1980 in another study that took place in Kapuk, Java and 14 of these isolates were sequenced in this study (11 of these isolates were descendants of the DjAr703 isolate and three of these isolates share a most recent common ancestor with the FU isolate mentioned above) (Olson *et al*., 1985b). Three of the 17 GII Indonesian viruses (JKT1724, JKT2254 and JKT2267) were collected in villages near Gerung, West Lombok during March of 1979 (Olson *et al*., 1985a), where a previous study indicated a low human seroprevalence to JEV (Olson *et al*., 1983). The JKT1724 isolate was obtained from a pool of *Cx. tritaeniorhynchus* feeding on domestic buffalo or resting in buffalo stables, the JKT2254 isolate was made from a pool of *Anopheles annularis* collected from buffalo stables, and the JKT2267 isolate was made from a pool of *Anopheles vagus* collected by CDC light traps (Olson *et al*., 1985a). Interestingly, the minimum frequencies of JEV infection in zoophilic *Anopheles* sp. were higher than in *Cx. tritaeniorhynchus (Olson et al., 1985a)*. It has been previously suggested that the relatively low minimum frequency of JEV infection in *Cx. tritaeniorhynchus* coupled with the absence of swine from Lombok may have been responsible for the low seroprevalence of JEV among humans residing in this area (Olson *et al*., 1985a).

5.4.3 GIII of JEV in Indonesia

Two of the 24 Indonesian JEV isolates sequenced in this study belonged to GIII (JKT27-085 and JKT27-087 [both Central Java, January-1987]) and share the A157T node-defining amino acid substitution. These two isolates grouped closely with the Equine isolate (Japan, 1947) and together these three isolates share the S51V and S276N node-defining amino acid substitutions. Sites 51 and 276 also exhibited evidence of co-evolution. It is interesting to note that they lie adjacent to each other within domain I of the three-dimensional structure of the E protein of JEV. The close phylogenetic and

evolutionary relationships between the JKT27-085, JKT27-087 and Equine isolates may indicate that the viruses responsible for the major JE epidemics occurring in Japan in 1924, 1935 and 1948 (Innis, 1995) evolved in Indonesia and were subsequently transported by migratory birds traveling along the East Asian-Australasian flyway to Japan.

5.4.4 GIV of JEV in Indonesia

Two of the 24 Indonesian JEV isolates sequenced in this study belonged to GIV (JKT7089 [Bantul, Java, June-1981] and JKT7180 [Central Java, July-1981]). These two isolates possessed six amino acid substitutions in the E protein when compared to the previously sequenced JKT6468 (Flores, 1981) isolate (T126I, I159V, R260G, Q369K, E389D and T490I). Notably, these two GIV isolates, as well as the other five GIV isolates were all collected from mosquitoes (three isolates were made from mosquito pools, three isolates were made from *Cx. tritaeniorhynchus* pools and one isolate was made from a *Culex vishnui* pools in Indonesia (four isolates were collected in Java, two isolates were collected in Bali and one isolate was collected in Flores) between 1980 and 1981 (one isolate was collected in 1980 and six isolates were collected in 1981). As GIV has not been collected since 1981, it remains unknown whether this genotype is still circulating in Indonesia or it has become extinct. The reasons underlying the failure of GIV to spread beyond the Indonesian archipelago are unknown, but could be hypothesized to be due one or more factors: the vector competence of *Cx. tritaeniorhynchus*, the primary vector for JEV throughout Asia, for GIV of JEV may be lower compared to the other three virus genotypes, GIV may preferentially infect and disseminate to the salivary glands in a mosquito species that does not exist throughout Asia, the replicative ability of GIV in birds may be poor implying that birds infected with

this genotype may not produce sufficient viremias to transport the virus to mainland Asia, the GIV transmission cycle may involve an amplifying host that is a non-migratory bird and/or the GIV transmission cycle may involve a non-avian amplifying host that is either non-migratory or is geographically confined to Indonesia.

5.4.5 Conclusions

It is interesting to note that three genotypes of JEV circulated throughout Indonesia over a time-span of 14 years, whereas with the exception of one instance (Schuh *et al*., 2010), GIII circulated exclusively throughout Northern and Western Asia for at least 45 years (collection of the prototype GIII isolate in Japan in 1935 to the collection of the GI YN79-Bao83 GI isolate in China in 1979). Although GI has recently emerged and displaced GIII as the most frequently isolated virus genotype throughout most of Asia (Nga *et al*., 2004; Nitatpattana *et al*., 2008), a GI virus has never been isolated in Indonesia. However, several GI isolates have been collected in neighboring northern Malaysia (Tsuchie *et al*., 1997), and isolates representative of genotypes II, III and V have been collected throughout Malaysia (Mohammed *et al*., 2011; Tsuchie *et al*., 1994; Tsuchie *et al*., 1997). The tropical climate of the Indonesia-Malaysia region coupled with the vast array of distinct fauna and flora present throughout the region may have facilitated the emergence and evolution of JEV. This is consistent with the extensive genetic diversity observed among isolates collected from this region, as well with the previously proposed hypothesis that JEV originated from an ancestral virus in the Indonesia-Malaysia region (Mohammed *et al*., 2011; Solomon *et al*., 2003).

Table 5.1: Details of the JEV isolates used in this study.

Isolate	Origin	Year	Host	Genotype	GenBank accession number
Ishikawa[2]	Japan	1998	Mosquito	GI	ABO51292
KV1899[2]	Korea	1999	Swine	GI	AY316157
JEV/sw/Mie/40/2004[2]	Japan	2004	Swine	GI	AB241118
XJ69[2]	China	2007	Mosquito	GI	EU880214
DjAr703[1]	West Java	1974	*Cx. quiquefasciatus*	GII	JQ429287
Bennett[2]	Korea	1951	Human	GII	FJ872376
WTP-70-22[2]	Malaysia	1970	Mosquito	GII	HQ223286
JKT654[3]	Kapuk, Java	1978	*Cx. tritaeniorhynchus*	GII	HQ223287
JKT657[3]	Kapuk, Java	1978	*Cx. tritaeniorhynchus*	GII	L42157
JKT1105[3]	Kapuk, Java	1979	*Cx. gelidus*	GII	L42155
JKT2219[3]	Kapuk, Java	1979	*Cx. tritaeniorhynchus*	GII	L42165
JKT2363[3]	Kapuk, Java	1979	*Cx. tritaeniorhynchus*	GII	L42163
JKT5441 (U70406)[2]	Indonesia	1981	Mosquito	GII	U70406
FU[2]	Australia	1995	Human	GII	AF217620
JKT220507[1]	Jakarta, Java	Jan-79	Mosquito	GII	JQ429291
JKT811[1]	Kapuk, Java	Jan-79	*Cx. tritaeniorhynchus*	GII	JQ429303
JKT1110[1]	Kapuk, Java	Feb-79	*Cx. tritaeniorhynchus*	GII	JQ429288
JKT1724[1]	Lombok	Mar-79	*Cx. tritaeniorhynchus*	GII	JQ429304
JKT1749[1]	Kapuk, Java	Mar-79	*Cx. tritaeniorhynchus*	GII	JQ429305
JKT2254[1]	Lombok	Mar-79	*An. annularis*	GII	JQ429293
JKT2267[1]	Lombok	Mar-79	*An. vagus*	GII	JQ429294
JKT1729[1]	Kapuk, Java	Apr-79	*Cx. tritaeniorhynchus*	GII	JQ429289
JKT1754[1]	Kapuk, Java	Apr-79	*Cx. tritaeniorhynchus*	GII	JQ429290
JKT2212[1]	Kapuk, Java	Oct-79	*Cx. tritaeniorhynchus*	GII	JQ429292

Isolate	Origin	Year	Host	Genotype	GenBank accession number
JKT2303[1]	Kapuk, Java	Nov-79	*Cx. tritaeniorhynchus*	GII	JQ429295
JKT2329[1]	Kapuk, Java	Nov-79	*Cx. tritaeniorhynchus*	GII	JQ429298
JKT2352[1]	Kapuk, Java	Nov-79	*Cx. tritaeniorhynchus*	GII	JQ429299
JKT2362[1]	Kapuk, Java	Nov-79	*Cx. tritaeniorhynchus*	GII	JQ429296
JKT2380[1]	Kapuk, Java	Nov-79	*Cx. vishnui*	GII	JQ429297
JKT4312[1]	Kapuk, Java	Dec-79	*Cx. gelidus*	GII	JQ429300
JKT4331[1]	Kapuk, Java	Dec-79	*Cx. tritaeniorhynchus*	GII	JQ429301
JKT4332[1]	Kapuk, Java	Dec-79	*Cx. tritaeniorhynchus*	GII	JQ429302
JKT5441[1]	Indonesia	Jun-80	Mosquito	GII	JQ429306
Matsunaga[2]	Japan	1939	Human	GIII	FJ872381
Roum[2]	Korea	1946	Human	GIII	FJ872377
Equine[2]	Japan	1947	Equid	GIII	FJ872378
Beijing-1[2]	China	1949	Mosquito	GIII	L48961
SA14[2]	China	1954	Mosquito	GIII	JEU14163
HV1[2]	Taiwan	1958	Human	GIII	AF098735
JaGAr01[2]	Japan	1959	Mosquito	GIII	AF069076
JaTH160[2]	Japan	1960	Human	GIII	AB269326
Ling[2]	Taiwan	1965	Human	GIII	L78128
JaOH0566[2]	Japan	1966	Human	GIII	AY508813
GP78[2]	India	1978	Human	GIII	AF075723
JKT1724 (U70404)[2]	Lombok	1979	Mosquito	GIII	U70404
JKT1749 (U70405)[2]	Kapuk, Java	1979	Mosquito	GIII	U70405
JaOArS982[2]	Japan	1982	Mosquito	GIII	NC_001437
K87P39[2]	Korea	1987	Mosquito	GIII	AY585242
CH1392[2]	Taiwan	1990	Mosquito	GIII	AF254452
T1P1[2]	Taiwan	1997	Mosquito	GIII	AF254453

Isolate	Origin	Year	Host	Genotype	GenBank accession number
05734[2]	India	2005	Human	GIII	EF623988
JKT27-085[1]	Central Java	Jan-87	Mosquito	GIII	JQ429307
JKT27-087[1]	Central Java	Jan-87	Mosquito	GIII	JQ429308
"Korea Jap B[2]	Korea	Circa 1950	Unknown	GIII	FJ872379
V9-3901[2]	Japan	Circa 1950	Human	GIII	FJ872382
V9-3902[2]	Japan	Circa 1950	Human	GIII	FJ872383
V9-4399[2]	Japan	Circa 1950	Human	GIII	FJ872380
JKT8442[3]	Bali	1980	Cx. tritaeniorhynchus	GIV	L42159
JKT6468[3]	Flores	1981	*Cx. tritaeniorhynchus*	GIV	AY184212
JKT7003[3]	Java	1981	Mosquito	GIV	L42161
JKT7887[3]	Java	1981	Mosquito	GIV	L42160
JKT9092[3]	Bali	1981	Mosquito	GIV	L42158
JKT7089[1]	Bantul, Java	Jun-81	*Cx. vishnui*	GIV	JQ429309
JKT7180[1]	Central Java	Jul-81	*Cx. tritaeniorhynchus*	GIV	JQ429310
MVE-1-51[4]	Australia	1951	Human	MVEV	AF161266

[1]Indonesian viruses sequenced in this study. [2]The nucleotide sequences of these viruses were retrieved from GenBank to generate phylogenies. [3]The nucleotide sequences of these Indonesian JEV isolates were downloaded from GenBank to determine the overall distribution of Indonesian JEV isolates according to year of collection and genotype. [4]All viruses are JEV except for MVE-1-51, which is an isolate of MVEV.

Table 5.2: Distribution of Indonesian JEV isolates according to year of collection and genotype.

	Year						
Genotype	**1974**	**1978**	**1979**	**1980**	**1981**	**1987**	**Total**
II	1	1	22	1	0	0	25 (73.5%)
III	0	0	0	0	0	2	2 (5.9%)
IV	0	0	0	1	6	0	7 (20.6%)
Total	1 (2.9%)	1 (2.9%)	22 (64.7%)	2 (5.9%)	6 (17.6%)	2 (5.9%)	34 (100.0%)

Indonesian JEV isolates for which there was nucleotide sequence information available, including isolates sequenced in this study and isolates for which the nucleotide sequence was retrieved from GenBank.

Table 5.3: DEPS analysis of the JEV E protein sequences.

Residue	p-value	Bias	Proportion (%)	No. of sites
A	< 0.00	3.09	12.5	3
I	< 0.00	23.05	3.07	3
M	< 0.00	51.29	1.75	3
N	< 0.00	34.3	2.17	2
P	< 0.00	83.26	1.35	2
T	< 0.00	4.31	16.63	2
V	< 0.00	20.97	3.34	2

Table 5.4: Amino acid sites within the E protein of the JEV isolates that were found to be under directional selection.

Site	Composition	MRCA	Target	Inferred substitutions[1]	DEPS EBF
51	$S_{35}V_2T_1$	S	V	$S_0 \leftrightarrow_1 T$, $S_0 \leftrightarrow_2 V$	298.3
76	$T_{36}M_2$	T	M	$M_2 \leftrightarrow_0 T$	692.7
123	$S_{33}N_3R_2$	S	N	$N_3 \leftrightarrow_0 S$, $R_2 \leftrightarrow_0 S$	6656.3
129	$T_{33}M_4L_1$	T	M/T	$L_1 \leftrightarrow_0 T$, $M_4 \leftrightarrow_1 T$	$>10^5/>10^5$
146	$T_{32}A_5S_1$	T	A/T	$A_5 \leftrightarrow_0 T$, $S_1 \leftrightarrow_0 T$	200.6/122.6
169	$V_{34}I_4$	V	I	$I_4 \leftrightarrow_0 V$	$>10^5$
222	$A_{19}S_{18}L_1$	A	A	$A_0 \leftrightarrow_1 L$, $A_{14} \leftrightarrow_6 S$	2558.1
227	$S_{34}P_4$	S	P	$P_4 \leftrightarrow_0 S$	$>10^5$
230	$S_{34}N_4$	S	N	$N_4 \leftrightarrow_0 S$	$>10^5$
367	$A_{19}S_{19}$	A	A	$A_{11} \leftrightarrow_{36} S$	1637.4
375	$M_{36}I_2$	M	I	$I_2 \leftrightarrow_0 M$	126.2
400	$A_{35}P_2E_1$	A	P	$A_0 \leftrightarrow_1 E$, $A_0 \leftrightarrow_2 P$	1594.9
474	$V_{33}I_5$	V	I	$I_5 \leftrightarrow_0 V$	$>10^5$
483	$L_{36}M_2$	L	M	$L_0 \leftrightarrow_2 M$	318.8
484	$A_{35}V_3$	A	V	$A_0 \leftrightarrow_3 V$	1654.1

[1]Amino acid substitutions inferred when $A_b \leftrightarrow_c D$ indicates b substitutions from A to D and c substitutions from D to A.

Table 5.5: Spidermonkey analysis of the JEV E protein alignment.

Site 1	Site 2	Number of isolates	Posterior probability	Supporting replicates (%)
S51V	S276N	2	0.90	99
S222A	T327S	19	0.98	99
K398R	T363A	2	0.94	99

Figure 5.1: Map of the Indonesian archipelago. The islands comprising Indonesia are shown in white, red text indicates the islands where JEV isolates included in this study were collected and black text indicates the cities in Java where JEV isolates included in this study were collected. Twenty-seven of the Indonesian isolates were from the island of Java (one from Jakarta, 19 from Kapuk, one from West Java, three from Central Java, one from Bantul and two from unspecified locations in Java), two were from the island of Bali, three were from the island of Lombok, one was from the island of Flores, and one was from an unspecified location in Indonesia.

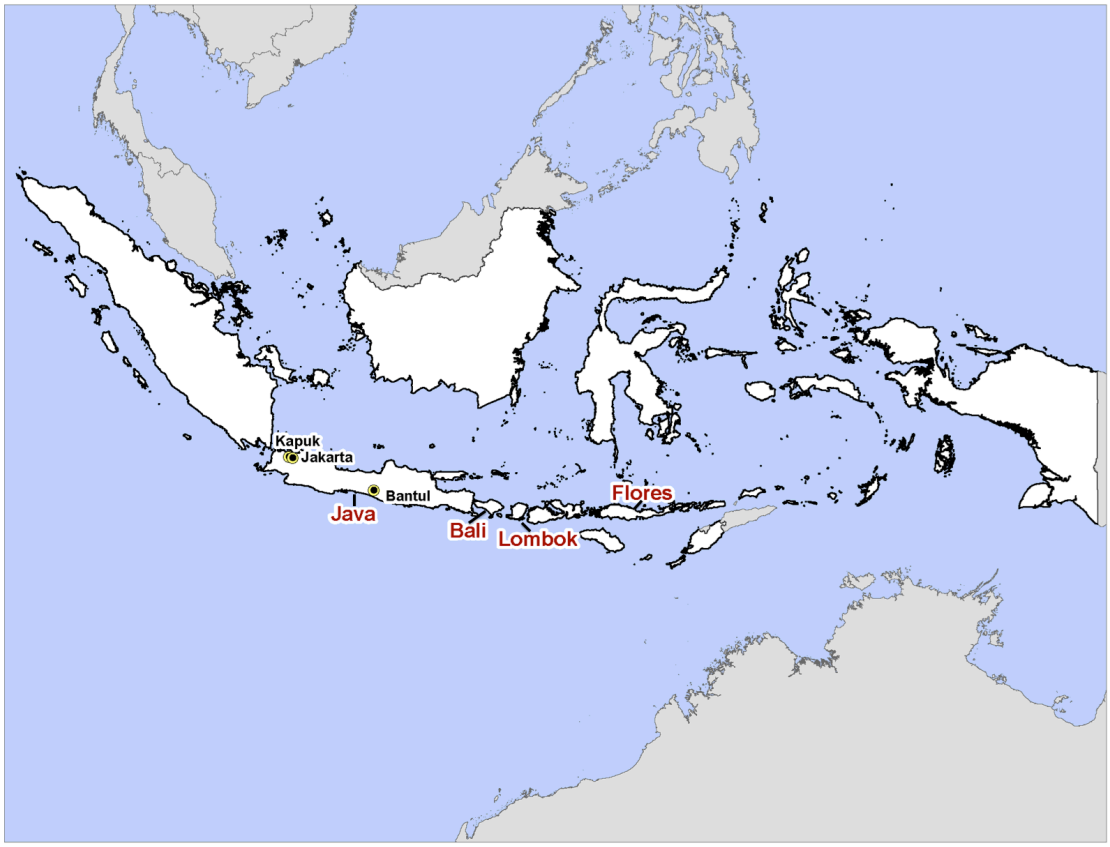


Figure 5.2: NJ phylogeny based on nucleotide sequence information derived from the E gene of the JEV isolates. The trees was rooted using the MVE-1-51 isolate of MVEV, which is a member of the Japanese encephalitis serocomplex, but has been removed to allow for better visualization of branch lengths. Horizontal branch lengths are proportional to the genetic distance between strains and the scale at the lower-left of the tree indicates the number of nucleotide substitutions per site. GI-IV are represented to the right of the tree. Bootstrap percentages based on 1,000 replicates are indicated to the left of the genotype-defining nodes within the phylogeny, as well as additional selected nodes. The Indonesian isolates sequenced in this study are indicated by "1".

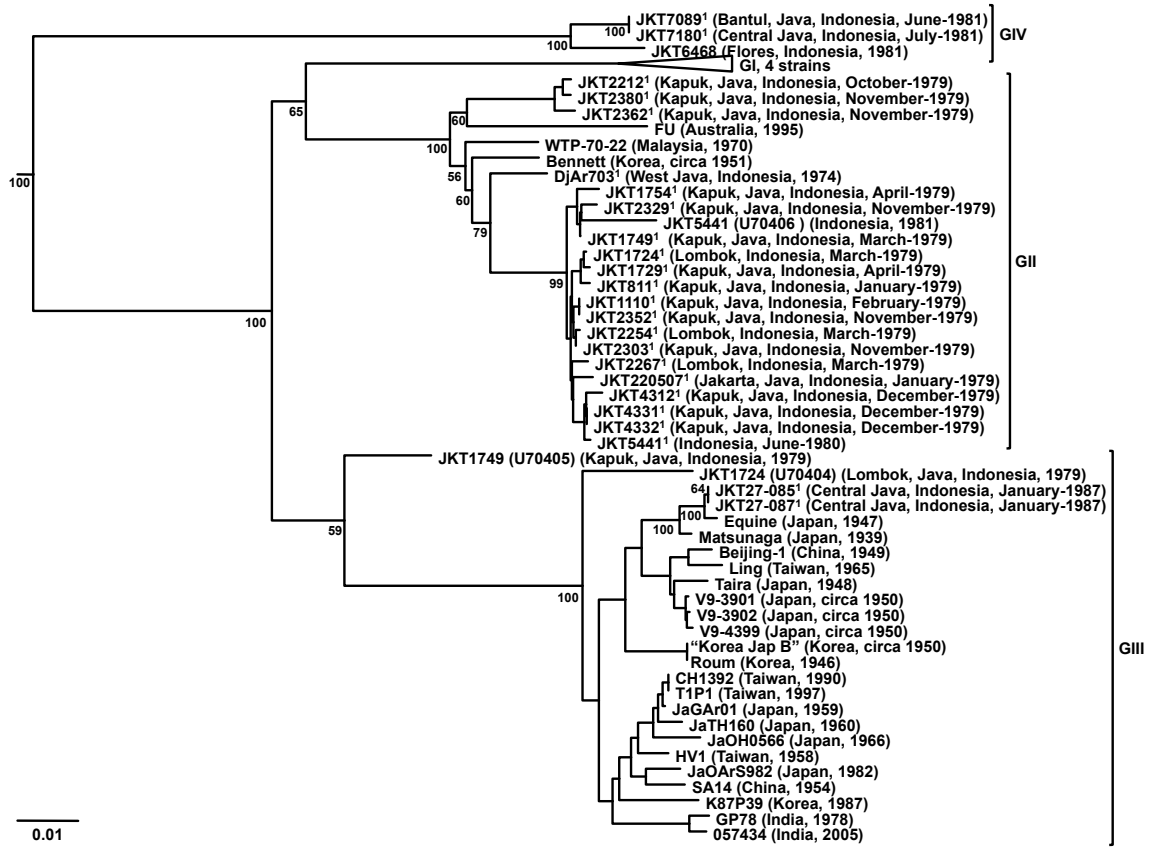
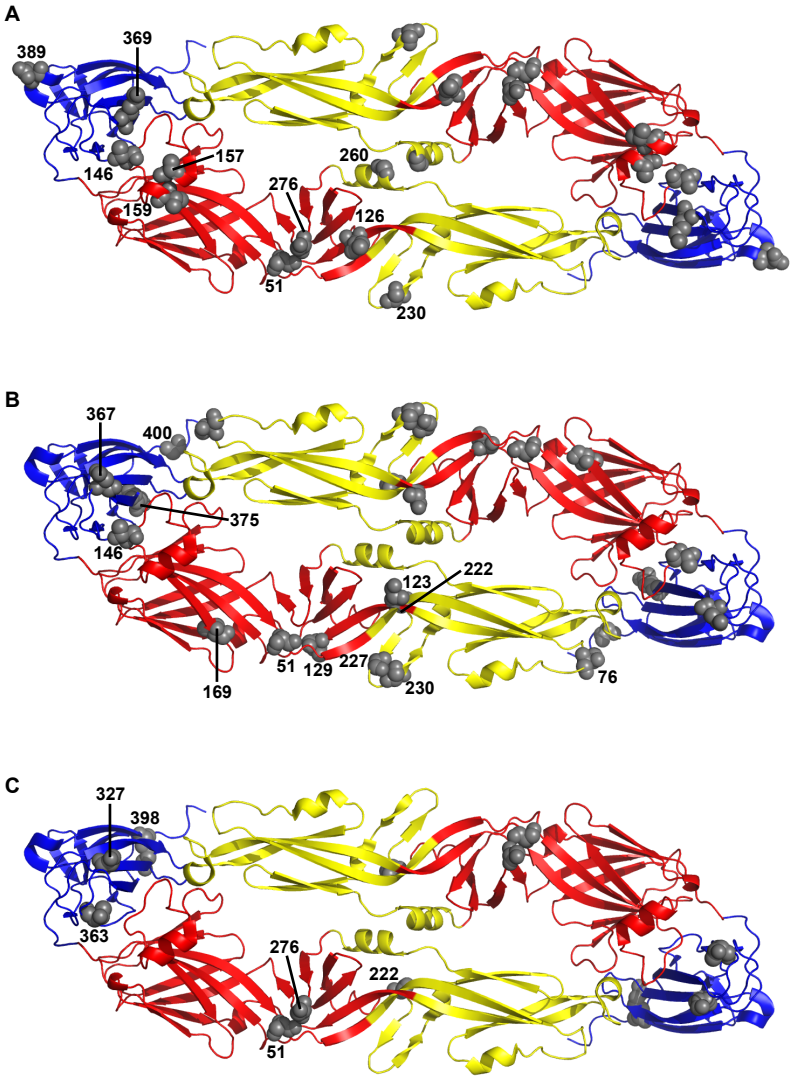


Figure 5.3: Important amino acids indicated on the E protein dimer structure of JEV: A) Node-defining amino acid substitutions specific to the Indonesian viruses sequenced in this study, B) Sites identified by the DEPS analysis to exhibit evidence of directional evolution, and C) Sites detected by the Spidermonkey analysis to display evidence of co-evolution. Domain I is indicated in red, domain II in yellow and domain III in blue. The substitutions are indicated in gray and are only numbered on the lower monomer.



Chapter 6: Phylogeography of Japanese encephalitis virus: Genotype is associated with climate

6.1 ABSTRACT

Japanese encephalitis virus (JEV) is found throughout most of Asia, extending from maritime Siberia in the north to Australia in the south, and as far as Pakistan to the west and Saipan to the east. Transmission of the virus in temperate zones is epidemic with the majority of cases occurring in summer months, while transmission in tropical zones is endemic and occurs year-round at lower rates. In recent years, genotype I of the virus has displaced genotype III as the dominant JEV genotype and genotype V has re-emerged after almost 60 years of undetected virus circulation. However, little is known about the evolutionary history, and the geographical distribution and the epidemiology of the five virus genotypes. To fill this gap, Bayesian phylogeographic and categorical data analysis techniques were applied to the largest JEV sequence dataset compiled to date, representing the envelope (E) gene of 487 isolates collected from 12 countries over 75 years. In addition to elucidating the evolutionary history of the virus on a spatiotemporal scale, this study demonstrated that genotype III and the recently emerged genotype I-b are temperate genotypes that are likely maintained year-round in northern latitudes by either hibernating mosquitoes, transovarial transmission in mosquitoes, poikilothermic vertebrates and/or bats, while genotype I-a and genotype II are tropical genotypes likely maintained primarily through mosquito-bird and/or mosquito-swine transmission cycles. This suggests that the spread and establishment of GI-b throughout Asia may have been due to its ability to efficiently overwinter in temperate Asia. Finally, this study identified genotype-defining amino acid substitutions within the E protein that may have played a critical role in the adaptation of these viral genotypes to their respective environments.

6.2 INTRODUCTION

Japanese encephalitis virus (JEV) circulates throughout most of Asia, with the northern limit of virus activity extending into maritime Siberia. In recent years the geographical distribution of JEV has expanded, reaching east into Saipan in 1990 (Paul *et al*., 1993), west into Pakistan in 1992 (Igarashi *et al*., 1994) and south into the Torres Strait of Australia in 1995 (Hanna *et al*., 1996). Transmission of the virus in temperate zones is epidemic with the majority of cases occurring in summer or monsoon season months, while transmission in tropical zones is endemic and occurs year-round at lower rates (Innis, 1995). Previous investigations noted genotype I (GI) and genotype III (GIII) viruses were collected mostly in temperate zones, while genotype II (GII) and genotype IV (GIV) isolates were collected mostly in tropical zones (Chen *et al*., 1990; Chen *et al*., 1992). However, the statistical significance of this observation has never been tested using a comprehensive dataset of JEV isolates, with information regarding the isolates genotype and location of collection.

Not only has JEV expanded its geographical distribution in recent years causing outbreaks of encephalitis in immunologically naïve populations, the molecular epidemiology of the virus has changed as well. From the isolation of the prototype Nakayama strain of JEV in 1935 until recently, GIII was the most frequently isolated genotype throughout Asia. However, over the past two decades, multiple reports have indicated that GI has displaced GIII as the most frequently isolated virus genotype in a number of Asian countries including China (Wang *et al*., 2007), Thailand (Nitatpattana *et al*., 2008), South Korea (Nam *et al*., 1996), Japan (Ma *et al*., 2003), Malaysia (Tsuchie *et al*., 1997), Vietnam (Nga *et al*., 2004), India (Fulmali *et al*., 2011), and Taiwan (Chen, 2011). Further, following the isolation of the genotype V (GV) Muar isolate (Uchil & Satchidanandam, 2001) in 1952 from an encephalitic patient originating in Malaysia, the

genotype remained undetected for almost 60 years until a pool of *Culex tritaeniorhynchus* collected in the Tibetan Province of China in 2009 yielded the GV XZ0934 isolate (Li *et al*., 2011a) and a pool of *Culex bitaeniorhynchus* collected in South Korea in 2010 yielded the GV 10-1827 isolate (Takhampunya *et al*., 2011). A recent evolutionary study utilizing sequence information derived from the open reading frame (ORF) of 35 JEV isolates (22 GIII isolates) revealed that JEV diverged from its ancestral virus around 1500 (Mohammed *et al*., 2011) and an earlier evolutionary study using 18 genomic JEV sequences (14 GIII isolates) proposed that this divergence event occurred in the Indonesia-Malaysia region (Solomon *et al*., 2003). Due to the small viral sequence sample sizes, neither of these studies were able to robustly examine the evolution, epidemiology or geographical distribution of the genotypes of JEV (Mohammed *et al*., 2011; Solomon *et al*., 2003). Inconsistent with the results of previous studies (Mohammed *et al*., 2011; Solomon *et al*., 2003), a recent study utilizing 98 genomic sequences, 76 of which were derived from Chinese virus isolates, estimated that JEV diverged from its ancestral virus around 300 after death (AD) (Pan *et al*., 2011). This inconsistency was likely due to the slow evolutionary rate estimated for the Chinese JEV study (Pan *et al*., 2011) relative to previous JEV evolutionary studies (Mohammed *et al*., 2011; Solomon *et al*., 2003), as well other flavivirus evolutionary studies (Jenkins *et al*., 2002). Prior to the initiation of the work presented in this chapter, no studies have utilized a comprehensive dataset of molecular sequences to examine the phylogeography and epidemiology of the virus genotypes.

Although there is a paucity of ORF sequences of wild-type isolates, extensive sequencing of the E gene of both old and new JEV isolates in recent years has resulted in a large, spatiotemporally distributed collection of viral sequence data. Therefore, in this study a phylogeographic analysis was performed on a dataset consisting of envelope (E)

gene sequence information derived from 487 JEV isolates (the largest JEV sequence dataset assembled to date) to address the following key questions: 1) When and where did the virus and its genotypes originate, and what is their geographical range? 2) Is there an association between genotype and climate of virus collection (temperate versus tropical zones)? 3) What coding substitutions were involved in the phylogenetic divergence of the virus genotypes?

6.3 RESULTS

6.3.1 Recombination analysis

Recombination can invalidate the results of coalescent analyses. Therefore, the JEV E gene alignment was checked for the presence of recombination. The K82P01 (Korea, 1982, GenBank accession no. U34926) and K91P55 (Korea, 1991, GenBank accession no. U34928) isolates were confirmed as recombinants (Table 6.1). As these isolates were not available from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) to re-sequence, it could not be determined whether these recombinant sequences resulted from true recombination events that occurred in nature or were the result of laboratory sequence assembly errors. Therefore, these two sequences were removed from the dataset, leaving a final dataset of 487 sequences.

6.3.2 Descriptive analysis of the JEV dataset

The largest collection of JEV sequences assembled to date (n = 487) was used to investigate the phylogeography of the virus, the association between genotype and climate and molecular determinants of phylogenetic divergence (Table 6.2). Of the 487 isolates in the JEV dataset, 15 (3.08%) belonged to GI-a, 219 (44.97%) belonged to GI-b (see section 6.3.4 for a definition of GI-a and GI-b), 28 (5.75%) belonged to GII, 219 (44.97%) belonged to GIII, 3 (0.62%) belonged to GIV and 3 (0.62%) belonged to GV

(Table 6.3). The majority of the viruses within the JEV E gene dataset were isolated from mosquitoes (57.91%) in the last decade (63.04%) from Japan (42.51%), and were classified as temperate (north of the Tropic of Cancer (23.5°N) (83.37%) (Table 6.3).

6.3.3 Rate of molecular evolution

The overall evolutionary rate estimated from the JEV E gene country dataset was 5.27×10^{-4} substitutions/site/year (95% highest posterior density [HPD]: 3.92×10^{-4}, 6.52×10^{-4}) and the rate estimated for the JEV E gene climate dataset was 5.48×10^{-4} substitutions/site/year (95% HPD: 4.24×10^{-4}, 6.67×10^{-4}). These estimates are consistent with that recently reported based on a dataset of 35 JEV ORF sequences (mean: 4.35×10^{-4} substitutions/site/year, 95% HPD: 3.49×10^{-4}, 5.30×10^{-4}) (Mohammed *et al*., 2011).

6.3.4 Spatiotemporal chronology of the evolution of JEV

Figure 6.1 shows the geographical distribution of the JEV sequences included in this study according to the country of collection and Figure 6.2 shows the geographical distribution of the JEV sequences included in this study according to the climate collection. Country and climate Bayesian maximum clade credibility (MCC) phylogenies are shown in Figures 6.3 and 6.4, respectively. Not only are the major topologies of the country and climate Bayesian MCC phylogenies supported by each other, they are also supported by phylogenies constructed using neighbor-joining (NJ) (Figure 6.5) and maximum-likelihood (ML) (Figure 6.6) methods, and are similar to recently published phylogenies generated from both ORF and E gene sequence information derived from GI-V of the virus (Li *et al*., 2011a; Mohammed *et al*., 2011; Takhampunya *et al*., 2011). All four of the phylogenies inferred in this study (country Bayesian MCC, climate Bayesian MCC, NJ and ML) support the division of GI into two clusters, GI-a and GI-b, where GI-a is defined as 15 isolates sampled in Cambodia, Thailand and Australia

between 1967 and 2005 and GI-b includes 219 isolates sampled from Vietnam, Thailand, Japan, Korea, China and Taiwan between 1979 and 2009.

In Figures 6.3 and 6.4, key nodes within the phylogenies are designated by numbers and are supported by the posterior probability (PP) values indicated in Tables 6.4 and 6.5, respectively. Branch tips correspond to the date of collection of each of the virus isolates and branch lengths correspond to the time since divergence. Terminal branches within the phylogenies are colored according to the geographic location of the taxon at the tip, while internal braches are colored according to the most probable location of their parental node. Divergence dates of the most recent common ancestor (MRCA) and state PP values for each of the 12 countries are presented in Table 6.4 for the key nodes within the country Bayesian MCC phylogeny (Figure 6.3), and divergence dates of the MRCA and state PP values in support of tropical and temperate climates of divergence are presented in Table 6.4 for the key nodes within the climate Bayesian MCC phylogeny (Figure 6.4).

The country Bayesian MCC phylogeny (Figure 6.3) and the country map (Figure 6.1) show that GV includes three isolates sampled in China, South Korea and Malaysia between 1952 and 2010, GIV includes three isolates sampled in Indonesia only between 1980 and 1981, GIII includes 234 isolates sampled in China, India, Indonesia, Japan, Korea, Sri Lanka, Taiwan and Vietnam between 1935 and 2009, GII includes 28 isolates sampled in Australia, Indonesia, Korea and Malaysia between 1951 and 1999, GI-a includes 15 isolates sampled in Cambodia, Thailand and Australia between 1967 and 2005, and GI-b includes 219 isolates sampled from Vietnam, Thailand, Japan, Korea, China and Taiwan between 1979 and 2009. Phylogeographic analysis estimated that JEV (node PP: 1.00) diverged from the MRCA in 1504 (95% HPD: 1089, 1794) possibly in Malaysia (state PP: 0.19) or Indonesia (state PP: 0.18) (Figure 6.3, Table 6.4). This

estimated divergence time of the MRCA of JEV is consistent with the estimate recently reported based on a dataset of 35 JEV ORF sequences (1559; 95% HPD: 1509, 1635), which only contained one GV isolate (Mohammed *et al*., 2011). Further, the finding that the MRCA most likely diverged in Malaysia or Indonesia is in agreement with previous suggestions regarding the origin of JEV (Solomon *et al*., 2003).

Subsequent to the divergence of JEV, the MRCA of GIV + GIII + GII + GI (node PP: 0.60) diverged in 1695 (95% HPD: 1516, 1844) possibly in Indonesia (state PP: 0.21), followed by the divergence of the MRCA of GIII + GII + GI (node PP: 0.94) in 1801 (95% HPD: 1701, 1880) possibly in Korea (state PP: 0.20) or Japan (state PP: 018), and the MRCA of GII + GI (node PP: 1.00) in 1867 (95% HPD: 1799, 1920) possibly in Korea (state PP: 0.24) or Indonesia (state PP: 0.22) (Figure 6.3, Table 6.4).

Of the five genotypes of JEV, the MRCA of GIII (node PP: 1.00) diverged first in 1890 (95% HPD: 1857, 1916) possibly in Japan (state PP: 0.57), followed by the divergence of the MRCA of GV (node PP: 1.00) in 1892 (95% HPD: 1813, 1938) possibly in Malaysia (state PP: 0.40), the MRCA of GII (node PP: 1.00) in 1909 (95% HPD: 1867, 1939) possibly in Indonesia (state PP: 0.40), the MRCA of GI (node PP: 1.00) in 1933 (95% HPD: 1908, 1957) possibly in Vietnam (state PP: 0.44) and lastly the MRCA of GIV (node PP: 1.00) in 1967 (95% HPD: 1948, 1977) possibly in Indonesia (state PP: 0.98) (Figure 6.3, Table 6.4).

The MRCA of GI-a (node PP: 0.88) diverged in 1948 (95% HPD: 1927, 1962) possibly in Thailand (state PP: 0.43), followed by the divergence of the MRCA of GI-b (node PP: 1.00) in 1959 (95% HPD: 1941, 1971) possibly in Vietnam (state PP: 0.56) (Figure 6.3, Table 6.4).

The MRCA of the recently emerged GV isolates (XZ0934 [China, 2009] and 10-1827 [South Korea, 2010]; node PP: 1.00) diverged in 1996 (95% HPD: 1982, 2006) possibly in Korea (state PP: 0.51) (Figure 6.3, Table 6.4).

The most striking observation noted from viewing the climate Bayesian MCC phylogeny (Figure 6.4) and the climate map (Figure 6.2) is that GV includes isolates sampled from temperate and tropical locations, GIV includes isolates sampled from only tropical locations, GIII includes isolates sampled primarily from temperate locations, GII includes isolates sampled primarily from tropical locations, GI-a includes isolates sampled primarily from tropical locations and GI-b includes isolates sampled primarily from temperate locations. As the node PP values and divergence dates of the MRCA of the key nodes within the country and climate Bayesian MCC phylogenies are similar (Tables 6.4 and 6.5), only the probable locations of the key nodes within the climate Bayesian MCC phylogeny will be presented here. The state PP values in support of either a tropical or temperate location of divergence of the MRCA are similar for JEV (tropical state PP: 0.53, temperate state PP: 0.47) GIV + GIII + GII + GI (tropical state PP: 0.52, temperate state PP: 0.48) and GIII + GII + GI (tropical state PP: 0.47, temperate state PP: 0.53) and therefore the most probable climates of divergence could not be determined for these nodes (Figure 6.4, Table 6.5). However, the MRCA of GII + GI most likely diverged in tropical Asia (state PP: 0.73), followed by the divergence of the MRCA of GIII most likely in temperate Asia (state PP: 0.97), the MRCA of GV most likely in tropical Asia (state PP: 0.65), the MRCA of GII most likely in tropical Asia (state PP: 0.77), the MRCA of GI most likely in tropical Asia (state PP: 0.87), the MRCA of GI-a most likely in tropical Asia (state PP: 0.97), the MRCA of GI-b most likely in tropical Asia (state PP: 0.67), the MRCA of GIV most likely in tropical Asia (state PP: 0.99), and lastly the MRCA of the recently emerged GV isolates (XZ0934 [China, 2009] and 10-

1827 [South Korea, 2010]) (95% HPD: 1986, 2007) most likely in temperate Asia (state PP: 0.99) (Figure 6.4, Table 6.5).

6.3.5 Relationship between genotype and climate

To evaluate the statistical significance of the relationship between genotype and climate that was observed in the climate Bayesian MCC phylogeny (Figure 6.4) and climate map (Figure 6.2) for GIII, GII, GI-a and GI-b, the sequences within the JEV dataset were categorized according to genotype and climate (Table 6.6). A Fisher's exact test was used to test the null hypothesis of no association between genotype and climate. Based on $\alpha = 0.05$, the null hypothesis was rejected and it was concluded that there was a statistically significant relationship between genotype and climate (Fischer's exact test: 173.48; Exact two-sided p-value: < 0.00). Using a critical z-value = ±1.96 for $\alpha = 0.05$, post-hoc analysis revealed that GIII included significantly more isolates sampled from temperate climates than expected (adjusted standardized residual: 4.0), GII included significantly more isolates sampled from tropical climates than expected (adjusted standardized residual: 12.4), GI-a included significantly more isolates sampled from tropical climates than expected (adjusted standardized residual: 9.3) and GI-b included significantly more isolates sampled from temperate climates than expected (adjusted standardized residual: 5.2) (Table 6.6).

6.3.6 Coding substitutions underlying the phylogenetic divergence of JEV

The GV viruses shared 37 coding substitutions (domain I: 14, domain II: 11, domain III: 9, stem-anchor region: 3; Conservative: 24, non-conservative: 13), the GIV viruses shared four coding substitutions (domain I: K38R [conservative], domain III: Q327L [non-conservative], SA region: A466V [conservative] and L482M [conservative]), the GIII viruses shared one coding substitution (domain III: L327S [non-

conservative]), the GII viruses shared no coding substitutions and the GI viruses shared one coding substitution (domain I: T129M [non-conservative]), and the GI-b viruses shared one coding substitution (domain I: I141V [conservative]) (Table 6.7, Figure 6.7). Viruses of GIII + GII + GI shared three coding substitutions (domain I: I56S [conservative] and I169V [conservative], SA region: I473V [conservative]), viruses of GII + GI shared one coding substitution (domain III: S327T [conservative]) and two amino acid sites (domain I: Site 222 [non-conservative], domain III: Site 366 [non-conservative]) were found to toggle between serine and asparagine residues throughout the evolutionary history of JEV (Table 6.7, Figure 6.8).

6.3.7 Genetic comparison of the three GV sequences

Pairwise comparisons of both E-gene and -protein sequences were computed to determine the genetic relatedness of the three GV isolates. Based on nucleotide composition, the Muar isolate (Malaysia, 1952) is 9.8% divergent from the XZ0934 (China, 2009) isolate and 10.1% divergent from the 10-1827 isolate (South Korea, 2010), and the XZ0934 and 10-1827 isolates are 2.7% divergent from each other. Based on amino acid composition, the Muar isolate is 1.2% divergent from both the XZ0934 and 10-1827 isolates, and the XZ0934 and 10-1827 isolates are 0.4% divergent from each other.

6.4 DISCUSSION

In recent years, members of the JEV serocomplex have emerged and established in previously unoccupied geographic regions. West Nile virus (WNV) was recognized for the first time in North America in 1999 when it caused an epidemic of encephalitis in New York City and viruses isolated from this epidemic were most likely of African origin (May *et al.*, 2011). Since 1999, the virus has spread and established itself

throughout most of the continental United States. Previously restricted to Sub-Saharan Africa, Usutu virus (USUV) emerged in Austria in 2001 causing a massive blackbird die-off (Weissenbock *et al*., 2002) and the virus has since been detected in several European counties. Japanese encephalitis virus has continued to expand its geographical boarders of activity, reaching east into Saipan in 1990 (Paul *et al*., 1993), west into Pakistan in 1992 (Igarashi *et al*., 1994), and south into the Torres Strait of Australia in 1995 (Hanna *et al*., 1996). In addition to the detection of JEV in historically naïve geographic regions in recent years, the molecular epidemiology of the virus has changed as evidenced by the displacement of GIII by GI throughout most of Asia (Chen *et al*., 2011; Ma *et al*., 2003; Nam *et al*., 1996; Nga *et al*., 2004; Nitatpattana *et al*., 2008; Tsuchie *et al*., 1997; Wang *et al*., 2007) and the emergence of GV after approximately 60 years of undetected viral circulation (Li *et al*., 2011a; Takhampunya *et al*., 2011). Although general phylogenetic and evolutionary studies had considered the time and place of divergence of JEV from its ancestral virus (limited by the small number of viral sequences) (Mohammed *et al*., 2011; Pan *et al*., 2011; Solomon *et al*., 2003), no studies had utilized a comprehensive viral sequence dataset to examine the phylogeography and epidemiology of this significant neurotropic virus and its genotypes. Therefore in this study, the largest JEV sequence dataset compiled to date, including 487 E gene sequences sampled from 12 countries over 75 years, was utilized to investigate the origins, history, dispersal and epidemiology of the viral genotypes on two spatial levels (country and climate). Additionally, the specific evolutionary events leading to the current geographical distribution of the virus genotypes were examined.

6.4.1 The E gene of JEV is a robust evolutionary proxy of the ORF

Ideally, ORF viral sequence data would have been utilized in this study. However, for JEV these are only sparsely available and are not spatiotemporally distributed. For example, a recently published evolutionary analyses that erroneously estimated that JEV diverged from it ancestral virus in 300 used a dataset consisting of 98 JEV genomic sequences, 67 of which were sampled from China (GIV: 1 sequence [1981; Indonesia], GIII: 54 sequences [1935-2004; China, India, Japan and Taiwan], GII: 1 sequence [1995; Australia], GI: 42 sequences [1977-2009; China, Japan and Korea]) (Pan *et al*., 2011). However, it was demonstrated here that the E gene is a good evolutionary proxy of the ORF as the evolutionary rates estimated in this study using the E gene JEV sequences (country and climate datasets) approximated the evolutionary rate previously estimated using a sequence alignment derived from the ORF of 35 JEV isolates (Mohammed *et al*., 2011). Further, the use of densely sampled JEV E gene sequence data allowed for the performance of a robust phylogeographic analyses that provided reliable insights into the geographic distribution, origins, spread and epidemiology of the virus genotypes.

6.4.2 JEV evolved around 1500 in the Indonesia-Malaysia region

In accordance with previous analyses, it was estimated here that the MRCA of JEV diverged around 1500 (Mohammed *et al*., 2011) most likely in the Indonesia-Malaysia region (Solomon *et al*., 2003). Although the sum of the PP values supporting the divergence of the MRCA of JEV in the Indonesia-Malaysia region was only 0.38, the highest PP value in support of the MRCA having diverged from another geographical location was 0.10 (Korea). As emphasized previously, all virus genotypes have been found in the Indonesia-Malaysia region and large epidemics suggestive of JEV have never been reported to occur in this region (Solomon *et al*., 2003). These lines of evidence are consistent with the virus having evolved in the Indonesia-Malaysia region

(Solomon *et al*., 2003). Interestingly, based on the results of an amino acid signature analysis, others have suggested that Asian JEV and Australian Murray Valley encephalitis virus (MVEV) may have evolved from a virus related to the African USUV in the Southeast Asia-Australasia region (Bakonyi *et al*., 2004).

6.4.3 Order of genotype divergence

The order of divergence of the five genotypes inferred here using a dataset of 487 E gene sequences (order: JEV, GIII, GV, GII, GI and GIV) differed from that previously inferred using a relatively sparse dataset of 35 ORF sequences (order: JEV, GIV, GIII, GII and GI) (Mohammed *et al*., 2011). This previous JEV evolutionary study was unable to infer true divergence dates for the MRCA of GII, GIV and GV as the dataset only included one isolate for each of these genotypes (Mohammed *et al*., 2011). Rather, the divergence dates reported for the MRCA of GII (1821) and GIV (1736) are indicative of the time of divergence of a single isolate, not a viral genotype (Mohammed *et al*., 2011). The mean divergence dates of the MRCA of GIII (mean: 1890; 95% HPD: 1857, 1916) and GI (mean: 1933; 95% HPD: 1908, 1957) calculated here are in agreement with those previously calculated using the ORF dataset (GIII: 1851, GI: 1900) (Mohammed *et al*., 2011). However, 95% HPD values for the divergence dates of the MRCA of the genotypes were omitted from the previous study, thus precluding appropriate comparisons between the two studies (Mohammed *et al*., 2011).

6.4.4 Genotype III is significantly associated with temperate climates

Phylogeographic analyses estimated that the MRCA of GIII diverged in 1890 in temperate Asia (most likely in Japan). The 95% HPD values surrounding the estimated date of the MRCA include 1871, which is the date that epidemics of summer encephalitis suggestive of JE were first recorded in Japan (Lewis *et al*., 1947). Following the isolation

of the prototype Nakayama strain of JEV (a GIII virus) from Japan in 1935, GIII has been found throughout most of Asia including China, India, Indonesia, Korea, Japan, Sri Lanka, Taiwan and Vietnam.

The climate Bayesian MCC phylogeny and map revealed that GIII was primarily comprised of isolates sampled from temperate regions, and statistical analyses indicated that GIII did indeed include significantly more temperate isolates than expected under the null hypothesis of no relationship between genotype and climate (i.e., under the null hypothesis GIII consists of the same number of tropical and temperate isolates). The paucity of GIII viruses sampled from tropical regions and the genetic relatedness of GIII viruses sampled years apart suggests that the annual re-introduction of GIII viruses from tropical regions to temperate regions by migratory birds or wind-blow mosquitoes does not seem to play a large role in the epidemiology of GIII. Rather, GIII is most likely maintained year-to-year by either hibernating mosquitoes, transovarial transmission in mosquitoes, poikilothermic vertebrates, and/or bats. Supporting this hypothesis, *Cx. tritaeniorhynchus* (the primary vector of JEV throughout Asia) have been reported to overwinter as adults in Japan (Nabeshima *et al*., 2009), JEV has been isolated from overwintering *Culex pipiens* in Korea (Rosen, 1986) and it has been demonstrated that *Cx. tritaeniorhynchus*, as well as other *Culex sp*., can transmit JEV following experimental hibernation (Hurlbut, 1950). Transovarial transmission of JEV to the F1 adult stage was experimentally demonstrated in *Cx. tritaeniorhynchus*, *Culex annulus*, *Culex quinquefasciatus*, and *Armigeres subalbatus* (Rosen *et al*., 1980; Rosen *et al*., 1989). Interestingly, antibody to JEV (hemagglutination inhibition assay and virus neutralization test) has been demonstrated in several poikilothermic vertebrates found in temperate Asia, including lizards (*Takydromus tachydromoides*) (Doi *et al*., 1983), snakes (*Naja naja*, *Bungarus fasciatus*, *Ptyas korros* and *Elaphe rufodorsata*) (Shortridge

et al., 1977; Shortridge *et al*., 1974), turtles (*Trionyx sinensis*) (Shortridge *et al*., 1975) and frogs (*Rana tigrina*) (Shortridge *et al*., 1977). Additionally, several lizard species (*Eumeces latiscutatus*, *Eumeces barbouri* and *Eumeces marginatus oshimensis*) have been experimentally infected with JEV (Oya *et al*., 1983), experimental hibernation studies have demonstrated that *Takydromus tachydromoides* is able to maintain JEV throughout the winter (Doi *et al*., 1983), and experimental transmission of JEV (two GIII isolates) has been shown from infected mosquitoes (*Culex pipiens fatigans* and *Culex pipiens pallens*) to uninfected lizards (*Takydromus tachydromoides* and *Eumeces latiscutatus*) and from infected lizards to mice through mosquitoes (Oya *et al*., 1983). In temperate Asia, JEV has been isolated from various species of insectivorous and frugivorous bats, including *Rousettus leschenaultii* (Wang *et al*., 2009), *Murina aurata* (Wang *et al*., 2009), *Miniopterus schreibersii fuliginosus* (Sulkin *et al*., 1970), *Rhinolophus cornutus* (Sulkin *et al*., 1970). The virus was isolated throughout the winter months from *Miniopterus schreibersii fuliginosus* and *Rhinolophus cornutus* bats in Japan, demonstrating that bats are important reservoir hosts of JEV that are able to sustain infection year-round (Sulkin *et al*., 1970). Moreover, an experimental study found that *Eptesicus fuscus and Myotis lucifigus* bats infected with JEV and then subjected to temperatures experienced during hibernation were able to maintain viremias for up to 108 days (Sulkin & Allen, 1974). From 1935 until recently, GIII was the dominant genotype of JEV throughout Asia and was the only genotype isolated from temperate Asia (with the exception of the Bennett isolate of GII) (Schuh *et al*., 2010). Therefore, it is highly likely that all of the JEV isolations made from the poikilothermic vertebrates and bats referred to above were GIII viruses and that all of the experimental infections were performed with GIII viruses. The geographical dominance of this genotype may have

been due to the unique ability of GIII viruses to infect a wide range of vertebrate and invertebrate hosts that were able to maintain the virus year-round in temperate Asia.

The phylogenetic divergence of GIII is defined by a non-conservative serine substitution at amino acid site 327 of the E protein, which is located in the exposed lateral surface of domain III, a region thought to be involved in receptor binding. The amino acid variability of the JEV genotypes at site 327 has been observed previously (Mohammed *et al*., 2011; Solomon *et al*., 2003) and it has been proposed that this substitution may have contributed to the wider dispersal of GIII by altering vector and/or host preference (Solomon *et al*., 2003).

6.4.5 Genotype V has recently re-emerged after 60 years of undetected virus circulation

The MRCA of the most ancestral of the five genotypes, GV, diverged around 1892 in tropical Asia, most likely in Malaysia. In Malaysia, JEV was first described in the 1940s when an outbreak occurred during the Second World War among British prisoners of war (Cruickshank, 1951). It is possible that GV may have circulated undetected in tropical Asia for much longer, causing only sporadic cases of encephalitis that were mistaken for cerebral malaria or other encephalitic diseases.

Surprisingly, after almost 60 years of undetected virus circulation, a pool of *Cx. tritaeniorhynchus* collected in the Tibetan Province of China in 2009 yielded the GV XZ0934 isolate (Li *et al*., 2011a) and a pool of *Culex bitaeniorhynchus* collected in South Korea in 2010 yielded the GV 10-1827 isolate (Takhampunya *et al*., 2011). The MRCA of the XZ0934 and 10-1827 isolates diverged around 1996 in temperate Asia, most likely in the Korean peninsula. Interestingly, despite surveillance neither JEV nor *Cx. tritaeniorhynchus* had ever been detected in Tibet prior to 2009 (Li *et al*., 2011b),

suggesting that GV of JEV may have been dispersed into Tibet shortly before it was initially isolated in 2009.

The three GV viruses shared 37 nonsynonymous substitutions within the E protein when compared to the other genotypes, 13 of which were non-conservative. This is consistent with the Muar strain's distinct serological classification based on its reactivity with a set of monoclonal antibodies (Kobayashi *et al*., 1984). None of the GV isolates have been characterized using polyclonal antibodies derived from other members of the JEV serocomplex. Such studies may provide interesting information regarding the antigenic relationships between this ancestral JEV genotype and other closely related viruses, such as MVEV and USUV.

The relatively high nucleotide sequence divergence between the Muar isolate and the XZ0934 and 10-1827 isolates (9.8% and 10.1%, respectively) coupled with the relatively low nucleotide sequence divergence between the XZ0934 and the 10-1827 isolates suggests that the evolution of the newly emerged GV isolates was shaped primarily by genetic drift.

6.4.6 Genotype II is significantly associated with tropical climates

Genotype II diverged from the MRCA around 1909 in tropical Asia, most likely in the Indonesia-Malaysia region. The Bennett isolate, made in Korea circa 1951 represents the only example of a GII virus collected outside of tropical Asia (Schuh *et al*., 2010). Therefore, as predicted, statistical analysis demonstrated that GII included significantly more isolates sampled from temperate regions than expected. Virus sequences derived from isolates collected north of the Tropic of Cancer (23.5°N) were classified as temperate, while isolates collected south of the Tropic of Cancer were classified as tropical. The GII viruses did not possess any genotype-defining coding

substitutions within the E protein that may explain why this genotype has circulated primarily in tropical Asia.

6.4.7 Genotype I-b, a temperate genotype, has recently displaced GIII as the dominant viral genotype of JEV throughout Asia

The MRCA of GI diverged around 1933 in tropical Asia (most likely in Vietnam), followed by the divergence of the MRCA GI-a (15 isolates collected in Cambodia, Thailand and Australia) around 1948 in tropical Asia (most likely in Thailand) and the MRCA of the GI-b (219 isolates collected between 1979 and 2009 in both tropical and temperate Asia) around 1959 in tropical Asia (most likely in Vietnam).

The climate Bayesian MCC phylogeny and map revealed that all of the GI-a isolates were collected in tropical Asia and the vast majority of the GI-b isolates were collected in temperate Asia. As anticipated, statistical analysis demonstrated that GI-a did include significantly more isolates sampled from tropical regions than expected and GI-b included significantly more isolates sampled from temperate regions than expected. Like GIII, GI-b may be maintained in temperate Asia throughout the winter months in hibernating mosquitoes, transovarial transmission in mosquitoes, poikilothermic vertebrates, and/or bats. This suggests that the spread and establishment of GI-b throughout Asia may have been due to its ability to efficiently overwinter in temperate Asia.

The phylogenetic divergence of GI is defined by a non-conservative methionine substitution at amino acid site 129 of the E protein (domain I) and the phylogenetic divergence of GI-b is defined by a conservative valine substitution at residue 141 of the E protein (domain I). This substitution, and/or other substitutions found elsewhere in the genome may have provided a phenotypic advantage to GI-b viruses that led to the spread and establishment of this genotype throughout Asia, and the subsequent displacement of

GIII as the dominant JEV genotype. Unfortunately, no genomic sequences of GI-a viruses are available to compare with the published GI-b genomic sequences.

6.4.8 Genotype IV is geographically confined to Indonesia

Although GIV is one of the more ancestral genotypes, the MRCA of GIV diverged last around 1967 in tropical Asia, most likely in Indonesia. Genotype IV includes seven isolates (only three of these include E gene sequence information) collected from mosquitoes only on three islands encompassing the Indonesian archipelago between 1980 and 1981. The reasons why GIV is confined to Indonesia are unknown, but could be due to a narrow host/vector range for GIV, the vector competence of *Cx. tritaeniorhynchus* for GIV may be low, the primary vector of GIV may be a mosquito that is confined to Indonesia, the replicative ability of GIV in birds may be low, and/or the GIV transmission cycle may involve a non-migratory amplifying host.

6.4.9 Conclusions

By applying Bayesian phylogeographic and categorical data analysis techniques to a large JEV sequence dataset it was demonstrated here that GIII and GI-b are temperate genotypes maintained year-round in northern latitudes likely by either hibernating mosquitoes, transovarial transmission in mosquitoes, poikilothermic vertebrates and/or bats, while GI-a and GII are tropical genotypes likely maintained via mosquito-bird and/or mosquito-swine transmission cycles. This suggests that the spread and establishment of GI-b throughout Asia may have been due to its ability to efficiently overwinter in temperate Asia. Further, this study identified genotype-defining amino acids within the E protein that may have played a critical role in the adaptation of these viral genotypes to their respective environments. As highlighted by the recent emergence of WNV into the western hemisphere and USUV into the European continent, the

invasion of JEV into previously unoccupied regions is a real threat. Many areas of the world have JEV-competent vectors and ardeid birds, and unlike WNV and USUV, JEV also utilizes domestic swine as amplifying hosts, which can drive epidemics by producing an abundance of infected mosquitoes. The ability of the newly emerged GI-b virus to be maintained year-round in temperate climates suggests that if this virus genotype were introduced into JEV-naïve regions it would likely become established. Therefore, to predict what geographic areas at the greatest risk of a JEV incursion it is imperative to obtain a further understanding of the host and vector range of GI-b.

Table 6.1: Detected recombination events. .

Recombinant	Minor parent	Major parent	Nucleotide breakpoint positions	Algorithm	p-value
K82P01	JaNAr32-04	JE-82	768-1095	RDP	< 0.00
				GENECONV	< 0.00
				Bootscan	< 0.00
				Maxchi	< 0.00
				Chimera	< 0.00
K91P55	K87P39	JaNAr32-04	1-298	RDP	< 0.00
				GENECONV	< 0.00
				Bootscan	< 0.00
				Maxchi	> 0.05
				Chimera	< 0.00

Table 6.2: Details of the JEV sequences examined in this study.

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
M859/Cambodia/1967/Mosquito	Cambodia	Tropical	1967	Mosquito	GI-a	
KE-93-83	Thailand	Tropical	1983	Mosquito	GI-a	
TS00	Badu Island, Australia	Tropical	2000	Swine	GI-a	EF434785
JE_RT_36	Ratchaburi, Thailand	Tropical	2003	Mosquito	GI-a	DQ087975
JE_CP_49	Chumphon, Thailand	Tropical	2004	Swine	GI-a	DQ087974
JE_CP_67	Chumphon, Thailand	Tropical	2004	Swine	GI-a	DQ087972
JE_KK_80	Khon Khen, Thailand	Tropical	2004	Unknown	GI-a	DQ111784
JE_KK_82	Khon Khen, Thailand	Tropical	2004	Unknown	GI-a	DQ111785
JE_KK_R83	Khon Khen, Thailand	Tropical	2004	Unknown	GI-a	DQ111787
JE_KK_R87	Khon Khen, Thailand	Tropical	2004	Unknown	GI-a	DQ111788
JE_KK_R88	Khon Khen, Thailand	Tropical	2004	Unknown	GI-a	DQ111786
JE_PK52	Phuket, Thailand	Tropical	2004	Unknown	GI-a	DQ84229
JE_CM_1196	Chiang Mai, Thailand	Tropical	2005	Unknown	GI-a	DQ238602
JE_KK_577	Khon Khen, Thailand	Tropical	2005	Unknown	GI-a	DQ238601
JE_KK_580	Khon Khen, Thailand	Tropical	2005	Unknown	GI-a	DQ238600
YN79-Bao83	Yunnan, China	Temperate	1979	Mosquito	GI-b	DQ404128
YN82-BN8219	Yunnan, China	Temperate	1982	Mosquito	GI-b	DQ404129
YN83-83199	Yunnan, China	Temperate	1983	Mosquito	GI-b	DQ404131
YN83-Meng83-54	Yunnan, China	Temperate	1983	Midge	GI-b	DQ404130
YN85-L86-99	Yunnan, China	Temperate	1985	Mosquito	GI-b	DQ404132
YN86-86266	Yunnan, China	Temperate	1986	Unknown	GI-b	DQ404134
YN86-B8639	Yunnan, China	Temperate	1986	Mosquito	GI-b	DQ404133
JE-91	Korea	Temperate	1991	Mosquito	GI-b	GQ415355

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
K93A07	South Korea	Temperate	1993	Mosquito	GI-b	FJ938230
K94P05	Korea	Temperate	1994	Mosquito	GI-b	AF045551
K94A07	South Korea	Temperate	1994	Mosquito	GI-b	FJ938216
JaTAn 1/94	Tokyo, Japan	Temperate	1994	Swine	GI-b	AB237171
95-167	Japan	Temperate	1995	Swine	GI-b	AY377579
95-91	Japan	Temperate	1995	Swine	GI-b	AY377578
95P99	Oita, Japan	Temperate	1995	Swine	GI-b	FJ943471
K95A07	South Korea	Temperate	1995	Mosquito	GI-b	FJ938218
K96A07	South Korea	Temperate	1996	Mosquito	GI-b	FJ938219
97P82	Oita, Japan	Temperate	1997	Swine	GI-b	FJ943472
Ishikawa	Ishikawa, Japan	Temperate	1998	Mosquito	GI-b	AB051292
JEV/wb/Okinawa/1/1998	Okinawa, Japan	Temperate	1998	Swine	GI-b	AB306941
99P103	Oita, Japan	Temperate	1999	Swine	GI-b	FJ943473
99P104	Oita, Japan	Temperate	1999	Swine	GI-b	FJ943474
KV1899	South Korea	Temperate	1999	Swine	GI-b	AF474075
SH-101	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY555761
SH-53	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY555757
SH-80	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY243841
SH-81	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY555758
SH-83	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY555759
SH-90	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY243835
SH-96	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY555760
K01-GN	South Korea	Temperate	2001	Mosquito	GI-b	FJ938220
K01-JB	South Korea	Temperate	2001	Mosquito	GI-b	FJ938221
K01-JN	South Korea	Temperate	2001	Mosquito	GI-b	FJ938222

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
VN88	Vietnam	Tropical	2001	Swine	GI-b	AY376464
JEV/sw/Chiba/88/2002	Chiba, Japan	Temperate	2002	Swine	GI-b	AB112705
JEV/sw/Hiroshima/25/2002	Hiroshima, Japan	Temperate	2002	Swine	GI-b	AB231465
JaNAr0102	Japan	Temperate	2002	Mosquito	GI-b	AY377577
JEV/sw/Kagawa/24/2002	Kagawa, Japan	Temperate	2002	Swine	GI-b	AB112706
JEV/sw/Kagawa/27/2002	Kagawa, Japan	Temperate	2002	Swine	GI-b	AB112707
LN02-102	Liaoning, China	Temperate	2002	Mosquito	GI-b	DQ404085
LN02-104	Liaoning, China	Temperate	2002	Mosquito	GI-b	DQ404086
JEV/sw/Mie/41/2002	Mie, Japan	Temperate	2002	Swine	GI-b	AB112709
JEV/sw/Shizuoka/33/2002	Shizuoka, Japan	Temperate	2002	Swine	GI-b	AB112703
JEV/sw/Shizuoka/39/2002	Shizuoka, Japan	Temperate	2002	Swine	GI-b	AB112704
VN105	Vietnam	Tropical	2002	Mosquito	GI-b	AY376468
VN22	Vietnam	Tropical	2002	Swine	GI-b	AY376465
VN34	Vietnam	Tropical	2002	Mosquito	GI-b	AY376466
VN78	Vietnam	Tropical	2002	Mosquito	GI-b	AY376467
03P113	Oita, Japan	Temperate	2003	Swine	GI-b	FJ943475
03P120	Oita, Japan	Temperate	2003	Swine	GI-b	FJ943476
03P126	Oita, Japan	Temperate	2003	Swine	GI-b	FJ943477
03P145	Oita, Japan	Temperate	2003	Swine	GI-b	FJ943478
03P189	Oita, Japan	Temperate	2003	Swine	GI-b	FJ943479
JEV/sw/Okinawa/285/2003	Okinawa, Japan	Temperate	2003	Swine	GI-b	AB238693
SH03-103	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404096
SH03-105	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404097
SH03-109	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404098
SH03-115	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404099

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
SH03-124	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404100
SH03-127	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404101
SH03-128	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404102
SH03-129	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404103
SH03-130	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404104
JEV/eq/Tottori/2003	Tottori, Japan	Temperate	2003	Equid	GI-b	AB213007
HN04-11	Henan, China	Temperate	2004	Mosquito	GI-b	DQ404087
HN04-21	Henan, China	Temperate	2004	Mosquito	GI-b	DQ404088
HN04-40	Henan, China	Temperate	2004	Mosquito	GI-b	DQ404089
JaNAr07-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185144
JaNAr10-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185145
JaNAr13-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185146
JaNAr31-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185150
JaNAr32-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185151
JaNAr38-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185152
JEV/sw/Kagawa/35/2004	Kagawa, Japan	Temperate	2004	Swine	GI-b	AB231464
JEV/sw/Mie/34/2004	Mie, Japan	Temperate	2004	Swine	GI-b	AB231462
JEV/sw/Mie/40/2004	Mie, Japan	Temperate	2004	Swine	GI-b	AB231463
SC04-12	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404090
SC04-15	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404091
SC04-16	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404092
SC04-17	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404093
SC04-25	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404094
SC04-27	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404095
GX0519	Guangxi, China	Temperate	2005	Mosquito	GI-b	FJ161967

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
GX0523	Guangxi, China	Temperate	2005	Mosquito	GI-b	FJ161968
GX0558	Guangxi, China	Temperate	2005	Mosquito	GI-b	FJ161969
JE_KK_1116	Khon Khen, Thailand	Tropical	2005	Unknown	GI-b	DQ343290
05P75	Oita, Japan	Temperate	2005	Swine	GI-b	FJ943480
SH05-24	Shanghai, China	Temperate	2005	Mosquito	GI-b	DQ404108
K05-GS	South Korea	Temperate	2005	Mosquito	GI-b	FJ938223
Mo/Toyama/1089c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538603
Mo/Toyama/1089v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538604
Mo/Toyama/1148c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538605
Mo/Toyama/1148v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538606
Mo/Toyama/1149c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538607
Mo/Toyama/1155c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538608
Mo/Toyama/1155v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538609
Mo/Toyama/1157c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538610
Mo/Toyama/1158c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538611
Mo/Toyama/1158v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538612
Mo/Toyama/1160c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538613
Mo/Toyama/1161c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538614
Mo/Toyama/1161v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538615
Mo/Toyama/1222c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538616
Mo/Toyama/1222v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538617
Mo/Toyama/1256c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538618
Mo/Toyama/1256v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538619
Sw/Toyama/05197v/2005	Toyama, Japan	Temperate	2005	Swine	GI-b	AB538823
Sw/Toyama/05231v/2005	Toyama, Japan	Temperate	2005	Swine	GI-b	AB538824

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
Mo/Toyama/1018c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538601
Mo/Toyama/1018v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538602
CT-MO-P7	Vietnam	Tropical	2005	Swine	GI-b	HQ009266
LA_H06-05	Vietnam	Tropical	2005	Mosquito	GI-b	FJ185153
LA_H07-05	Vietnam	Tropical	2005	Mosquito	GI-b	FJ185154
LA-H-5330	Vietnam	Tropical	2005	Swine	GI-b	HQ009265
LAH_2079-05	Vietnam	Tropical	2005	Mosquito	GI-b	FJ185155
06P152	Oita, Japan	Temperate	2006	Swine	GI-b	FJ943481
06P183	Oita, Japan	Temperate	2006	Swine	GI-b	FJ943483
06P212	Oita, Japan	Temperate	2006	Swine	GI-b	FJ943484
HEN0701	Henan, China	Temperate	2007	Swine	GI-b	FJ156730
JaNAr06-07	Isahaya, Nagasaki Prefecture, Japan	Temperate	2007	Mosquito	GI-b	FJ185143
JaNAr14-07	Isahaya, Nagasaki Prefecture, Japan	Temperate	2007	Mosquito	GI-b	FJ185147
JaNAr15-07	Isahaya, Nagasaki Prefecture, Japan	Temperate	2007	Mosquito	GI-b	FJ185148
JaNAr17-07	Isahaya, Nagasaki Prefecture, Japan	Temperate	2007	Mosquito	GI-b	FJ185149
07P127	Oita, Japan	Temperate	2007	Swine	GI-b	FJ943487
07P83	Oita, Japan	Temperate	2007	Swine	GI-b	FJ943485
07P90	Oita, Japan	Temperate	2007	Swine	GI-b	FJ943486
Mo/Toyama/2347c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538658
Mo/Toyama/2441c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538659
Mo/Toyama/2462c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538660
Mo/Toyama/2506c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538661
Mo/Toyama/2507c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538662
Mo/Toyama/2513c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538663
Mo/Toyama/2513v/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538664

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
Mo/Toyama/2554c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538665
Mo/Toyama/2554v/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538666
Mo/Toyama/2556c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538667
Mo/Toyama/2556v/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538668
Mo/Toyama/2567c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538669
Mo/Toyama/2569c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538670
Mo/Toyama/2569v/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538671
Sw/Toyama/07232c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538825
Sw/Toyama/07234c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538826
Sw/Toyama/07240c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538827
Sw/Toyama/07292c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538828
Sw/Toyama/07292v/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538829
Sw/Toyama/07296c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538830
Sw/Toyama/07326c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538831
07VN310	Vietnam	Tropical	2007	Mosquito	GI-b	HM228922
07VN311	Vietnam	Tropical	2007	Mosquito	GI-b	HM228923
XJ69	Zhejiang, China	Temperate	2007	Mosquito	GI-b	EU258742
XJP613	Zhejiang, China	Temperate	2007	Mosquito	GI-b	EU258741
Japanese wild boar	Hyogo, Nishinomiya Prefecture, Japan	Temperate	2008	Swine	GI-b	AB481224
08P37	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943488
08P38	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943489
08P42	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943490
08P48	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943491
08P49	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943492
08P54	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943493

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
08P62	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943494
JEV/sw/Okinawa/154/2008	Okinawa, Japan	Temperate	2008	Swine	GI-b	AB471666
JEV/sw/Okinawa/254/2008	Okinawa, Japan	Temperate	2008	Swine	GI-b	AB471667
JEV/sw/Okinawa/372/2008	Okinawa, Japan	Temperate	2008	Swine	GI-b	AB471668
JEV/sw/Okinawa/377/2008	Okinawa, Japan	Temperate	2008	Swine	GI-b	AB471669
JEV/sw/Okinawa/402/2008	Okinawa, Japan	Temperate	2008	Swine	GI-b	AB471670
TPC0806c	Taipei County, Taiwan	Temperate	2008	Mosquito	GI-b	GQ260635
Mo/Toyama/2757c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538700
Mo/Toyama/2759c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538701
Mo/Toyama/2794c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538702
Mo/Toyama/2794v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538703
Mo/Toyama/2795c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538704
Mo/Toyama/2795v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538705
Mo/Toyama/2805c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538706
Mo/Toyama/2805v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538707
Mo/Toyama/2808c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538708
Mo/Toyama/2821c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538709
Mo/Toyama/2842c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538710
Mo/Toyama/2842v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538711
Mo/Toyama/2853c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538712
Mo/Toyama/2853v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538713
Mo/Toyama/2886c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538714
Mo/Toyama/2886v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538715
Mo/Toyama/2888c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538716
Mo/Toyama/2895c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538717

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
Mo/Toyama/2905c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538718
Mo/Toyama/2906c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538719
Mo/Toyama/2909c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538720
Mo/Toyama/2910c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538721
Mo/Toyama/2915c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538722
Mo/Toyama/2917c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538723
Mo/Toyama/2918c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538724
Mo/Toyama/2929c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538725
Mo/Toyama/2929v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538726
Mo/Toyama/2967c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538727
Mo/Toyama/2967v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538728
Mo/Toyama/2976c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538729
Mo/Toyama/2976v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538730
Mo/Toyama/2977c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538731
Mo/Toyama/2977v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538732
Mo/Toyama/2984c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538733
Mo/Toyama/2984v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538734
Mo/Toyama/2985c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538735
Mo/Toyama/2985v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538736
Mo/Toyama/2986c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538737
Mo/Toyama/2986v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538738
Mo/Toyama/2987c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538739
Mo/Toyama/2987v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538740
Sw/Toyama/08253c/2008	Toyama, Japan	Temperate	2008	Swine	GI-b	AB538832
YL0806f	Yilan County, Taiwan	Temperate	2008	Mosquito	GI-b	GQ260633

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
FQ24M-08	Yunnan, China	Temperate	2008	Mosquito	GI-b	HM204531
XP174M-08	Yunnan, China	Temperate	2008	Mosquito	GI-b	HM204527
09P123	Oita, Japan	Temperate	2009	Swine	GI-b	GU108334
09P141	Oita, Japan	Temperate	2009	Swine	GI-b	GU108335
LY5P-09	Shanxi, China	Temperate	2009	Human	GI-b	HM204530
JEV-CZ1	Sichuan, China	Temperate	2009	Mosquito	GI-b	HM234673
Mo/Toyama/3133c/2009	Toyama, Japan	Temperate	2009	Mosquito	GI-b	AB543738
Mo/Toyama/3140c/2009	Toyama, Japan	Temperate	2009	Mosquito	GI-b	AB543739
Mo/Toyama/3141c/2009	Toyama, Japan	Temperate	2009	Mosquito	GI-b	AB543740
LX10P-09	Yunnan, China	Temperate	2009	Human	GI-b	HM204528
LX29P-09	Yunnan, China	Temperate	2009	Human	GI-b	HM204529
Bennett	Korea	Temperate	1951	Human	GII	FJ872376
WTP-70-22	Malaysia	Tropical	1970	Mosquito	GII	HQ223286
DjAr703	West Java, Indonesia	Tropical	1974	Mosquito	GII	
JKT654	Indonesia	Tropical	1978	Mosquito	GII	HQ223287
JKT1724	Indonesia	Tropical	1979	Mosquito	GII	JQ429304
JKT1749	Indonesia	Tropical	1979	Mosquito	GII	JQ429305
JKT220507	Jakarta, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429291
JKT1110	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429288
JKT1729	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429289
JKT1754	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429290
JKT2212	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429292
JKT2303	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429295
JKT2329	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429298
JKT2352	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429299

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
JKT2362	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429296
JKT2380	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429297
JKT4312	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429300
JKT4331	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429301
JKT4332	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429302
JKT811	Kapuk, Java, Indonesia	Tropical	1979	Mosquito	GII	JQ429303
JKT2254	Lombok, Indonesia	Tropical	1979	Mosquito	GII	JQ429293
JKT2267	Lombok, Indonesia	Tropical	1979	Mosquito	GII	JQ429294
JKT5441	Indonesia	Tropical	1980	Mosquito	GII	JQ429306
FU	Australia	Tropical	1995	Human	GII	L43565
M15	Australia	Tropical	1995	Unknown	GII	L47439
M40	Australia	Tropical	1995	Unknown	GII	L47350
NO	Australia	Tropical	1995	Unknown	GII	L43566
CNS138-11	Malaysia	Tropical	1999	Human	GII	AY184213
Nakayama	Nakayama, Japan	Temperate	1935	Human	GIII	EF571853
Matsunaga	Japan	Temperate	1939	Human	GIII	FJ872381
Roum	Korea	Temperate	1946	Human	GIII	FJ872377
Taira	Japan	Temperate	1948	Human	GIII	FJ872384
Beijing-1	Beijing, China	Temperate	1949	Mosquito	GIII	L48961
Equine	Japan	Temperate	1949	Equid	GIII	FJ872378
K-29	Korea	Temperate	1949	Human	GIII	GQ415356
V9-3901	Japan	Temperate	1950	Human	GIII	FJ872382
V9-3902	Japan	Temperate	1950	Human	GIII	FJ872383
V9-4399	Japan	Temperate	1950	Human	GIII	FJ872380
Korea Jap B	Korea	Temperate	1950	Human	GIII	FJ872379

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
JaOArK151	Oita Prefecture, Japan	Temperate	1951	Unknown	GIII	AB028255
SA14	China	Temperate	1954	Mosquito	GIII	U14163
CBH	Fujian, China	Temperate	1954	Human	GIII	DQ404116
CZX	Fujian, China	Temperate	1954	Human	GIII	AY243828
G35	Fujian, China	Temperate	1954	Mosquito	GIII	AY243831
CTS	Fujian, China	Temperate	1955	Human	GIII	AY243830
LFM	Fujian, China	Temperate	1955	Human	GIII	AY243833
YLG	Fujian, China	Temperate	1955	Human	GIII	AY243837
ZMT	Fujian, China	Temperate	1955	Human	GIII	AY243840
ZSZ	Fujian, China	Temperate	1955	Human	GIII	AY243839
LYZ	Fujian, China	Temperate	1957	Human	GIII	AY243834
CH-13	Sichuan, China	Temperate	1957	Human	GIII	AY243829
HVI	Taiwan	Unknown	1958	Mosquito	GIII	AF098735
JaTH160	Tokyo, Japan	Temperate	1960	Human	GIII	AB269326
JaGAr01	Japan	Temperate	1965	Human	GIII	U44964
Ling	Ling, Taiwan	Temperate	1965	Human	GIII	L78128
TC	Taiwan	Unknown	1965	Mosquito	GIII	AF098736
TL	Taiwan	Unknown	1965	Mosquito	GIII	AF098737
JaOH0566	Japan	Temperate	1966	Human	GIII	AY029207
Kamiyama 1	Kamiyama, Japan	Temperate	1966	Human	GIII	S47265
JaOAr404	Oita Prefecture, Japan	Temperate	1968	Unknown	GIII	AB028250
Oita100-69	Oita Prefecture, Japan	Temperate	1969	Unknown	GIII	AB028269
691004	Sri Lanka	Tropical	1969	Human	GIII	Z34097
MIe731-70	Mie, Japan	Temperate	1970	Unknown	GIII	AB028271
JaNAr516-70	Nagasaki Prefecture, Japan	Temperate	1970	Unknown	GIII	AB028270

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
JaOAr363	Oita Prefecture, Japan	Temperate	1970	Unknown	GIII	AB028252
Tla	Heilongjiang, China	Temperate	1971	Human	GIII	AY243826
JaOAr72	Okinawa, Japan	Temperate	1972	Mosquito	GIII	AB569990
733913	Bankura, West Bengal, India	Tropical	1973	Human	GIII	EU372660
JaOAr73050	Okinawa, Japan	Temperate	1973	Swine	GIII	AB569987
JaOAr73055	Okinawa, Japan	Temperate	1973	Swine	GIII	AB569988
JaOAr73062	Okinawa, Japan	Temperate	1973	Swine	GIII	AB569989
JaOS73620	Okinawa, Japan	Temperate	1973	Swine	GIII	AB569978
JaOS73832	Okinawa, Japan	Temperate	1973	Swine	GIII	AB569979
JaOAr74010	Okinawa, Japan	Temperate	1974	Mosquito	GIII	AB569994
JaOAr74011	Okinawa, Japan	Temperate	1974	Mosquito	GIII	AB569995
JaOS74728	Okinawa, Japan	Temperate	1974	Swine	GIII	AB569992
JaOS74729	Okinawa, Japan	Temperate	1974	Swine	GIII	AB569993
JaOS74801	Okinawa, Japan	Temperate	1974	Swine	GIII	AB569996
JaOS75571	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569980
JaOS75642	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569981
JaOS75672	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569982
JaOS75722	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569983
JaOS75770	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569984
JaOS75833	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569985
JaOS75918	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569986
JaOAr76075	Okinawa, Japan	Temperate	1976	Mosquito	GIII	AB569991
GP78	Gorakhpur, India	Temperate	1978	Mosquito	GIII	AF075723
Liyujie	Yunnan, China	Temperate	1979	Human	GIII	FJ185039
80P136	Japan	Temperate	1980	Swine	GIII	FJ943462

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
80P205	Japan	Temperate	1980	Swine	GIII	FJ943463
81P241	Japan	Temperate	1981	Swine	GIII	FJ943464
81P244	Japan	Temperate	1981	Swine	GIII	FJ943465
JE-82	Korea	Temperate	1982	Mosquito	GIII	GQ415347
JaOArS982	Osaka, Japan	Temperate	1982	Mosquito	GIII	NC_001437
BN19	Yunnan, China	Temperate	1982	Mosquito	GIII	FJ185038
JE-83	Korea	Temperate	1983	Mosquito	GIII	GQ415348
cc27	Pingtun County, Taiwan	Tropical	1983	Mosquito	GIII	U44957
K83P34	South Korea	Temperate	1983	Mosquito	GIII	FJ938231
K83P44	South Korea	Temperate	1983	Mosquito	GIII	FJ938232
JE-84	Korea	Temperate	1984	Mosquito	GIII	GQ415349
CC94	Pingtun County, Taiwan	Tropical	1984	Mosquito	GIII	U44958
K84A071	South Korea	Temperate	1984	Mosquito	GIII	FJ938224
JE-85	Korea	Temperate	1985	Mosquito	GIII	GQ415350
CN80	Miaoli County, Taiwan	Temperate	1985	Mosquito	GIII	U44962
JaOArS4385	Oita Prefecture, Japan	Temperate	1985	Unknown	GIII	AB028258
JaOArS5485	Oita Prefecture, Japan	Temperate	1985	Unknown	GIII	AB028260
JaOArS7485	Oita Prefecture, Japan	Temperate	1985	Unknown	GIII	AB028259
NT109	Tiachung County, Taiwan	Temperate	1985	Mosquito	GIII	U44967
NT113	Tiachung County, Taiwan	Temperate	1985	Mosquito	GIII	U44968
CH109	Changhua County, Taiwan	Temperate	1986	Mosquito	GIII	U44959
JE-86	Korea	Temperate	1986	Mosquito	GIII	GQ415351
JaOArK3786	Oita Prefecture, Japan	Temperate	1986	Unknown	GIII	AB028261
JaOArK7286	Oita Prefecture, Japan	Temperate	1986	Unknown	GIII	AB028263
JaOArS1186	Oita Prefecture, Japan	Temperate	1986	Unknown	GIII	AB028262

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
VN206	Vietnam	Tropical	1986	Human	GIII	AY376460
VN207	Vietnam	Tropical	1986	Human	GIII	AY376461
JKT27-085	Central Java, Indonesia	Tropical	1987	Mosquito	GIII	JQ429307
JKT27-087	Central Java, Indonesia	Tropical	1987	Mosquito	GIII	JQ429308
CH392	Changhua County, Taiwan	Temperate	1987	Mosquito	GIII	U44961
JE-87	Korea	Temperate	1987	Mosquito	GIII	GQ415352
K87P39	Korea	Temperate	1987	Mosquito	GIII	U34927
SH-3	Shanghai, China	Temperate	1987	Human	GIII	AY243836
K87A07	South Korea	Temperate	1987	Mosquito	GIII	FJ938225
K87A071	South Korea	Temperate	1987	Mosquito	GIII	FJ938226
JE-88	Korea	Temperate	1988	Mosquito	GIII	GQ415353
JaOArK6688	Oita Prefecture, Japan	Temperate	1988	Unknown	GIII	AB028264
K88A07	South Korea	Temperate	1988	Mosquito	GIII	FJ938227
K88A071	South Korea	Temperate	1988	Mosquito	GIII	FJ938228
89P131	Japan	Temperate	1989	Swine	GIII	FJ943467
89P141	Japan	Temperate	1989	Swine	GIII	FJ943468
89P149	Japan	Temperate	1989	Swine	GIII	FJ943469
89P160	Japan	Temperate	1989	Swine	GIII	FJ943470
89P49	Japan	Temperate	1989	Swine	GIII	FJ943466
JE-89	Korea	Temperate	1989	Mosquito	GIII	GQ415354
JaOArK5789	Oita Prefecture, Japan	Temperate	1989	Unknown	GIII	AB028265
JaOArK6289	Oita Prefecture, Japan	Temperate	1989	Unknown	GIII	AB028266
K89A07	South Korea	Temperate	1989	Mosquito	GIII	FJ938229
VN49	Vietnam	Tropical	1989	Human	GIII	AY376462
VN50	Vietnam	Tropical	1989	Human	GIII	AY376463

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
CH1392	Changhua County, Taiwan	Temperate	1990	Mosquito	GIII	U44960
JaNAr0290	Nagasaki Prefecture, Japan	Temperate	1990	Mosquito	GIII	AY427794
JaNAr0590	Nagasaki Prefecture, Japan	Temperate	1990	Mosquito	GIII	AY427795
JaNAr0690	Nagasaki Prefecture, Japan	Temperate	1990	Mosquito	GIII	AY427796
JaNAr0990	Nagasaki Prefecture, Japan	Temperate	1990	Mosquito	GIII	AY427797
JaOArK3990	Oita Prefecture, Japan	Temperate	1990	Unknown	GIII	AB028267
JaOArK5990	Oita Prefecture, Japan	Temperate	1990	Unknown	GIII	AB028268
CH1949	Changhua, Taiwan	Temperate	1992	Unknown	GIII	AF030549
CH2195	Changhua, Taiwan	Temperate	1994	Unknown	GIII	AF030550
K94A071	South Korea	Temperate	1994	Mosquito	GIII	FJ938217
T1P1	Liu Chi Islet, Taiwan	Tropical	1997	Mosquito	GIII	AF254453
YN03-A151	Yunnan, China	Temperate	1998	Mosquito	GIII	DQ404136
14178	Lakhimpur, India	Temperate	2001	Human	GIII	EF623987
02-41	Fujian, China	Temperate	2002	Human	GIII	AY555763
02-43	Fujian, China	Temperate	2002	Human	GIII	AY555764
02-76	Fujian, China	Temperate	2002	Human	GIII	AY555765
02-84	Fujian, China	Temperate	2002	Human	GIII	AY555766
02-29	Fujian, China	Temperate	2002	Human	GIII	AY555762
02-102	Fujian, China	Temperate	2002	Human	GIII	AY555767
HLJ02-134	Heilongjiang, China	Temperate	2002	Midge	GIII	DQ404081
HLJ02-136	Heilongjiang, China	Temperate	2002	Midge	GIII	DQ404082
HLJ02-144	Heilongjiang, China	Temperate	2002	Mosquito	GIII	DQ404083
HLJ02-170	Heilongjiang, China	Temperate	2002	Mosquito	GIII	DQ404084
04940-4	Maharashtra, India	Tropical	2002	Mosquito	GIII	EF623989
TN207	Taiwan	Unknown	2002	Mosquito	GIII	EU683895

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
FJ03-31	Fujian, China	Temperate	2003	Human	GIII	DQ404117
FJ03-35	Fujian, China	Temperate	2003	Human	GIII	DQ404118
FJ03-39	Fujian, China	Temperate	2003	Human	GIII	DQ404119
FJ03-46	Fujian, China	Temperate	2003	Human	GIII	DQ404120
FJ03-56	Fujian, China	Temperate	2003	Human	GIII	DQ404121
FJ03-66	Fujian, China	Temperate	2003	Human	GIII	DQ404122
FJ03-67	Fujian, China	Temperate	2003	Human	GIII	DQ404123
FJ03-68	Fujian, China	Temperate	2003	Human	GIII	DQ404124
FJ03-69	Fujian, China	Temperate	2003	Human	GIII	DQ404125
FJ03-94	Fujian, China	Temperate	2003	Human	GIII	DQ404126
FJ03-97	Fujian, China	Temperate	2003	Human	GIII	DQ404127
GZ04-2	Guizhou, China	Temperate	2004	Mosquito	GIII	DQ404109
GZ04-4	Guizhou, China	Temperate	2004	Mosquito	GIII	DQ404110
GZ04-43	Guizhou, China	Temperate	2004	Mosquito	GIII	DQ404113
GZ04-71	Guizhou, China	Temperate	2004	Mosquito	GIII	DQ404114
GZ04-89	Guizhou, China	Temperate	2004	Mosquito	GIII	DQ404115
SH04-10	Shanghai, China	Temperate	2004	Mosquito	GIII	DQ404107
SH04-3	Shanghai, China	Temperate	2004	Mosquito	GIII	DQ404105
SH04-5	Shanghai, China	Temperate	2004	Mosquito	GIII	DQ404106
04VN75	Vietnam	Tropical	2004	Human	GIII	HQ009263
04VN79	Vietnam	Tropical	2004	Human	GIII	HQ009264
YNDL04-1	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404137
YNDL04-29	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404139
YNDL04-31	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404140
YNDL04-37	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404141

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
YNDL04-39	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404142
YNDL04-42	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404143
YNDL04-44	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404144
YNDL04-45	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404145
YNDL04-6	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404138
YNJH04-25-3	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404148
FJ05-139	Fujian, China	Temperate	2005	Human	GIII	GQ856661
FJ05-62	Fujian, China	Temperate	2005	Human	GIII	GQ856660
57434	Gorakhpur, India	Temperate	2005	Human	GIII	EF688625
HL0505a	Hualien County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260610
HL0506a	Hualien County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260615
KH0505a	Kaohsiung County, Taiwan	Tropical	2005	Mosquito	GIII	GQ260608
KH0505b	Kaohsiung County, Taiwan	Tropical	2005	Mosquito	GIII	GQ260609
TP0506a	Taipei County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260616
TC0506a	Tiachung County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260613
TC0506b	Tiachung County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260614
GP05	Uttar Pradesh, India	Temperate	2005	Human	GIII	FJ979830
YL0506a	Yilan County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260611
YL0506b	Yilan County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260612
CSF-2522	Taiwan	Unknown	2006	Human	GIII	GQ260621
TC0605a	Tiachung County, Taiwan	Temperate	2006	Mosquito	GIII	GQ260618
TC0605b	Tiachung County, Taiwan	Temperate	2006	Mosquito	GIII	GQ260619
YL0605a	Yilan County, Taiwan	Temperate	2006	Mosquito	GIII	GQ260617
YL0606a	Yilan County, Taiwan	Temperate	2006	Mosquito	GIII	GQ260620
CH0706a	Changhua County, Taiwan	Temperate	2007	Swine	GIII	GQ260624

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
FJ07-51	Fujian, China	Temperate	2007	Human	GIII	GQ856662
HL0706a	Hualien County, Taiwan	Temperate	2007	Mosquito	GIII	GQ260625
TN0705a	Tainan County, Taiwan	Tropical	2007	Swine	GIII	GQ260623
TPC0706b	Taipei City, Taiwan	Temperate	2007	Mosquito	GIII	GQ260627
TY0704a	Taoyuan County, Taiwan	Temperate	2007	Swine	GIII	GQ260622
TPC0706a	Taipei City, Taiwan	Temperate	2007	Mosquito	GIII	GQ260626
Mo/Chongmingdao/12/2008	Chongmingdao, China	Temperate	2008	Mosquito	GIII	GU253955
Mo/Deqing/11/2008	Deqing, China	Temperate	2008	Mosquito	GIII	GU253961
FJ08-48	Fujian, China	Temperate	2008	Human	GIII	GQ856663
FJ08-65	Fujian, China	Temperate	2008	Human	GIII	GQ856664
HLJ08-01	Heilongjiang, China	Temperate	2008	Swine	GIII	GQ495004
HLJ08-02	Heilongjiang, China	Temperate	2008	Swine	GIII	GQ495005
HL0805a	Hualien, Taiwan	Temperate	2008	Mosquito	GIII	GQ260628
Mo/Jintan/9/2008	Jintan, China	Temperate	2008	Mosquito	GIII	GU253954
Mo/Nanjing/10/2008	Nanjing, China	Temperate	2008	Mosquito	GIII	GU253951
TC0806a	Taichung County, Taiwan	Temperate	2008	Mosquito	GIII	GQ260630
TPC0806a	Taipei City, Taiwan	Temperate	2008	Mosquito	GIII	GQ260634
YL0805a	Yilan County, Taiwan	Temperate	2008	Mosquito	GIII	GQ260629
YL0806a	Yilan County, Taiwan	Temperate	2008	Mosquito	GIII	GQ260631
YL0806e	Yilan County, Taiwan	Temperate	2008	Mosquito	GIII	GQ260632
Mo/Haimen/3/2009	Haimen, China	Temperate	2009	Mosquito	GIII	GU253947
Mo/Haimen/6/2009	Haimen, China	Temperate	2009	Mosquito	GIII	GU253950
Mo/Qidong/4/2009	Haimen, China	Temperate	2009	Mosquito	GIII	GU253948
JEV/eq/Haryana/H225/2009	Haryana, India	Temperate	2009	Equid	GIII	GQ387646
JEV/eq/India/H225/2009	Hisar, India	Temperate	2009	Equid	GIII	HQ018880

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
Sw(blood)/Jintan/15/2009	Jintan, China	Temperate	2009	Swine	GIII	GU253953
Sw(sperm)/Ningbo/13/2009	Ningbo, China	Temperate	2009	Swine	GIII	GU253956
Mo/Qidong/5/2009	Qidong, China	Temperate	2009	Mosquito	GIII	GU253949
Sw(sperm)/Rizhao/8/2009	Rizhao, China	Temperate	2009	Swine	GIII	GU253960
Sw(blood)/Rugao/14/2009	Rugao, China	Temperate	2009	Swine	GIII	GU253957
JEV-NJ1	Sichuan, China	Temperate	2009	Mosquito	GIII	HM234674
Mo/Yixing/2/2009	Yixing, China	Temperate	2009	Mosquito	GIII	GU253959
Mo/Zhenjiang/1/2009	Zhenjiang, China	Temperate	2009	Mosquito	GIII	GU253958
JKT7089	Bantul, Java, Indonesia	Tropical	1981	Mosquito	GIV	JQ429309
JKT7180	Central Java, Indonesia	Tropical	1981	Mosquito	GIV	JQ429310
JKT6468	Flores, Indonesia	Tropical	1981	Mosquito	GIV	AY184212
Muar	Malaysia	Tropical	1952	Human	GV	HM596272
XZ0934	China	Temperate	2009	Mosquito	GV	JF915894
10-1827	South Korea	Temperate	2010	Mosquito	GV	JN587258

Table 6.3: Descriptive analysis of the JEV dataset.

		n	% of total
Genotype	GI-a	15	3.08
	GI-b	219	44.97
	GII	28	5.75
	GIII	219	44.97
	GIV	3	0.62
	GV	3	0.62
	Total	487	100.00
Host	Equid	4	0.82
	Human	67	13.76
	Midge	3	0.62
	Mosquito	282	57.91
	Swine	98	20.12
	Unknown	33	6.78
	Total	487	100.00
Decade	1930s	2	0.41
	1940s	5	1.03
	1950s	19	3.90
	1960s	11	2.26
	1970s	48	9.86
	1980s	62	12.73
	1990s	33	6.78
	2000s	307	63.04
	Total	487	100.00
Origin	Australia	5	1.03
	Cambodia	1	0.21
	China	126	25.87
	India	8	1.64
	Indonesia	26	5.34
	Japan	207	42.51
	Korea	34	6.98
	Malaysia	3	0.62
	Sri Lanka	1	0.21
	Taiwan	44	9.03
	Thailand	14	2.87
	Vietnam	18	3.70
	Total	487	100.00
Climate	Temperate	406	83.37
	Tropical	76	15.61
	Unknown	5	1.03
	Total	487	100.00

Table 6.4: Country phylogeographic analysis.

Node[1]	Group	Node PP	Date of the MRCA (95% HPD)	State PP											
				Australia	Cambodia	China	India	Indonesia	Japan	Korea	Malaysia	Sri Lanka	Taiwan	Thailand	Vietnam
1	JEV	1.00	1504 (1089, 1794)	0.08	0.05	0.07	0.03	0.18	0.09	0.10	0.19	0.05	0.03	0.06	0.07
2	GIV + GIII + GII + GI	0.60	1695 (1516, 1844)	0.06	0.03	0.08	0.02	0.21	0.11	0.14	0.16	0.04	0.02	0.05	0.08
3	GIII + GII + GI	0.94	1801 (1701, 1880)	0.04	0.02	0.13	0.01	0.14	0.18	0.20	0.10	0.02	0.01	0.04	0.11
4	GII + GI	1.00	1867 (1799, 1920)	0.07	0.02	0.05	0.00	0.22	0.07	0.24	0.12	0.02	0.00	0.05	0.14
5	GIII	1.00	1890 (1857, 1916)	0.00	0.00	0.26	0.00	0.00	0.57	0.16	0.00	0.00	0.00	0.00	0.01
6	GV	1.00	1892 (1813, 1938)	0.06	0.01	0.03	0.01	0.22	0.04	0.12	0.40	0.03	0.01	0.03	0.04
7	GII	1.00	1909 (1867, 1939)	0.05	0.01	0.01	0.00	0.40	0.03	0.18	0.22	0.02	0.00	0.01	0.07
8	GI	1.00	1933 (1908, 1957)	0.06	0.06	0.05	0.00	0.03	0.02	0.09	0.02	0.01	0.00	0.22	0.44
9	GI-a	0.88	1948 (1927, 1962)	0.08	0.18	0.01	0.00	0.01	0.00	0.03	0.02	0.01	0.00	0.43	0.23
10	GI-b	1.00	1959 (1941, 1971)	0.00	0.00	0.27	0.00	0.00	0.04	0.12	0.00	0.00	0.00	0.01	0.56
11	GIV	1.00	1967 (1948, 1977)	0.00	0.00	0.00	0.00	0.98	0.00	0.00	0.02	0.00	0.00	0.00	0.00
12	XZ0934/10-1827	1.00	1996 (1982, 2006)	0.00	0.00	0.28	0.00	0.00	0.15	0.51	0.00	0.00	0.00	0.00	0.05

[1]As indicated in the country Bayesian MCC phylogeny.

Table 6.5: Climate phylogeographic analysis.

Node[1]	Group	Node PP	Date of the MRCA (95% HPD)	State PP	
				Temperate	Tropical
1	JEV	1.00	1552 (1207, 1791)	0.47	0.53
2	GIV + GIII + GII + GI	0.56	1708 (1540, 1839)	0.48	0.52
3	GIII + GII + GI	0.96	1809 (1704, 1884)	0.53	0.47
4	GII + GI	1.00	1876 (1818, 1923)	0.27	0.73
5	GIII	1.00	1893 (1863, 1918)	0.97	0.03
6	GV	1.00	1890 (1809, 1939)	0.35	0.65
7	GII	1.00	1913 (1877, 1939)	0.23	0.77
8	GI	1.00	1939 (1916, 1956)	0.13	0.87
9	GI-a	1.00	1951 (1936, 1964)	0.03	0.97
10	GI-b	1.00	1960 (1972, 1944)	0.33	0.67
11	GIV	1.00	1965 (1942, 1978)	0.01	0.99
12	XZ0934/10-1827	1.00	1999 (1986, 2007)	0.99	0.01

[1]As indicated in the climate Bayesian MCC phylogeny.

Table 6.6: Cross-tabulation of genotype and climate.

Genotype		Climate		
		Temperate	**Tropical**	**Row total**
GI-a	Count	0	15	15
	Expected count	12.73	2.27	15.00
	% within genotype	0.00	100.00	100.00
	% within climate	0.00	20.83	3.15
	% of total	0.00	3.15	3.15
	Residual	-12.73	12.73	
	Adjusted standardized residual	-9.32	9.32	
GI-b	Count	206	13	219
	Expected count	185.87	33.13	219.00
	% within genotype	94.06	5.94	100.00
	% within climate	50.99	18.06	46.01
	% of total	43.28	2.73	46.01
	Residual	20.13	-20.13	
	Adjusted standardized residual	5.17	-5.17	
GII	Count	1	27	28
	Expected count	23.76	4.24	28.00
	% within genotype	3.57	96.43	100.00
	% within climate	0.25	37.50	5.88
	% of total	0.21	5.67	5.88
	Residual	-22.76	22.76	
	Adjusted standardized residual	-12.38	12.38	
GIII	Count	197	17	214
	Expected count	181.63	32.37	214.00
	% within genotype	92.06	7.94	100.00
	% within climate	48.76	23.61	44.96
	% of total	41.39	3.57	44.96
	Residual	15.37	-15.37	
	Adjusted standardized residual	3.95	-3.95	
Column total	Count	404	72	476
	Expected count	404.00	72.00	476.00
	% within genotype	84.87	15.13	100.00
	% within climate	100.00	100.00	100.00
	% of total	84.87	15.13	100.00

Table 6.7: Genotype-defining coding substitutions within the E protein.

Domain	Site	GV	GIV	GIII	GII	GI	GI-a	GI-b	Type
I	15	V	A	A	A	A	A	A	Conservative
	38	K	R	K	K	K	K	K	Conservative
	51	T	S	S	S	S	S	S	Conservative
	52	E	Q	Q	Q	Q	Q	Q	Non-conservative
II	58	T	S	S	S	S	S	S	Conservative
	64	T	S	S	S	S	S	S	Conservative
	66	A	T	T	T	T	T	T	Non-conservative
	83	T	E	E	E	E	E	E	Non-conservative
	84	R	K	K	K	K	K	K	Conservative
	91	Y	F	F	F	F	F	F	Conservative
	120	V	S	S	S	S	S	S	Non-conservative
I	122	S	T	T	T	T	T	T	Conservative
	123	H	S	S	S	S	S	S	Non-conservative
	128	K	R	R	R	R	R	R	Conservative
	129	I	T	T	T	M	M	M	Non-conservative
	141	V	I	I	I	I	I	V	Conservative
	149	A	S	S	S	S	S	S	Non-conservative
	156	T	T	S	S	S	S	S	Conservative
	159	I	V	V	V	V	V	V	Conservative
	169	I	I	V	V	V	V	V	Conservative
	188	M	L	L	L	L	L	L	Conservative
	196	F	L	L	L	L	L	L	Non-conservative
	204	L	M	M	M	M	M	M	Conservative
	219	N	H	H	H	H	H	H	Non-conservative
	222	S	A	A	S	S	S	S	Non-conservative
II	226	L	T	T	T	T	T	T	Non-conservative
	232	N	A	A	A	A	A	A	Non-conservative
	238	I	L	L	L	L	L	L	Conservative
	261	A	G	G	G	G	G	G	Non-conservative
III	311	S	A	A	A	A	A	A	Non-conservative
	327	Q	L	S	T	T	T	T	Non-conservative/Conservative
	329	T	S	S	S	S	S	S	Conservative
	331	T	S	S	S	S	S	S	Conservative
	340	S	V	V	V	V	V	V	Non-conservative
	348	L	M	M	M	M	M	M	Conservative
	365	T	S	S	S	S	S	S	Conservative
	366	A	S	A	S	S	S	S	Non-conservative
	374	L	M	M	M	M	M	M	Conservative
	382	F	Y	Y	Y	Y	Y	Y	Conservative
	402	S	T	T	T	T	T	T	Conservative
Stem-anchor	408	T	S	S	S	S	S	S	Conservative

Domain	Site	GV	GIV	GIII	GII	GI	GI-a	GI-b	Type
	466	A	V	A	A	A	A	A	Conservative
	473	I	I	V	V	V	V	V	Conservative
	482	L	M	L	L	L	L	L	Conservative
	492	L	V	V	V	V	V	V	Conservative

Figure 6.1: Geographical distribution of the JEV sequences included in this study according to country of collection. The coloring of the chart corresponds to the map and shows the relative proportions of viral sequences sampled from each country according to genotype. Of note, GI viruses have also been isolated in India, Laos and Malaysia, GII viruses have also been isolated in Papua New Guinea and Thailand, and GIII viruses have also been isolated in Malaysia, Myanmar, Nepal, the Philippines, the former Soviet Union and Thailand. These virus isolates were not included in this study, as the E gene of isolates from these countries have not been sequenced.

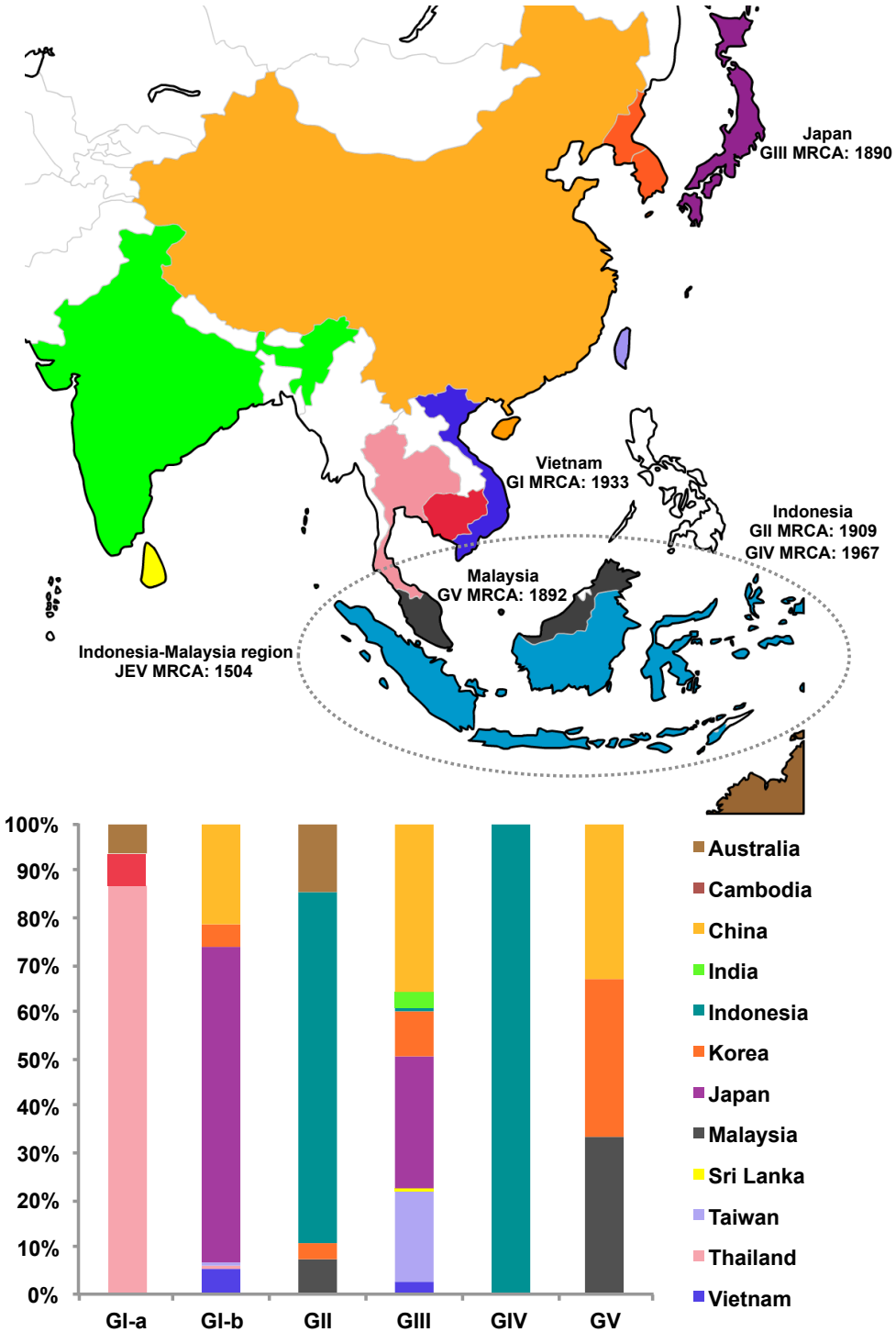
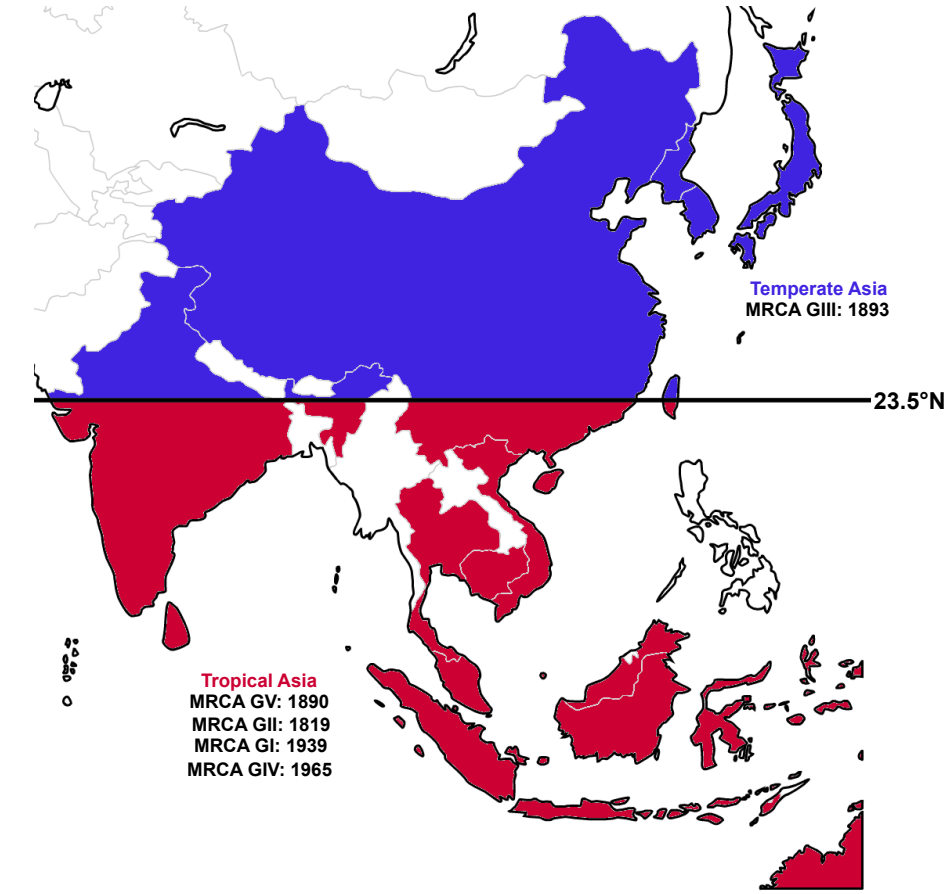

Japan
GIII MRCA: 1890
Vietnam
GI MRCA: 1933
Indonesia
GII MRCA: 1909
GIV MRCA: 1967
Malaysia
GV MRCA: 1892
Indonesia-Malaysia region
JEV MRCA: 1504
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
GI-a
GI-b
GII
GIII
GIV
GV
Australia
Cambodia
China
India
Indonesia
Korea
Japan
Malaysia
Sri Lanka
Taiwan
Thailand
Vietnam

Figure 6.2: Geographical distribution of the JEV sequences included in this study according to climate of collection. The coloring of the chart corresponds to the map and shows the relative proportions of viral sequences sampled from each climate according to genotype.


Temperate Asia
MRCA GIII: 1893
23.5°N
Tropical Asia
MRCA GV: 1890
MRCA GII: 1819
MRCA GI: 1939
MRCA GIV: 1965

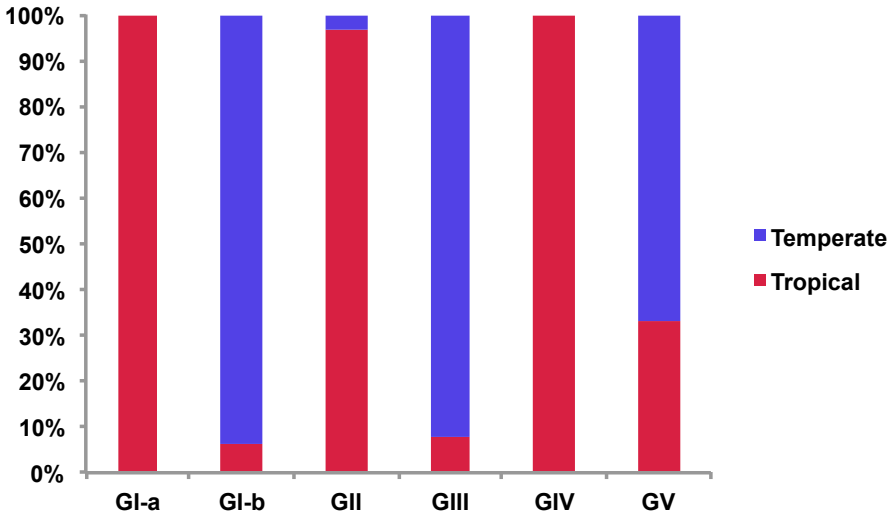

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
GI-a
GI-b
GII
GIII
GIV
GV
Temperate
Tropical

Figure 6.3: Country Bayesian MCC phylogeny of the JEV isolates. GI-V are represented to the right of the tree. Branch tips correspond to the date of collection of each of the virus isolates. Branch lengths correspond to lengths of time, as measured by the scale underneath the tree. Terminal branches are colored according to the sampling location of the taxon at the tip, while internal braches are colored according to the most probable location of their parental node. The branch colors correspond to those used in the map and legend. The numbers at the nodes correspond to the country phylogeographic analysis data presented in Table 6.4.

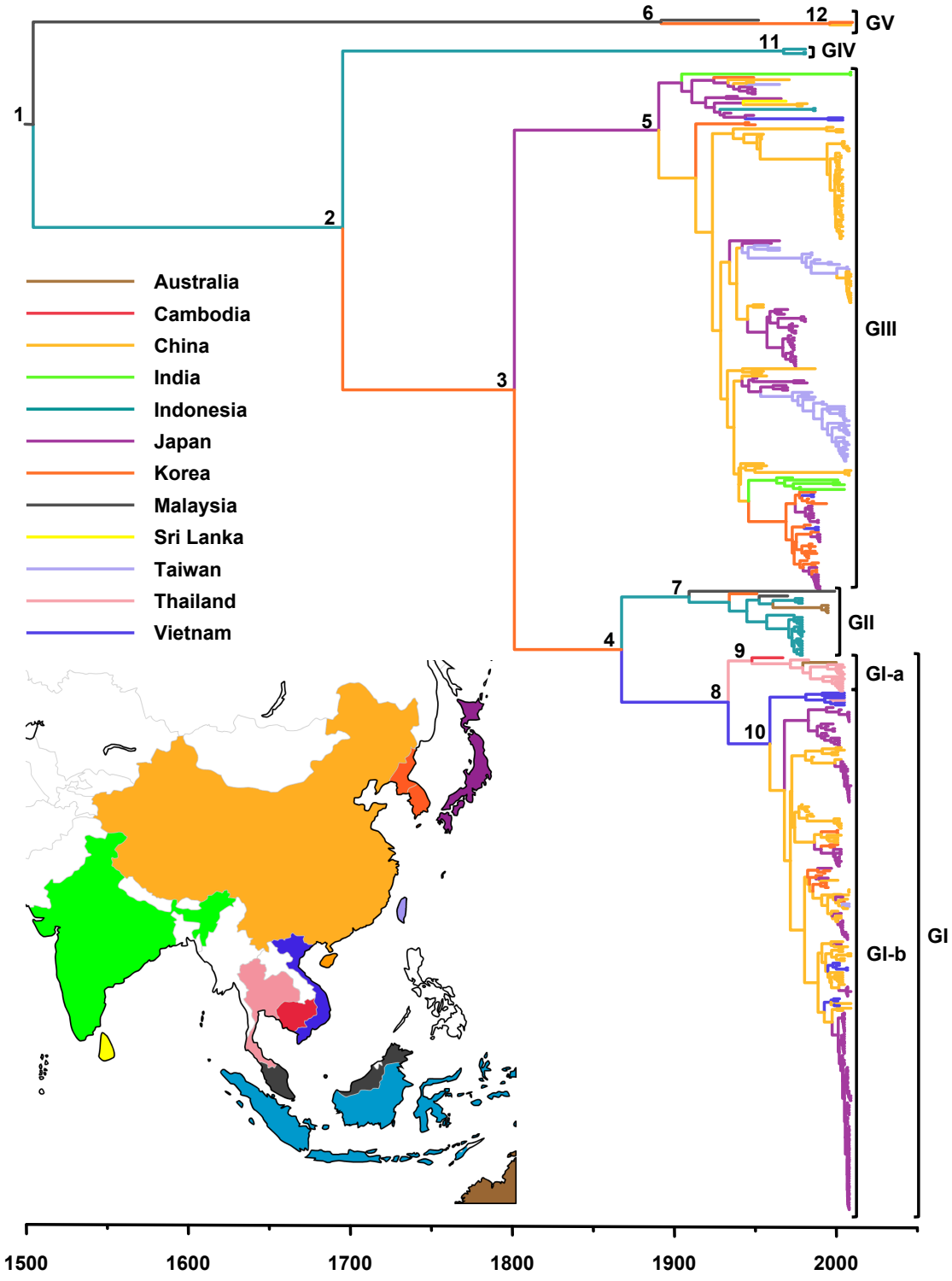

Australia
Cambodia
China
India
Indonesia
Japan
Korea
Malaysia
Sri Lanka
Taiwan
Thailand
Vietnam
1
2
3
4
5
6
7
8
9
10
11
12
GV
GIV
GIII
GII
GI-a
GI-b
GI
1500
1600
1700
1800
1900
2000

Figure 6.4: Climate Bayesian MCC phylogeny of the JEV isolates. GI-V are represented to the right of the tree. Branch tips correspond to the date of collection of each of the virus isolates. Branch lengths correspond to lengths of time, as measured by the scale underneath the tree. Terminal branches are colored according to the sampling location of the taxon at the tip, while internal braches are colored according to the most probable location of their parental node. The branch colors correspond to those used in the map and legend. The numbers at the nodes correspond to the climate phylogeographic analysis data presented in Table 6.5.

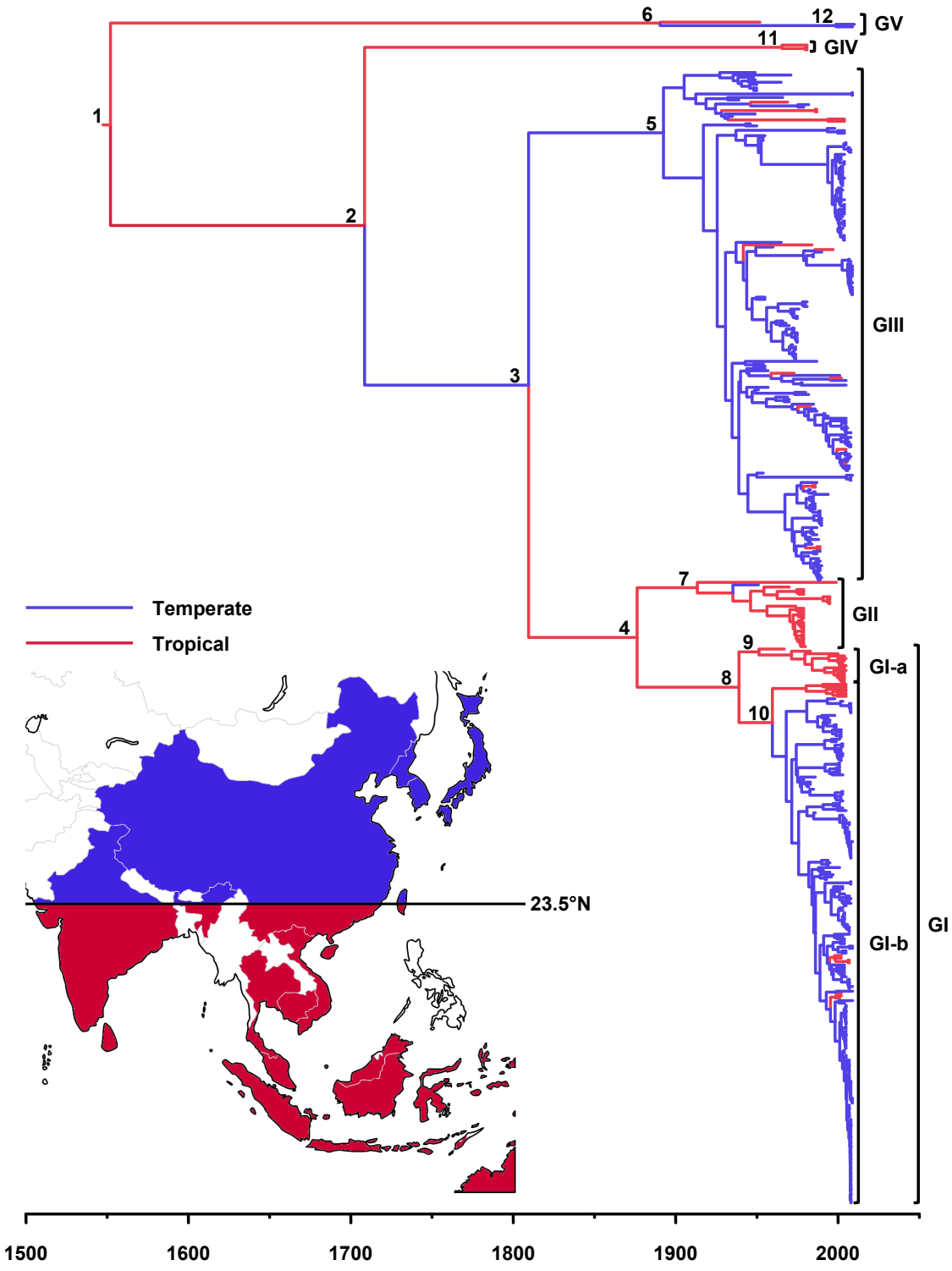

6
12
GV
11
GIV
1
5
2
GIII
3
7
GII
4
9
GI-a
8
10
GI-b
GI
Temperate
Tropical
23.5°N
1500
1600
1700
1800
1900
2000

Figure 6.5: NJ phylogeny of the JEV isolates. The trees was rooted using the MVE-1-51 isolate of MVEV, which is a member of the JEV serocomplex, but has been removed to allow for better visualization of branch lengths. GI-V are represented to the right of the tree. Bootstrap percentages based on 1,000 replicates are indicated at key nodes within the phylogeny. Horizontal branch lengths are proportional to the genetic distance between isolates and the scale beneath the tree indicates the number of nucleotide substitutions per site.

Figure 6.6: ML phylogeny of the JEV isolates. The trees was rooted using the MVE-1-51 isolate of MVEV, which is a member of the JEV serocomplex, but has been removed to allow for better visualization of branch lengths. GI-V are represented to the right of the tree. Bootstrap percentages based on 100 replicates are indicated at key nodes within the phylogeny. Horizontal branch lengths are proportional to the genetic distance between isolates and the scale beneath the tree indicates the number of nucleotide substitutions per site.

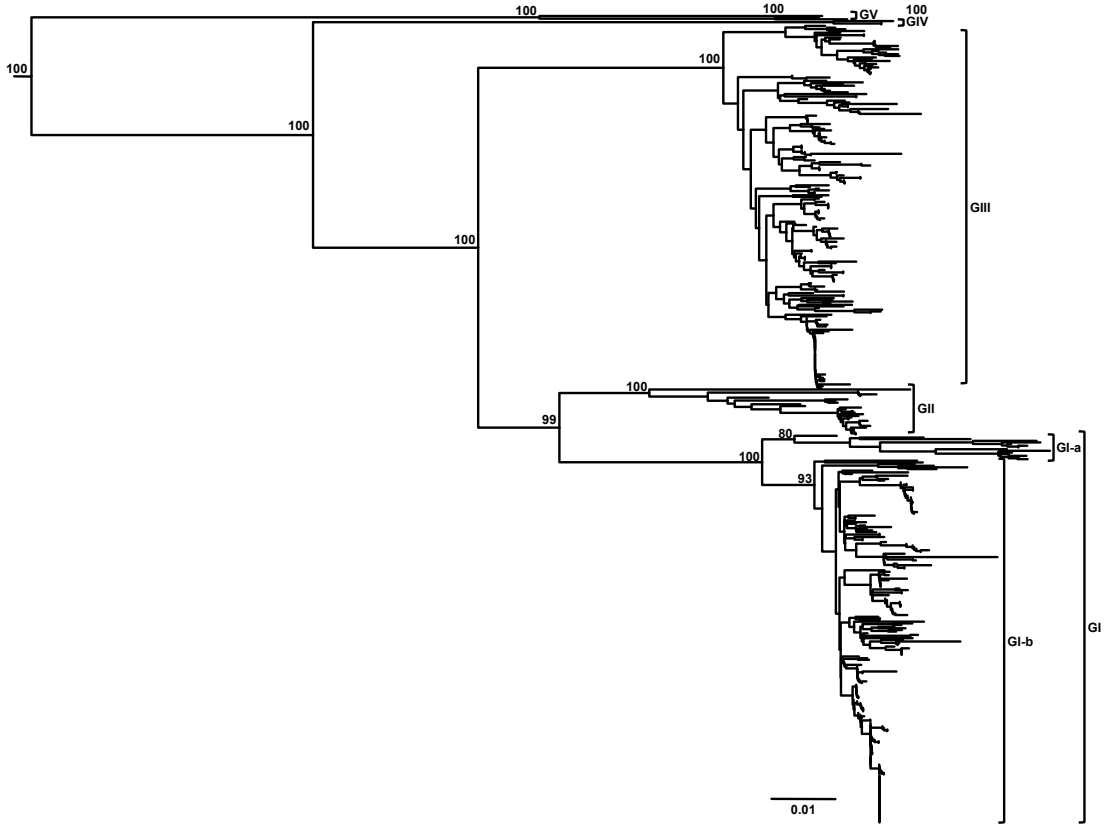


Figure 6.7: Location of the genotype-defining coding substitutions on the structure of the JEV E protein dimer: A) GV-defining substitutions, B) GIV-defining substitutions, C) GIII-defining substitutions, and D) GI-defining substitutions. Domain I is highlighted in red, domain II is highlighted in yellow and domain III is highlighted in blue. The substitutions are indicated in gray and are only numbered on the lower monomer.

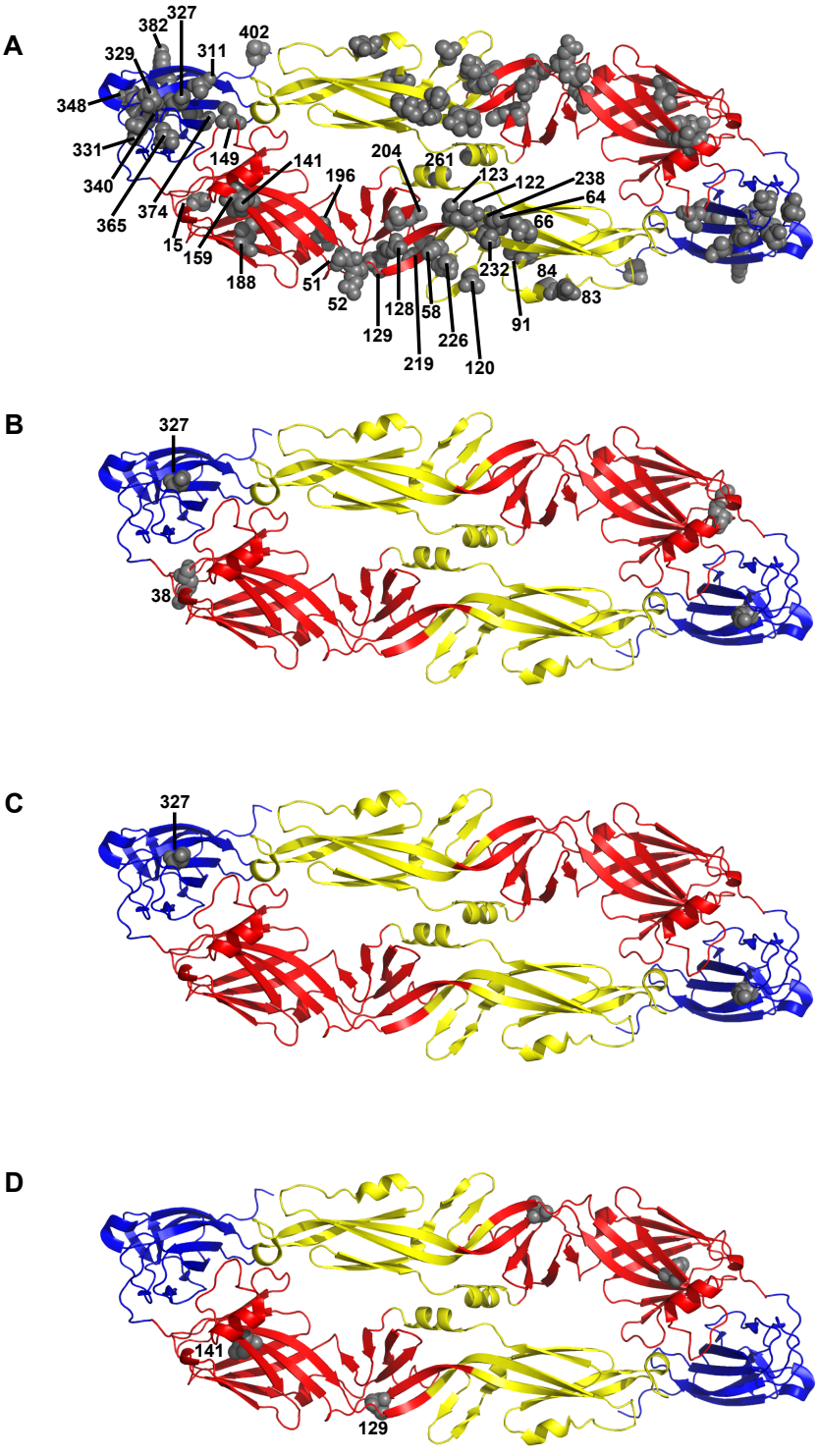
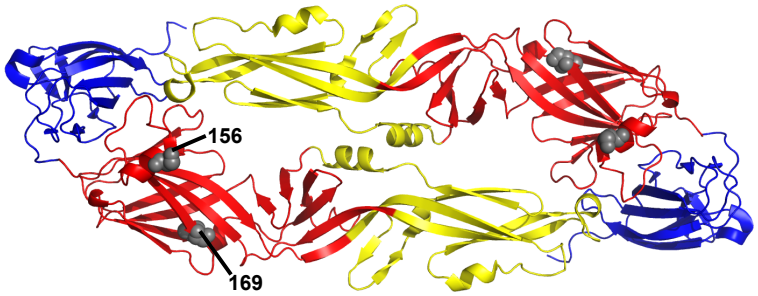
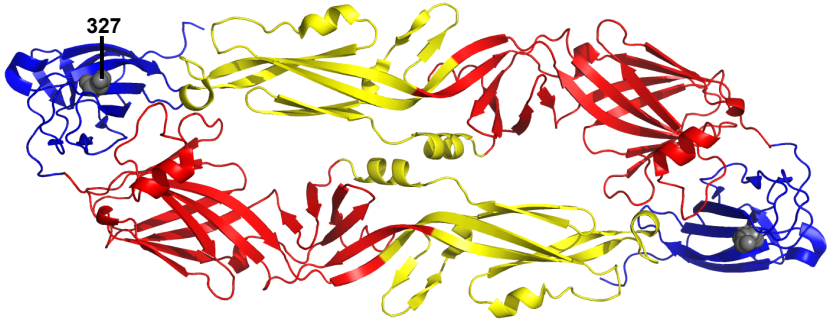

A
382
327
402
329
311
348
331
340
365
374
149
141
196
204
261
123
122
238
64
66
15
159
188
51
52
128
58
129
219
226
120
232
84
83
91
B
327
38
C
327
D
141
129

Figure 6.8: Location of the genotype-defining coding substitutions on the structure of the JEV E protein dimer: A) GIII + GII + GI-defining substitutions B) GII + GI-defining substitutions, and C) Toggling sites. Domain I is highlighted in red, domain II is highlighted in yellow and domain III is highlighted in blue. The substitutions are indicated in gray and are only numbered on the lower monomer.

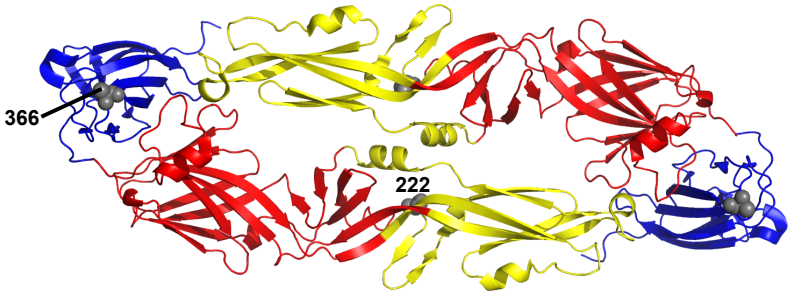
A



B



C



Chapter 7: Dynamics of the emergence and establishment of a newly dominant genotype of Japanese encephalitis virus throughout Asia

7.1 ABSTRACT

In recent years, genotype I (GI) of Japanese encephalitis virus (JEV) has displaced genotype III (GIII) as the dominant virus genotype throughout Asia. However, no studies have examined the epidemiological and evolutionary dynamics underlying this geographically expansive genotype displacement, nor have any studies aimed to identify what phenotypic advantages are responsible for the displacement. Therefore, in this study the largest collection of GIII and GI envelope (E) gene-derived viral sequences assembled to date were utilized to reconstruct the spatiotemporal chronology of the genotype displacement, and to determine the evolutionary and epidemiological dynamics underlying this significant event. Genotype I consists of two clades, G1-a and G1-b with the latter associated with displacement of GIII as the dominant JEV genotype throughout Asia in the 1990s. A decade prior to the genotype displacement, the relative genetic diversity of GI had surpassed that of GIII, thereby conferring a selective advantage (compatible with elevated fitness) to GI. It is plausible that an increase in genetic diversity would have increased the viral population's ability to adapt to new selective pressures. Phylogeographic analysis revealed that GI-a diverged in Thailand and has remained confined to tropical Asia, whereas GI-b diverged in Vietnam and was then dispersed northwards to China, where it was subsequently dispersed to Japan, Korea and Taiwan. To determine what phenotypic advantages may have facilitated the genotype displacement, the *in vitro* multiplication kinetics of GIII and GI isolates were compared in avian and mosquito cells. No differences in viral multiplication were observed among genotype III and I viruses in avian cells; however the GI-b isolate had significantly higher infectious titers in mosquito cells from 24-48 hours post infection compared to the GI-a and GIII isolates. In nature, an increased multiplicative ability of GI-b viruses compared to GIII viruses at early time points in mosquitoes may have resulted in a shortened EIP

that led to an increased number of GI enzootic transmission cycles and the subsequent displacement of GIII.

7.2 INTRODUCTION

Genotype III (GIII) of Japanese encephalitis virus (JEV) has been the source of numerous JE epidemics throughout history and was the predominantly isolated genotype throughout most of Asia from 1935 until recently. The initial isolate of genotype (GI) was collected in Cambodia in 1967 and GI remained undetected for 16 years until another isolate was identified in China in 1979. In recent years, multiple reports have indicated that GI has displaced GIII as the most frequently isolated virus genotype in a number of Asian countries including China (Wang *et al*., 2007), Thailand (Nitatpattana *et al*., 2008), South Korea (Nam *et al*., 1996), Japan (Ma *et al*., 2003), Malaysia (Tsuchie *et al*., 1997), Vietnam (Nga *et al*., 2004), India (Fulmali *et al*., 2011), and Taiwan (Chen *et al*., 2011). Although, two recent evolutionary studies have examined the genotype displacement in China (Chen, 2011; Pan *et al*., 2011), no studies have reconstructed the spatiotemporal chronology of the emergence and establishment of GI throughout Asia, nor have any studies identified genetic determinants underlying the genotype displacement as it unfolded across Asia. Further, even though this geographically expansive genotype displacement is suggestive of a major difference in fitness between GIII and GI viruses, the *in vitro* multiplication kinetics of GIII and GI viruses have never been compared in avian or mosquito cells. An increased *in vitro* multiplicative ability of GI viruses compared to GIII viruses in avian and/or mosquito cells may correlate to an increased number of mosquito-avian-mosquito transmission cycles in nature, thereby explaining how GI displaced GIII throughout most of Asia.

To this end, 453 GIII and GI envelope (E) gene-derived JEV sequences sampled from 11 countries over 75 years were compiled (the largest JEV sequence dataset assembled to date). The E protein gene was selected because there is a paucity of genomic sequences from countries other than China, and the E protein is the major constituent of the mature virion surface, is under continual selection pressure, plays a critical role in infectivity and immunity, is phylogenetically informative, and has been established as a robust evolutionary proxy of the polyprotein (see Chapter 6). To gain insight on the factors driving the emergence and establishment of GI throughout Asia, the viral sequences were utilized to reconstruct the chronology and evolutionary dynamics of these events on a spatiotemporal scale using Bayesian coalescent methods. A series of adaptive evolutionary algorithms were then employed to elucidate genetic determinants associated with the emergence of GI. This study also examined whether the selective advantage of GI viruses could be due to their increased multiplicative ability in avian and/or mosquito cells.

7.3 RESULTS

7.3.1 Descriptive analyses of the GIII and GI datasets

To investigate the phylogeography, phylodynamics and evolutionary pressures underlying the genotype displacement, the largest collection of GIII (n=219) and GI (n=234) viral sequences compiled to date was utilized (Table 7.1). These GIII and GI viral sequences were extracted from the JEV E gene dataset utilized in Chapter 6. Most of the viruses within the GIII dataset were isolated from mosquitoes (46.58%) collected in China (35.62%) within the last decade (43.8%), and most of the viruses within the GI dataset were isolated from mosquitoes (65.38%) collected in Japan (62.39%) within the last decade (89.32%) (Table 7.2). Figure 7.1 shows the relative proportions of GIII and

GI isolates according to the decade of collection (based on the datasets compiled in this study). Between the 1930s and the 1950s only GIII isolates were identified from samples collected, and during the 1960s through the 1980s GIII was the predominantly collected virus genotype (GIII: 1960s [90.91%], 1970s [96.30%], 1980s [87.93%]). The initial GI isolate (M859/Cambodia/1967/Mosquito) was collected in 1967, one additional GI isolate was collected in the 1970s (YN79-Bao83, China: Yunnan, 1979), and seven GI isolates were collected in the 1980s in China and Thailand. In the 1990s, GI displaced GIII as the most frequently collected genotype (GI [57.14%] versus GIII [42.86%]). Genotype III isolates were collected throughout the last decade; however GI was the predominantly collected genotype (GI [68.52%] versus GIII [31.48%]).

7.3.2 Comparison of the Bayesian skyline reconstructions for GIII and GI

Bayesian skyline reconstructions were inferred to compare the demographic histories of GIII and GI (Figure 7.2; Table 7.3). The reconstructions plot the effective number of infections, a measure of relative genetic diversity, as a function of time. The Bayesian skyline reconstruction for GIII shows that the relative genetic diversity increased from 1916 to 1950, decreased from 1951 to 1972, increased from 1973 to 1979, decreased from 1980 to 2000, increased from 2000 to 2002, decreased from 2003 to 2008, and increased in 2009 (Figure 7.2A). The Bayesian skyline reconstruction for GI shows that the relative genetic diversity remained stable from 1956 to 1970, increased from 1971 to 2001, rapidly decreased from 2002 to 2009, and increased from 2008 to 2009 (Figure 7.2B). The superimposition of the Bayesian skyline reconstructions for GIII and GI demonstrates that by 1983 the relative genetic diversity of GI superseded that of GIII (a decade prior to the genotype displacement) (Figure 7.2C), thereby conferring

GI a selective advantage (compatible with elevated fitness) that led to the subsequent displacement of GIII.

7.3.3 Spatiotemporal reconstruction of the phylogeny of GI

To elucidate the timing and geographic pattern of dispersal of GI throughout Asia, the phylogeny of GI was reconstructed on a spatiotemporal scale. Figure 7.3 shows the geographical distribution of GI, as determined based on the isolates used in this study. It should be noted that although not included in this study, due to the absence of E gene sequence information, GI viruses have also been isolated in Malaysia in 1994, in Laos in 1993, and in India in 2009 (other regions of the virus genome were sequenced or the E gene was only partially sequenced [available on GenBank]). The GI Bayesian maximum clade credibility (MCC) phylogeny inferred from 234 isolates collected between 1967 and 2009 is shown in Figure 7.4. The key nodes within the phylogeny are numbered and supported by the posterior probability (PP) values indicated in Table 7.4. Branch tips correspond to the date of collection of each of the virus isolates and branch lengths correspond to the time since divergence. Terminal branches within the phylogenies are colored according to the geographic location of the taxon at the tip, while internal braches are colored according to the most probable location of their parental node. Divergence dates of the most recent common ancestor (MRCA) and location state PP values for each of the eight countries included in the analysis are presented in Table 7.4 for the key nodes within the phylogeny (Figure 7.4).

As previously demonstrated (see Chapter 6), the GI Bayesian MCC phylogeny inferred from 234 isolates collected between 1967 and 2009 is divided into two geographically and genetically distinct clusters, GI-a (15 isolates collected in tropical Asia [Cambodia, Thailand and Australia] between 1967 and 2005; all isolates possessed a

valine at site 141 of the E protein) and GI-b (219 isolates collected primarily in temperate Asia [Vietnam, Thailand, Japan, Korea, China and Taiwan] between 1979 and 2009; all sites possessed an isoleucine at site 141 of the E protein) (Figure 7.4).

The MRCA of GI diverged in 1907 (95% highest posterior density [HPD]: 1836, 1956) in the Cambodia-Thailand-Vietnam region, and from this ancestral virus the MRCA of GI-a diverged in Thailand in 1940 (95% HPD: 1903, 1964) and the MRCA of GI-b diverged in Vietnam in 1953 (95% HPD: 1930, 1970) (Figures 7.3 and 7.4, Table 7.4). A virus related to the ancestral GI-a Thai virus was dispersed to Cambodia (MRCA: 1940, 95% HPD: 1903, 1964) and a GI-a virus that originated in Thailand was dispersed to Australia (MRCA: 1972, 95% HPD: 1953, 1985), while a virus related to the ancestral GI-b Vietnamese virus was dispersed to China (MRCA: 1964, 95% HPD: 1951, 1974) (Figures 7.3 and 7.4). Finally, viruses related to the MRCA of Chinese GI-b isolates were dispersed to Japan (MRCA: 1977, 95% HPD: 1966, 1988), Korea (MRCA: 1983, 95% HPD: 1974, 1990) and Taiwan (MRCA: 2003, 95% HPD: 2000, 2005) (Figures 7.3 and 7.4). Interestingly, GI had already seeded most of Asia by 1983, as evidenced by the MRCA of isolates from every country included in the analysis, except Taiwan, having diverged prior to this date (Figure 7.4).

It appears that once GI-b viruses were dispersed to temperate Asia, the viruses were maintained year-to-year in local transmission cycles. For example, the phylogeny indicates that GI-b viruses were maintained for at least 15 years in Japan (1994-2008), 24 years in China (1979-2003) and 4 years in Korea (1993-1996) (Figure 7.4).

The Bayesian stochastic search variable selection (BSSVS) procedure was used to identify the links between countries within the GI dataset generated by the continuous-time Markov chain (CTMC) method that explained the most likely virus dispersal patterns. Then Bayes factor (BF) tests were calculated to determine the statistical

significance of the viral dispersal pathways between the countries by integrating over the posterior sample of GI Bayesian phylogenies. Consistent with the relationships observed among the viruses in the GI Bayesian MCC phylogeny, the strongest pathways of viral dispersal were between China and Japan (BF: > 15,838), followed by China and Korea (BF: 443), China and Vietnam (BF: 321), Australia and Thailand (BF: 8), and China and Taiwan (BF: 6).

7.3.4 Analysis of GI phylogeographic structure

To determine whether GI genetic diversity was primarily driven by local virus transmission or extensive virus migration, the association index (AI), parsimony score (PS), unique fraction (UniFrac), nearest taxa (NT), net relatedness (NR) and phylogenetic diversity (PD) methods were used to test the null hypothesis of no overall association between phylogeny and sampling location. Based on a p-value < 0.05, the null hypothesis was rejected and it was concluded that there was an overall statistically significant relationship between phylogeny and sampling location for GI (Table 7.5). The maximum exclusive single-state clade size (MC) statistic was then utilized to test the null hypothesis of no association between phylogeny and sampling location according to country. Again, the null hypothesis was rejected and it was concluded that there was a statistically significant relationship between phylogeny and sampling location according to country for GI (except for Cambodia, Australia and Taiwan due to few isolates) (Table 7.5), suggesting that the observed phylogenetic diversity was primarily driven by local virus transmission rather than extensive viral dispersal between countries.

7.3.5 Comparison of evolutionary parameters estimated from the GIII and GI datasets

Elevated evolutionary rates can result in virus populations that are able to readily adapt to new environments. To determine if this was the case for GI, evolutionary parameters (including the evolutionary rate) estimated from the GI nucleotide sequence alignment were compared to those estimated from the GIII nucleotide sequence alignment (Table 7.6). Although not statistically different, the overall evolutionary rate for GI (mean: 5.05 x 10^{-4} nucleotide substitutions/site/year; 95% HPD: 3.44, 6.58) was 1.57-fold faster than GIII (mean: 3.21 x 10^{-4} nucleotide substitutions/site/year; 95% HPD: 2.66, 3.76) (Table 7.6). This increased evolutionary rate was not due to an elevated number of non-synonymous substitutions (d_N) for GI (170.02) compared to GIII (294.54), but rather resulted from an elevated number of synonymous substitutions (d_S) for GI (852.01) compared to GIII (823.52) (Table 7.6). Congruently, a statistically significant 1.80-fold increase in the global d_N/d_S ratio was observed for GIII (mean: 0.18; 95% CI: 0.16, 0.20) compared to GI (mean: 0.10; 95% CI: 0.08, 0.11) and a 1.54-fold increase in the ratio of the evolutionary rate at codon positions one and two (CP_{12}) to the evolutionary rate at codon position three (CP_3) was observed for GIII (mean: 0.20; 95% HPD: 0.16, 0.23) compared to GI (mean: 0.13; 95% HPD: 0.11, 0.16) (Table 7.6). The CP_{12}/CP_3 ratio has been shown to be a proxy of the d_N/d_S ratio as most first and all second position single substitutions are non-synonymous, whilst many third position substitutions are synonymous.

Of note, the mean evolutionary rates obtained from the E gene sequence files for GI and GIII are similar to those obtained using ORF sequence files that were limited by a lack of sequence data (GIII [18 sequences]: 2.65 x 10^{-4} nucleotide substitutions/site/year

[95% HPD: 5.42 x 10^{-5}, 4.50 x 10^{-4}]; GI [9 sequences]: 7.05 x 10^{-4} nucleotide substitutions/site/year [95% HPD: 1.73 x 10^{-7}, 1.68 x 10^{-3}).

7.3.6 Adaptive evolutionary analyses of the GIII and GI E protein gene sequence datasets

To provide insight on the genetic basis of the geographically expansive genotype displacement, a series of algorithms to detect adaptive evolution within the GIII and GI E protein gene sequence datasets were utilized. The single-likelihood ancestor counting (SLAC) and fixed effects likelihood (FEL) methods were used to detect sites under diversifying positive selection by estimating the d_N/d_S ratio at every codon in the E gene sequence alignments. The SLAC method did not identify any sites that displayed signatures of positive selection in either the GIII or GI alignments, however the FEL method identified three positively selected sites in the GIII alignment (codons 227, 261 and 306) and one positively selected site in the GI alignment (codon 15) (Table 7.7). The internal FEL (IFEL) method identified two positively selected sites within the GIII alignment (codons 51 and 227) and one positively selected site within the GI alignment (codon 126) at the population level (along internal branches of the phylogeny) (Table 7.7).

Although codon-based methods that estimate the d_N/d_S ratio at every codon in the alignment are powerful tools, they require repeated fixation of non-synonymous changes at specific sites to infer positive selection. Hence, the presence of episodic, or directional selection is difficult to detect even though many of the molecular adaptations associated with viral emergence are of this type (Holmes, 2009). Therefore, the directional evolution in protein sequences (DEPS) method was applied to test for evidence of preferential substitution toward a given residue at individual positions within the GIII and GI E protein alignments. The GIII DEPS analysis revealed elevated substitution rates towards

four residues (R, M, P and G) (Table 7.8). Of these four residues, directional evolution towards M was subjected to the strongest bias (89.30; the relative rate of amino acid substitution across the alignment towards the target site), but affected the smallest proportion of sites (1.00%; the percentage of sites that evolve under a directional model versus a non-directional model); whereas evolution towards G was subject to the weakest bias (14.15), but affected the largest proportion of sites (5.00%) (Table 7.8). Six sites were identified to be involved in this directional evolution (62, 76, 123, 209, 227 and 306) (Table 7.9). The GI DEPS analysis revealed elevated substitution rates towards four residues (N, T, V and Y) (Table 7.8). Of these four residues, directional evolution towards T was subjected to the strongest bias (19.15), but affected the smallest proportion of sites (1.58%); whereas evolution towards V was subjected to the weakest bias (3.21), but affected the largest proportion of sites (19.96%) (Table 7.8). Six sites were identified to be involved in this directional evolution (15, 87, 123, 129, 141 and 360) (Table 7.9).

The amino acids within the three-dimensional structure of the E protein of JEV are not arranged linearly and the E protein exists as a dimer consisting of two monomers sitting head-to-toe, consequently many of the residues functionally interact with one another. Therefore, in order to maintain a functional E protein the evolution of amino acid residues are co-dependent. The Spidermonkey algorithm identified 14 pairs of sites within the GIII alignment (76-209, 76-306, 126-492, 132-143, 176-51, 227-51, 276-51, 318-229, 364-123, 364-363, 364-408, 398-363, 418-51, 487-312, 141-129 and 360-89) and two pairs of sites within the GI protein alignment (89-360 and 129-141) that exhibited evidence of co-evolution (Table 7.10).

All sites for which molecular adaptation was detected are listed in Table 7.11 and are indicated on the E protein dimer structure of JEV (Figure 7.5). Sites displaying evidence of molecular adaptation in the GI E gene-protein alignment are indicated on GI

Bayesian MCC phylogenies with branches colored according to the amino acid residue present (Figure 7.6). Sites 123 (GIII: S-R [non-conservative substitution]; GI: S-N/K [non-conservative substitutions) and 126 (GIII: I-V [conservative substitution]; GI: I-T/V [non-conservative/conservative substitutions]) were identified to be under molecular adaptation in both the GIII and GI alignments. Site 141 (I-V) is the only site that displayed evidence of molecular adaptation and defined GI-b, the cluster that emerged from tropical Asia into temperate Asia and has since established itself as the dominant JEV genotype.

7.3.7 *In vitro* multiplication kinetics of GI and GIII viruses in avian and mosquito cells

In an effort to determine what phenotypic advantages facilitated the JEV genotype displacement, the *in vitro* multiplication kinetics of isolates representative of GI-IV (Table 7.12) were compared in avian duck embryo fibroblast (DEF) and *Ae. albopictus* C6/36 cells inoculated at a multiplicity of infection (MOI) of 0.1.

The genotype displacement may have been due to an increased multiplicative ability of GI-b viruses in avians that sustained normal body temperatures following JEV infection. Additionally, or alternatively, the genotype displacement may have resulted from the ability of GI-b viruses to multiply more efficiently in avians that developed elevated body temperatures following infection with JEV (e.g., GI-b viruses are not temperature sensitive in avians). Either of these scenarios may have resulted in an increased number of avian-mosquito-avian transmission cycles and would explain how GI-b was able to displace GIII as the dominant genotype throughout Asia. It is important to note that avians are endothermic an on average maintain core body temperatures ranging from 40 to 44°C, though temperature varies according to species, activity levels of individual avians, and age. Great blue herons (*Ardea herodias*), which are the largest

of the herons and considered a maintenance host of JEV, have a normal core body temperature of 41°C (Waterstraat *et al*., 1999). Therefore, DEF cells were utilized to evaluate two predictions regarding the phenotypic basis of the genotype displacement: 1) DEF cells infected with a GI-b isolate and incubated at 37°C will exhibit increased viral multiplication compared to cells infected with a GI-a isolate, as well as isolates representative of GII-IV and 2) DEF cells infected with a GI-b isolate and incubated at 41 and 44°C will exhibit increased viral multiplication compared to cells infected with GI-a isolate, as well as isolates representative of GII-IV. No multiplication differences in DEF cells were observed among isolates representative of GI-IV incubated at 37 or 41°C (Figure 7.7). Significantly, unlike lineage I West Nile virus (WNV) isolates, a flavivirus closely related to JEV, none of the isolates representative of GI-IV were able to replicate in DEF cells incubated at 44°C (Kinney *et al*., 2006).

It is also plausible that an increased multiplicative ability of GI-b viruses in mosquitoes would have resulted in an increased number of mosquito-avian-mosquito transmission cycles, in turn leading to the displacement of GIII by GI as the dominant Asian genotype of JEV. Therefore, it was hypothesized that C6/36 cells infected with a GI-b isolate and incubated at 28°C would exhibit increased viral multiplication compared to cells infected with a GI-a isolate, as well as isolates representative of GII-IV. It was found that the GI-b isolate (JE-91) had significantly higher infectious titers compared to the GI-a isolate (KE-93-83) and GIII isolates (Taira and Matsunaga), as well as isolates representative of GII-IV, from 24 hours post-infection (HPI) to 48 HPI (Figure 7.8; Tables 7.13 and 7.14). At 24 HPI, JE-91 had infectious titers 19.0-fold higher compared to the KE-93-83 (p-value < 0.00), 13.3-fold higher compared to Taira (p-value < 0.00) and 12.9-fold higher compared to Matsunaga (p-value < 0.00) (Figure 7.8, Table 7.14). At 36 HPI, JE-91 had infectious titers 14.6-fold higher compared to KE-93-83 (p-value <

0.00), 11.8-fold higher compared to Taira (p-value: 0.02) and 11.6-fold higher compared to Matsunga (p-value 0.01) (Figure 7.8, Table 7.14). At 48 HPI, JE-91 had infectious titers 14.3-fold higher compared to KE-93-83 (p-value < 0.00), 12.1-fold higher compared to Taira (p-value: 0.01) and 11.1-fold higher compared to Matsunaga (p-value < 0.00) (Figure 7.8, Table 7.14).

7.4 DISCUSSION

In recent years, multiple publications have documented that GI has displaced GIII as the most frequently isolated genotype of JEV throughout Asia (Chen *et al*., 2011; Fulmali *et al*., 2011; Ma *et al*., 2003; Nam *et al*., 1996; Nga *et al*., 2004; Nitatpattana *et al*., 2008; Tsuchie *et al*., 1997; Wang *et al*., 2007) and two evolutionary studies examined the genotype displacement using isolates sampled only from or primarily from China (Chen, 2011; Pan *et al*., 2011). However, no studies had examined the epidemiological dynamics underlying this geographically expansive genotype displacement using sequence data sampled from across Asia. To fill this gap, 453 GIII and GI E gene-derived JEV sequences sampled from 11 countries over 75 years were compiled (the largest JEV sequence dataset assembled to date). Ideally, genomic data would have been utilized in the analyses. However, the genomes of only 43 GI isolates collected between 1977 and 2009 have been sequenced and 39 of these isolates were collected in China. Further, none of these 43 GI isolates are members of GI-a. For GIII, there are 53 genomic sequences available for GIII isolates collected between 1935 and 2004, however 44 of these isolates were collected in China. A series of state-of-the-art coalescent analyses were applied to the E gene sequence datasets to determine the chronology of events leading up to the genotype displacement, the timing and geographical directionality of the emergence and establishment of GI throughout Asia, and sites within the E protein of GI isolates that

were subject to various selective pressures. Additionally, in an effort to identify what phenotypic advantages facilitated the geographically expansive genotype displacement, the *in vitro* multiplication kinetics of GI and GIII isolates were compared in avian and mosquito cells.

7.4.1 GI displaced GIII as the dominant JEV genotype in the 1990s

Descriptive analyses of the GIII and GI datasets revealed that by the 1990s GI had displaced GI as the dominant JEV genotype throughout most of Asia.

7.4.2 Selective advantage conferred to GI a decade prior to the genotype displacement

The Bayesian skyline reconstruction of the demographic history of GIII revealed two prolonged periods of decreasing relative genetic diversity (1951 to 1972 and 1980 to 2000) separated by a brief period of increasing relative genetic diversity (1973 to 1979), whereas the Bayesian skyline reconstruction of GI revealed a prolonged period of increasing relative genetic diversity (1971 to 2001). By 1983, the relative genetic diversity of GI had surpassed that of GIII. It is conceivable that the loss of genetic variation experienced by GIII during the two prolonged periods of decreasing genetic diversity reduced the population's ability to adapt to new selective pressures. While at the same time, it is plausible that the increase in the relative genetic diversity of GI led to fitness gains that resulted in the emergence and expansion of GI viruses and the subsequent displacement of GIII. Genotype I fitness gains may have resulted in viruses with an increased replicative ability in ardeid wading birds (the primary virus-amplifying vertebrate host in the enzootic transmission cycle of JEV) leading to birds with viremias of increased magnitude and/or duration, in-turn resulting in an increased number of enzootic transmission cycles. Longer duration viremias in birds would also have allowed

the birds to transport the virus to more distant geographic locations and may explain the rapid increase in the geographical distribution of GI. It is also possible that GI fitness gains may have resulted in viruses that could better infect, disseminate and be transmitted by *Cx. tritaeniorhynchus* (the primary vector of JEV throughout Asia), leading to an increased number of enzootic transmission cycles.

7.4.3 GI-b emerged in Vietnam, dispersed northwards to China, and was then dispersed from China to Japan, Korea and Taiwan

The reconstruction of the GI phylogeny on a spatiotemporal scale revealed two geographically and genetically distinct clusters, GI-a (15 isolates collected in tropical Asia between 1967 and 2005; all isolates possessed a valine at residue 141 of the E protein) and GI-b (219 isolates collected primarily in temperate Asia between 1979 and 2009; possessed an isoleucine at residue 141 of the E protein).

Following the divergence of the MRCA of GI in the Cambodia-Thailand-Vietnam region in 1907 (95% HPD: 1836, 1956), the MRCA of GI-a diverged in Thailand in 1940 (95% HPD: 1903, 1964), and the MRCA of GI-b diverged in Vietnam in 1953 (95% HPD: 1930, 1970). A virus related to the MRCA of GI-b dispersed northwards from Vietnam to China (MRCA: 1964, 95% HPD: 1951, 1974), and this virus genotype was then dispersed from China to Japan (MRCA: 1977, 95% HPD: 1966, 1988), Korea (MRCA: 1983, 95% HPD: 1974, 1990) and Taiwan (MRCA: 2003, 95% HPD: 2000, 2005). With the exception of Taiwan, GI-b had seeded most of temperate Asia by 1983. Interestingly, 1983 is also the date when the relative genetic diversity of GI surpassed that of GIII, suggesting that a selective advantage was conferred to GI around the same time the virus genotype seeded most of Asia. The relative genetic diversity of GI continued to increase throughout the 1980s into the 2000s as the virus genotype emerged

into naïve areas, adapted to its new environment and established itself as the dominant genotype of JEV.

7.4.4 GI genetic diversity primarily driven by local virus transmission

The results of the phylogeographic analysis indicated that once GI-b viruses were dispersed to temperate Asia (Japan, China and Korea), the viruses were maintained year-to-year in local transmission cycles. Not surprisingly, we found a statistically significant relationship between phylogeny and sampling location, overall and according to individual country (except for Cambodia [1 sequence], Australia [1 sequence] and Taiwan [2 sequences]). However, the relatively small number of sequences for these three countries may have biased the results. The results of these analyses suggest that in all but three countries, the observed genetic diversity among the GI isolates was primarily driven by local virus transmission cycles rather than extensive viral dispersal between countries via migratory birds, wind-blown mosquitoes, or long-distance public transportation (e.g., airplanes and trains). This is similar to what has been previously observed for GIII and GI-b viruses, suggesting both virus genotypes are maintained year-to-year in temperate Asia in local transmission cycles likely by hibernating mosquitoes, transovarial transmission in mosquitoes, hibernating poikilothermic vertebrates and/or bats (see Chapter 6). It is plausible that GI-b may have rapidly emerged from tropical Asia and spread throughout temperate Asia due to an acquired ability to be maintained across a wide range of temperatures, including the lower temperatures of temperate climates.

In addition to viral factors, environmental and host factors may have also contributed to the emergence of GI throughout Asia. Japanese encephalitis-endemic and -epidemic regions have experienced an unprecedented human population growth over the

last half-century. The population of Eastern, Southeastern and South-Central Asia has more than tripled in the last 60 years, increasing from 2.1 billion in 1950 to 6.6 billion in 2010 (United Nations, 2012). Asia has also been an area of many wars over the last century (e.g., World War I [194-1918], Chinese Civil War [1927-1950], World War II [1939-1945], Korean War [1950-1953] and Vietnam War [1955-1975], Cambodian Civil War [1967-1975], and Cambodian-Vietnamese War [1975-1989]), resulting in mass population movements and changes to the ecosystem (e.g., deforestation, defoliation and destruction of social structures). The most common larval habitat of *Cx. tritaeniorhynchus* is rice fields, and therefore flooded rice production systems have been associated with JE outbreaks. Approximately 91% of the world's rice is produced in Asia (Keiser *et al*., 2005), and in recent years rice production systems have increased in both cropping area and intensity. From 1963 to 2003, the total rice harvested area of all JE endemic and epidemic countries increased 22% from 1,102,459 km^2 to 1,345,000 km^2 (Keiser *et al*., 2005), and over the same time period the total yearly rice production in these countries increased 134% from 226 million tons to 529 million tons (Keiser *et al*., 2005). Domestic swine serve as a major amplifying host of JEV, and in Asia swine are typically reared in open, unroofed pigpens some of which are close to houses. In recent years, pig husbandry has grown exponentially in many Asian countries. Between 1990 and 2005, pork production (related to the risk of acquiring JE) has increased in Cambodia (+47%), China (+87%), South Korea (+69%), Laos (+21%), Myanmar (+381%), Nepal (+12%), the Philippines (+18%), Sri Lanka (+21%), Thailand (+80%) and Vietnam (+147%) (Erlanger *et al*., 2009).

7.4.5 Elevated evolutionary rate of GI due to an increased number of synonymous substitutions

As elevated evolutionary rates can lead to virus populations that are able to readily adapt to new environments, the overall evolutionary rates for the E gene of GIII and GI were compared and it was found that although not statistically significant, the overall evolutionary rate for GI was 1.57-fold faster compared to GIII (similar to evolutionary rates estimated from ORF GIII and GI sequence data). This increased evolutionary rate was due to an elevated number of synonymous substitutions for GI compared to GIII. In agreement, the global d_N/d_S ratio and the CP_{12}/CP_3 ratio for GI was 1.80 (statistically significant) and 1.54-fold faster, respectively, compared to GIII.

7.4.6 Eight residues within the E protein associated with the adaptive evolution of GI

A recent study identified five positively selected residues (6, 166, 312, 433 and 498) within an E gene alignment comprised of 45 GI JEV sequences derived from isolates collected from China only between 1979 and 2009 using the codon-based relaxed effects likelihood method (Chen, 2011). To provide insight on the genetic basis of the geographically expansive emergence and establishment of GI throughout Asia, five methods (SLAC, FEL, IFEL, DEPS and Spidermonkey) were utilized to identify evidence of adaptive evolution within the E protein of GIII and GI isolates throughout Asia between 1935 and 2009. Using these five methods, adaptive evolution was detected at 23 unique sites within the GIII alignment and at nine unique sites within the GI alignment.

Interestingly, sites 123 (domain I; GIII: serine to arginine [non-conservative substitution]; GI: serine to asparagine/lysine [non-conservative substitutions) and 126 (domain I; GIII: isoleucine to valine [conservative substitution]; GI: isoleucine to

threonine/valine [non-conservative/conservative substitutions]) were identified as displaying evidence of adaptive evolution in both the GIII and GI alignments. Site 123 has not been associated with alterations in virus phenotype; however, site 126 (lysine to glutamic acid) is a determinant of dengue-2 virus mouse neurovirulence (Bray *et al*., 1998; Gualano *et al*., 1998). Further, site 126 (isoleucine) along with sites 52, 136 and 275 comprise the neutralization epitope (located at the junction of domains I and II of the E protein) for the JEV-specific neutralizing antibody 503 (Morita *et al*., 2001). Another study found that a major determinant of antibody binding of the JEV-specific neutralizing antibody B2 was an isoleucine at site 126 of the E protein (Goncalvez *et al*., 2008). The only site within domain III of the E protein of the GI alignment that was identified to have undergone adaptive evolution was site 360 (isoleucine), which comprises the neutralization epitope (along with sites 337 and 387) of the JEV-specific neutralizing antibody E3.3 (recognizes the DIII lateral ridge) (Luca *et al*., 2012). Both sites 126 and 360 of the E protein compromise previously mapped B cell epitopes, suggesting the occurrence of immune selection with co-circulating flaviviruses that also utilize avian hosts. West Nile virus and JEV have co-circulated in India for over 60 years, and Murray Valley encephalitis virus and JEV have been reported to co-circulate in Australia and eastern Indonesia.

Site 141 (domain I of the E protein) is the only site that displayed evidence of molecular adaptation and defined GI-b, the cluster that emerged from tropical Asia into temperate Asia and has since established itself as the dominant JEV genotype. All isolates comprising GI-a possessed valine at site 141 and all isolates comprising GI-b possessed isoleucine at site 141. It is possible that this amino acid substitution, and/or other substitutions in other regions of the genome, may have increased the fitness of GI-b viruses, leading to viruses with an increased replicative ability in avians or mosquitoes.

Either one of these scenarios may have resulted in an increased number of enzootic transmission cycles, in-turn leading to the emergence and establishment of GI-b as the dominant genotype of JEV.

7.4.7 GI-b had significantly higher infectious titers in mosquito cells from 24 to 48 HPI compared to GIII

In an effort to determine what phenotypic advantages facilitated the JEV genotype displacement, the *in vitro* multiplication kinetics of GIII and GI viruses (as well as viruses representative of GII and GIV) were compared in avian DEF and *Ae. albopictus* C6/36 cells. No multiplication differences in DEF cells were observed among isolates representative of GI-IV incubated at 37 or 41°C. However, in C6/36 cells it was found that the GI-b isolate (JE-91, Korea, 1991) had statistically significantly higher infectious titers compared to the GI-a isolate and the GIII isolates at 24, 36 and 48 HPI. Ideally, the C6/36 *in vitro* multiplication efficiency experiment would have been performed using more than one representative of GI-b. However, the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), which includes a collection of over 90 JEV isolates, does not possess any other GI-b isolates. Conceivably, an increased multiplication efficiency of GI-b viruses compared to GIII viruses at early time points in *Cx. tritaeniorhynchus* mosquitoes may have resulted in mosquitoes that became infected more rapidly thereby resulting in a shortened extrinsic incubation period (EIP: the interval between the ingestion of a virus by a mosquito and the mosquito's ability to transmit the pathogen to a susceptible vertebrate host). This would have led to an increased number of GI-b enzootic transmission cycles and may explain how GI-b emerged and established throughout Asia eventually displacing GIII as the dominant JEV genotype. Unfortunately, the University of Texas Medical Branch (UTMB) does not have a colony of *Cx. tritaeniorhynchus* to investigate this hypothesis and it is well known that

the eggs of this mosquito are difficult to transport from Asia. The results of the C6/36 multiplication kinetics experiments should be interpreted with caution. The C6/36 cell line was established from *Aedes albopictus* larvae homogenates (Igarashi, 1978), which may not accurately represent the cell types and expression profiles of the mosquito midgut epithelium and the other cell types associated with infection, dissemination, and transmission of JEV. Further, the results of a recent study indicate that C6/36 cells lack a functional RNA interference (RNAi) response, suggesting that these cells may not accurately model mosquito-arbovirus interactions at the molecular level (Brackney *et al*., 2010).

7.4.8 Conclusions

West Nile virus has also recently experienced a geographically expansive genotype displacement. The first WNV isolates introduced to New York City in 1999 were members of the NY99 genotype. During the subsequent spread of WNV across North America the WN02 genotype emerged and rapidly displaced the NY99 genotype. Two previous studies demonstrated that following peroral infection of *Culex pipiens* and *Culex tarsalis* the EIP of the WN02 genotype was up to four days shorter compared to the NY99 genotype (Ebel *et al*., 2004; Moudy *et al*., 2007). However, no differences in the replicative ability of isolates representative of NY99 and WN02 genotypes were observed in C6/36 cells (Moudy *et al*., 2007). Further, a recent study using different virus isolates and mosquitoes strains did not confirm a shorter EIP for the WN02 genotype compared to the NY99 genotype in *Culex tarsalis* (Anderson *et al*., 2012). Nevertheless, the results of these studies suggest a model whereby earlier transmission of WN02 genotype viruses in at least some strains of *Culex pipiens* and *Culex tarsalis* led to higher WN02 infection rates in avian hosts and the subsequent establishment of the WN02 genotype throughout

North America. A similar model may also explain how GI-b displaced GIII as the dominant JEV genotype throughout Asia.

Table 2.1: Details of the cell lines.

Cell line	ATCC number	Origin	Passage number(s)[1]
DEF	CCL-141	Duck (*Anas platyrhynchus domesticus*) embryo fibroblast cells	12, 13
C6/36	CRL-1660	Cells from the larvae of *Aedes albopictus*	11
Vero	CCL-81	Epithelial cells from the kidney of a normal adult African green monkey (*Cercopithecus aethiops*)	16, 17, 18
A549	CCL-185; Kindly provided by Terence Hill	Adenocarcinomic human alveolar basal epithelial cells initiated through explant culture of lung carcinomatous tissue from an adult Caucasian male	12

[1]Indicated in reference to the cell passage numbers used for the viral multiplication kinetics experiments.

Table 7.1: Details of the JEV isolates used in this study.

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
M859/Cambodia/1967/Mosquito	Cambodia	Tropical	1967	Mosquito	GI-a	
KE-93-83	Thailand	Tropical	1983	Mosquito	GI-a	
TS00	Badu Island, Australia	Tropical	2000	Swine	GI-a	EF434785
JE_RT_36	Ratchaburi, Thailand	Tropical	2003	Mosquito	GI-a	DQ087975
JE_CP_49	Chumphon, Thailand	Tropical	2004	Swine	GI-a	DQ087974
JE_CP_67	Chumphon, Thailand	Tropical	2004	Swine	GI-a	DQ087972
JE_KK_80	Khon Khen, Thailand	Tropical	2004	Unknown	GI-a	DQ111784
JE_KK_82	Khon Khen, Thailand	Tropical	2004	Unknown	GI-a	DQ111785
JE_KK_R83	Khon Khen, Thailand	Tropical	2004	Unknown	GI-a	DQ111787
JE_KK_R87	Khon Khen, Thailand	Tropical	2004	Unknown	GI-a	DQ111788
JE_KK_R88	Khon Khen, Thailand	Tropical	2004	Unknown	GI-a	DQ111786
JE_PK52	Phuket, Thailand	Tropical	2004	Unknown	GI-a	DQ84229
JE_CM_1196	Chiang Mai, Thailand	Tropical	2005	Unknown	GI-a	DQ238602
JE_KK_577	Khon Khen, Thailand	Tropical	2005	Unknown	GI-a	DQ238601
JE_KK_580	Khon Khen, Thailand	Tropical	2005	Unknown	GI-a	DQ238600
YN79-Bao83	Yunnan, China	Temperate	1979	Mosquito	GI-b	DQ404128
YN82-BN8219	Yunnan, China	Temperate	1982	Mosquito	GI-b	DQ404129
YN83-83199	Yunnan, China	Temperate	1983	Mosquito	GI-b	DQ404131
YN83-Meng83-54	Yunnan, China	Temperate	1983	Midge	GI-b	DQ404130
YN85-L86-99	Yunnan, China	Temperate	1985	Mosquito	GI-b	DQ404132
YN86-86266	Yunnan, China	Temperate	1986	Unknown	GI-b	DQ404134
YN86-B8639	Yunnan, China	Temperate	1986	Mosquito	GI-b	DQ404133
JE-91	Korea	Temperate	1991	Mosquito	GI-b	GQ415355

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
K93A07	South Korea	Temperate	1993	Mosquito	GI-b	FJ938230
K94P05	Korea	Temperate	1994	Mosquito	GI-b	AF045551
K94A07	South Korea	Temperate	1994	Mosquito	GI-b	FJ938216
JaTAn 1/94	Tokyo, Japan	Temperate	1994	Swine	GI-b	AB237171
95-167	Japan	Temperate	1995	Swine	GI-b	AY377579
95-91	Japan	Temperate	1995	Swine	GI-b	AY377578
95P99	Oita, Japan	Temperate	1995	Swine	GI-b	FJ943471
K95A07	South Korea	Temperate	1995	Mosquito	GI-b	FJ938218
K96A07	South Korea	Temperate	1996	Mosquito	GI-b	FJ938219
97P82	Oita, Japan	Temperate	1997	Swine	GI-b	FJ943472
Ishikawa	Ishikawa, Japan	Temperate	1998	Mosquito	GI-b	AB051292
JEV/wb/Okinawa/1/1998	Okinawa, Japan	Temperate	1998	Swine	GI-b	AB306941
99P103	Oita, Japan	Temperate	1999	Swine	GI-b	FJ943473
99P104	Oita, Japan	Temperate	1999	Swine	GI-b	FJ943474
KV1899	South Korea	Temperate	1999	Swine	GI-b	AF474075
SH-101	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY555761
SH-53	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY555757
SH-80	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY243841
SH-81	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY555758
SH-83	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY555759
SH-90	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY243835
SH-96	Shanghai, China	Temperate	2001	Mosquito	GI-b	AY555760
K01-GN	South Korea	Temperate	2001	Mosquito	GI-b	FJ938220
K01-JB	South Korea	Temperate	2001	Mosquito	GI-b	FJ938221
K01-JN	South Korea	Temperate	2001	Mosquito	GI-b	FJ938222

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
VN88	Vietnam	Tropical	2001	Swine	GI-b	AY376464
JEV/sw/Chiba/88/2002	Chiba, Japan	Temperate	2002	Swine	GI-b	AB112705
JEV/sw/Hiroshima/25/2002	Hiroshima, Japan	Temperate	2002	Swine	GI-b	AB231465
JaNAr0102	Japan	Temperate	2002	Mosquito	GI-b	AY377577
JEV/sw/Kagawa/24/2002	Kagawa, Japan	Temperate	2002	Swine	GI-b	AB112706
JEV/sw/Kagawa/27/2002	Kagawa, Japan	Temperate	2002	Swine	GI-b	AB112707
LN02-102	Liaoning, China	Temperate	2002	Mosquito	GI-b	DQ404085
LN02-104	Liaoning, China	Temperate	2002	Mosquito	GI-b	DQ404086
JEV/sw/Mie/41/2002	Mie, Japan	Temperate	2002	Swine	GI-b	AB112709
JEV/sw/Shizuoka/33/2002	Shizuoka, Japan	Temperate	2002	Swine	GI-b	AB112703
JEV/sw/Shizuoka/39/2002	Shizuoka, Japan	Temperate	2002	Swine	GI-b	AB112704
VN105	Vietnam	Tropical	2002	Mosquito	GI-b	AY376468
VN22	Vietnam	Tropical	2002	Swine	GI-b	AY376465
VN34	Vietnam	Tropical	2002	Mosquito	GI-b	AY376466
VN78	Vietnam	Tropical	2002	Mosquito	GI-b	AY376467
03P113	Oita, Japan	Temperate	2003	Swine	GI-b	FJ943475
03P120	Oita, Japan	Temperate	2003	Swine	GI-b	FJ943476
03P126	Oita, Japan	Temperate	2003	Swine	GI-b	FJ943477
03P145	Oita, Japan	Temperate	2003	Swine	GI-b	FJ943478
03P189	Oita, Japan	Temperate	2003	Swine	GI-b	FJ943479
JEV/sw/Okinawa/285/2003	Okinawa, Japan	Temperate	2003	Swine	GI-b	AB238693
SH03-103	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404096
SH03-105	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404097
SH03-109	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404098
SH03-115	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404099

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
SH03-124	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404100
SH03-127	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404101
SH03-128	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404102
SH03-129	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404103
SH03-130	Shanghai, China	Temperate	2003	Mosquito	GI-b	DQ404104
JEV/eq/Tottori/2003	Tottori, Japan	Temperate	2003	Equid	GI-b	AB213007
HN04-11	Henan, China	Temperate	2004	Mosquito	GI-b	DQ404087
HN04-21	Henan, China	Temperate	2004	Mosquito	GI-b	DQ404088
HN04-40	Henan, China	Temperate	2004	Mosquito	GI-b	DQ404089
JaNAr07-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185144
JaNAr10-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185145
JaNAr13-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185146
JaNAr31-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185150
JaNAr32-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185151
JaNAr38-04	Isahaya, Nagasaki Prefecture, Japan	Temperate	2004	Mosquito	GI-b	FJ185152
JEV/sw/Kagawa/35/2004	Kagawa, Japan	Temperate	2004	Swine	GI-b	AB231464
JEV/sw/Mie/34/2004	Mie, Japan	Temperate	2004	Swine	GI-b	AB231462
JEV/sw/Mie/40/2004	Mie, Japan	Temperate	2004	Swine	GI-b	AB231463
SC04-12	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404090
SC04-15	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404091
SC04-16	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404092
SC04-17	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404093
SC04-25	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404094
SC04-27	Sichuan, China	Temperate	2004	Mosquito	GI-b	DQ404095
GX0519	Guangxi, China	Temperate	2005	Mosquito	GI-b	FJ161967

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
GX0523	Guangxi, China	Temperate	2005	Mosquito	GI-b	FJ161968
GX0558	Guangxi, China	Temperate	2005	Mosquito	GI-b	FJ161969
JE_KK_1116	Khon Khen, Thailand	Tropical	2005	Unknown	GI-b	DQ343290
05P75	Oita, Japan	Temperate	2005	Swine	GI-b	FJ943480
SH05-24	Shanghai, China	Temperate	2005	Mosquito	GI-b	DQ404108
K05-GS	South Korea	Temperate	2005	Mosquito	GI-b	FJ938223
Mo/Toyama/1089c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538603
Mo/Toyama/1089v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538604
Mo/Toyama/1148c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538605
Mo/Toyama/1148v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538606
Mo/Toyama/1149c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538607
Mo/Toyama/1155c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538608
Mo/Toyama/1155v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538609
Mo/Toyama/1157c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538610
Mo/Toyama/1158c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538611
Mo/Toyama/1158v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538612
Mo/Toyama/1160c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538613
Mo/Toyama/1161c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538614
Mo/Toyama/1161v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538615
Mo/Toyama/1222c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538616
Mo/Toyama/1222v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538617
Mo/Toyama/1256c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538618
Mo/Toyama/1256v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538619
Sw/Toyama/05197v/2005	Toyama, Japan	Temperate	2005	Swine	GI-b	AB538823
Sw/Toyama/05231v/2005	Toyama, Japan	Temperate	2005	Swine	GI-b	AB538824

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
Mo/Toyama/1018c/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538601
Mo/Toyama/1018v/2005	Toyama, Japan	Temperate	2005	Mosquito	GI-b	AB538602
CT-MO-P7	Vietnam	Tropical	2005	Swine	GI-b	HQ009266
LA_H06-05	Vietnam	Tropical	2005	Mosquito	GI-b	FJ185153
LA_H07-05	Vietnam	Tropical	2005	Mosquito	GI-b	FJ185154
LA-H-5330	Vietnam	Tropical	2005	Swine	GI-b	HQ009265
LAH_2079-05	Vietnam	Tropical	2005	Mosquito	GI-b	FJ185155
06P152	Oita, Japan	Temperate	2006	Swine	GI-b	FJ943481
06P183	Oita, Japan	Temperate	2006	Swine	GI-b	FJ943483
06P212	Oita, Japan	Temperate	2006	Swine	GI-b	FJ943484
HEN0701	Henan, China	Temperate	2007	Swine	GI-b	FJ156730
JaNAr06-07	Isahaya, Nagasaki Prefecture, Japan	Temperate	2007	Mosquito	GI-b	FJ185143
JaNAr14-07	Isahaya, Nagasaki Prefecture, Japan	Temperate	2007	Mosquito	GI-b	FJ185147
JaNAr15-07	Isahaya, Nagasaki Prefecture, Japan	Temperate	2007	Mosquito	GI-b	FJ185148
JaNAr17-07	Isahaya, Nagasaki Prefecture, Japan	Temperate	2007	Mosquito	GI-b	FJ185149
07P127	Oita, Japan	Temperate	2007	Swine	GI-b	FJ943487
07P83	Oita, Japan	Temperate	2007	Swine	GI-b	FJ943485
07P90	Oita, Japan	Temperate	2007	Swine	GI-b	FJ943486
Mo/Toyama/2347c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538658
Mo/Toyama/2441c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538659
Mo/Toyama/2462c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538660
Mo/Toyama/2506c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538661
Mo/Toyama/2507c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538662
Mo/Toyama/2513c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538663
Mo/Toyama/2513v/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538664

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
Mo/Toyama/2554c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538665
Mo/Toyama/2554v/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538666
Mo/Toyama/2556c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538667
Mo/Toyama/2556v/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538668
Mo/Toyama/2567c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538669
Mo/Toyama/2569c/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538670
Mo/Toyama/2569v/2007	Toyama, Japan	Temperate	2007	Mosquito	GI-b	AB538671
Sw/Toyama/07232c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538825
Sw/Toyama/07234c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538826
Sw/Toyama/07240c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538827
Sw/Toyama/07292c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538828
Sw/Toyama/07292v/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538829
Sw/Toyama/07296c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538830
Sw/Toyama/07326c/2007	Toyama, Japan	Temperate	2007	Swine	GI-b	AB538831
07VN310	Vietnam	Tropical	2007	Mosquito	GI-b	HM228922
07VN311	Vietnam	Tropical	2007	Mosquito	GI-b	HM228923
XJ69	Zhejiang, China	Temperate	2007	Mosquito	GI-b	EU258742
XJP613	Zhejiang, China	Temperate	2007	Mosquito	GI-b	EU258741
Japanese wild boar	Hyogo, Nishinomiya Prefecture, Japan	Temperate	2008	Swine	GI-b	AB481224
08P37	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943488
08P38	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943489
08P42	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943490
08P48	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943491
08P49	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943492
08P54	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943493

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
08P62	Oita, Japan	Temperate	2008	Swine	GI-b	FJ943494
JEV/sw/Okinawa/154/2008	Okinawa, Japan	Temperate	2008	Swine	GI-b	AB471666
JEV/sw/Okinawa/254/2008	Okinawa, Japan	Temperate	2008	Swine	GI-b	AB471667
JEV/sw/Okinawa/372/2008	Okinawa, Japan	Temperate	2008	Swine	GI-b	AB471668
JEV/sw/Okinawa/377/2008	Okinawa, Japan	Temperate	2008	Swine	GI-b	AB471669
JEV/sw/Okinawa/402/2008	Okinawa, Japan	Temperate	2008	Swine	GI-b	AB471670
TPC0806c	Taipei County, Taiwan	Temperate	2008	Mosquito	GI-b	GQ260635
Mo/Toyama/2757c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538700
Mo/Toyama/2759c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538701
Mo/Toyama/2794c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538702
Mo/Toyama/2794v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538703
Mo/Toyama/2795c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538704
Mo/Toyama/2795v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538705
Mo/Toyama/2805c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538706
Mo/Toyama/2805v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538707
Mo/Toyama/2808c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538708
Mo/Toyama/2821c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538709
Mo/Toyama/2842c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538710
Mo/Toyama/2842v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538711
Mo/Toyama/2853c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538712
Mo/Toyama/2853v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538713
Mo/Toyama/2886c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538714
Mo/Toyama/2886v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538715
Mo/Toyama/2888c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538716
Mo/Toyama/2895c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538717

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
Mo/Toyama/2905c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538718
Mo/Toyama/2906c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538719
Mo/Toyama/2909c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538720
Mo/Toyama/2910c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538721
Mo/Toyama/2915c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538722
Mo/Toyama/2917c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538723
Mo/Toyama/2918c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538724
Mo/Toyama/2929c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538725
Mo/Toyama/2929v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538726
Mo/Toyama/2967c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538727
Mo/Toyama/2967v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538728
Mo/Toyama/2976c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538729
Mo/Toyama/2976v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538730
Mo/Toyama/2977c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538731
Mo/Toyama/2977v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538732
Mo/Toyama/2984c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538733
Mo/Toyama/2984v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538734
Mo/Toyama/2985c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538735
Mo/Toyama/2985v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538736
Mo/Toyama/2986c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538737
Mo/Toyama/2986v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538738
Mo/Toyama/2987c/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538739
Mo/Toyama/2987v/2008	Toyama, Japan	Temperate	2008	Mosquito	GI-b	AB538740
Sw/Toyama/08253c/2008	Toyama, Japan	Temperate	2008	Swine	GI-b	AB538832
YL0806f	Yilan County, Taiwan	Temperate	2008	Mosquito	GI-b	GQ260633

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
FQ24M-08	Yunnan, China	Temperate	2008	Mosquito	GI-b	HM204531
XP174M-08	Yunnan, China	Temperate	2008	Mosquito	GI-b	HM204527
09P123	Oita, Japan	Temperate	2009	Swine	GI-b	GU108334
09P141	Oita, Japan	Temperate	2009	Swine	GI-b	GU108335
LY5P-09	Shanxi, China	Temperate	2009	Human	GI-b	HM204530
JEV-CZ1	Sichuan, China	Temperate	2009	Mosquito	GI-b	HM234673
Mo/Toyama/3133c/2009	Toyama, Japan	Temperate	2009	Mosquito	GI-b	AB543738
Mo/Toyama/3140c/2009	Toyama, Japan	Temperate	2009	Mosquito	GI-b	AB543739
Mo/Toyama/3141c/2009	Toyama, Japan	Temperate	2009	Mosquito	GI-b	AB543740
LX10P-09	Yunnan, China	Temperate	2009	Human	GI-b	HM204528
LX29P-09	Yunnan, China	Temperate	2009	Human	GI-b	HM204529
Nakayama	Nakayama, Japan	Temperate	1935	Human	GIII	EF571853
Matsunaga	Japan	Temperate	1939	Human	GIII	FJ872381
Roum	Korea	Temperate	1946	Human	GIII	FJ872377
Taira	Japan	Temperate	1948	Human	GIII	FJ872384
Beijing-1	Beijing, China	Temperate	1949	Mosquito	GIII	L48961
Equine	Japan	Temperate	1949	Equid	GIII	FJ872378
K-29	Korea	Temperate	1949	Human	GIII	GQ415356
V9-3901	Japan	Temperate	1950	Human	GIII	FJ872382
V9-3902	Japan	Temperate	1950	Human	GIII	FJ872383
V9-4399	Japan	Temperate	1950	Human	GIII	FJ872380
Korea Jap B	Korea	Temperate	1950	Human	GIII	FJ872379
JaOArK151	Oita Prefecture, Japan	Temperate	1951	Unknown	GIII	AB028255
SA14	China	Temperate	1954	Mosquito	GIII	U14163
CBH	Fujian, China	Temperate	1954	Human	GIII	DQ404116

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
CZX	Fujian, China	Temperate	1954	Human	GIII	AY243828
G35	Fujian, China	Temperate	1954	Mosquito	GIII	AY243831
CTS	Fujian, China	Temperate	1955	Human	GIII	AY243830
LFM	Fujian, China	Temperate	1955	Human	GIII	AY243833
YLG	Fujian, China	Temperate	1955	Human	GIII	AY243837
ZMT	Fujian, China	Temperate	1955	Human	GIII	AY243840
ZSZ	Fujian, China	Temperate	1955	Human	GIII	AY243839
LYZ	Fujian, China	Temperate	1957	Human	GIII	AY243834
CH-13	Sichuan, China	Temperate	1957	Human	GIII	AY243829
HVI	Taiwan	Unknown	1958	Mosquito	GIII	AF098735
JaTH160	Tokyo, Japan	Temperate	1960	Human	GIII	AB269326
JaGAr01	Japan	Temperate	1965	Human	GIII	U44964
Ling	Ling, Taiwan	Temperate	1965	Human	GIII	L78128
TC	Taiwan	Unknown	1965	Mosquito	GIII	AF098736
TL	Taiwan	Unknown	1965	Mosquito	GIII	AF098737
JaOH0566	Japan	Temperate	1966	Human	GIII	AY029207
Kamiyama 1	Kamiyama, Japan	Temperate	1966	Human	GIII	S47265
JaOAr404	Oita Prefecture, Japan	Temperate	1968	Unknown	GIII	AB028250
Oita100-69	Oita Prefecture, Japan	Temperate	1969	Unknown	GIII	AB028269
691004	Sri Lanka	Tropical	1969	Human	GIII	Z34097
MIe731-70	Mie, Japan	Temperate	1970	Unknown	GIII	AB028271
JaNAr516-70	Nagasaki Prefecture, Japan	Temperate	1970	Unknown	GIII	AB028270
JaOAr363	Oita Prefecture, Japan	Temperate	1970	Unknown	GIII	AB028252
Tla	Heilongjiang, China	Temperate	1971	Human	GIII	AY243826
JaOAr72	Okinawa, Japan	Temperate	1972	Mosquito	GIII	AB569990

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
733913	Bankura, West Bengal, India	Tropical	1973	Human	GIII	EU372660
JaOAr73050	Okinawa, Japan	Temperate	1973	Swine	GIII	AB569987
JaOAr73055	Okinawa, Japan	Temperate	1973	Swine	GIII	AB569988
JaOAr73062	Okinawa, Japan	Temperate	1973	Swine	GIII	AB569989
JaOS73620	Okinawa, Japan	Temperate	1973	Swine	GIII	AB569978
JaOS73832	Okinawa, Japan	Temperate	1973	Swine	GIII	AB569979
JaOAr74010	Okinawa, Japan	Temperate	1974	Mosquito	GIII	AB569994
JaOAr74011	Okinawa, Japan	Temperate	1974	Mosquito	GIII	AB569995
JaOS74728	Okinawa, Japan	Temperate	1974	Swine	GIII	AB569992
JaOS74729	Okinawa, Japan	Temperate	1974	Swine	GIII	AB569993
JaOS74801	Okinawa, Japan	Temperate	1974	Swine	GIII	AB569996
JaOS75571	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569980
JaOS75642	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569981
JaOS75672	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569982
JaOS75722	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569983
JaOS75770	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569984
JaOS75833	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569985
JaOS75918	Okinawa, Japan	Temperate	1975	Swine	GIII	AB569986
JaOAr76075	Okinawa, Japan	Temperate	1976	Mosquito	GIII	AB569991
GP78	Gorakhpur, India	Temperate	1978	Mosquito	GIII	AF075723
Liyujie	Yunnan, China	Temperate	1979	Human	GIII	FJ185039
80P136	Japan	Temperate	1980	Swine	GIII	FJ943462
80P205	Japan	Temperate	1980	Swine	GIII	FJ943463
81P241	Japan	Temperate	1981	Swine	GIII	FJ943464
81P244	Japan	Temperate	1981	Swine	GIII	FJ943465

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
JE-82	Korea	Temperate	1982	Mosquito	GIII	GQ415347
JaOArS982	Osaka, Japan	Temperate	1982	Mosquito	GIII	NC_001437
BN19	Yunnan, China	Temperate	1982	Mosquito	GIII	FJ185038
JE-83	Korea	Temperate	1983	Mosquito	GIII	GQ415348
cc27	Pingtun County, Taiwan	Tropical	1983	Mosquito	GIII	U44957
K83P34	South Korea	Temperate	1983	Mosquito	GIII	FJ938231
K83P44	South Korea	Temperate	1983	Mosquito	GIII	FJ938232
JE-84	Korea	Temperate	1984	Mosquito	GIII	GQ415349
CC94	Pingtun County, Taiwan	Tropical	1984	Mosquito	GIII	U44958
K84A071	South Korea	Temperate	1984	Mosquito	GIII	FJ938224
JE-85	Korea	Temperate	1985	Mosquito	GIII	GQ415350
CN80	Miaoli County, Taiwan	Temperate	1985	Mosquito	GIII	U44962
JaOArS4385	Oita Prefecture, Japan	Temperate	1985	Unknown	GIII	AB028258
JaOArS5485	Oita Prefecture, Japan	Temperate	1985	Unknown	GIII	AB028260
JaOArS7485	Oita Prefecture, Japan	Temperate	1985	Unknown	GIII	AB028259
NT109	Tiachung County, Taiwan	Temperate	1985	Mosquito	GIII	U44967
NT113	Tiachung County, Taiwan	Temperate	1985	Mosquito	GIII	U44968
CH109	Changhua County, Taiwan	Temperate	1986	Mosquito	GIII	U44959
JE-86	Korea	Temperate	1986	Mosquito	GIII	GQ415351
JaOArK3786	Oita Prefecture, Japan	Temperate	1986	Unknown	GIII	AB028261
JaOArK7286	Oita Prefecture, Japan	Temperate	1986	Unknown	GIII	AB028263
JaOArS1186	Oita Prefecture, Japan	Temperate	1986	Unknown	GIII	AB028262
VN206	Vietnam	Tropical	1986	Human	GIII	AY376460
VN207	Vietnam	Tropical	1986	Human	GIII	AY376461
JKT27-085	Central Java, Indonesia	Tropical	1987	Mosquito	GIII	JQ429307

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
JKT27-087	Central Java, Indonesia	Tropical	1987	Mosquito	GIII	JQ429308
CH392	Changhua County, Taiwan	Temperate	1987	Mosquito	GIII	U44961
JE-87	Korea	Temperate	1987	Mosquito	GIII	GQ415352
K87P39	Korea	Temperate	1987	Mosquito	GIII	U34927
SH-3	Shanghai, China	Temperate	1987	Human	GIII	AY243836
K87A07	South Korea	Temperate	1987	Mosquito	GIII	FJ938225
K87A071	South Korea	Temperate	1987	Mosquito	GIII	FJ938226
JE-88	Korea	Temperate	1988	Mosquito	GIII	GQ415353
JaOArK6688	Oita Prefecture, Japan	Temperate	1988	Unknown	GIII	AB028264
K88A07	South Korea	Temperate	1988	Mosquito	GIII	FJ938227
K88A071	South Korea	Temperate	1988	Mosquito	GIII	FJ938228
89P131	Japan	Temperate	1989	Swine	GIII	FJ943467
89P141	Japan	Temperate	1989	Swine	GIII	FJ943468
89P149	Japan	Temperate	1989	Swine	GIII	FJ943469
89P160	Japan	Temperate	1989	Swine	GIII	FJ943470
89P49	Japan	Temperate	1989	Swine	GIII	FJ943466
JE-89	Korea	Temperate	1989	Mosquito	GIII	GQ415354
JaOArK5789	Oita Prefecture, Japan	Temperate	1989	Unknown	GIII	AB028265
JaOArK6289	Oita Prefecture, Japan	Temperate	1989	Unknown	GIII	AB028266
K89A07	South Korea	Temperate	1989	Mosquito	GIII	FJ938229
VN49	Vietnam	Tropical	1989	Human	GIII	AY376462
VN50	Vietnam	Tropical	1989	Human	GIII	AY376463
CH1392	Changhua County, Taiwan	Temperate	1990	Mosquito	GIII	U44960
JaNAr0290	Nagasaki Prefecture, Japan	Temperate	1990	Mosquito	GIII	AY427794
JaNAr0590	Nagasaki Prefecture, Japan	Temperate	1990	Mosquito	GIII	AY427795

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
JaNAr0690	Nagasaki Prefecture, Japan	Temperate	1990	Mosquito	GIII	AY427796
JaNAr0990	Nagasaki Prefecture, Japan	Temperate	1990	Mosquito	GIII	AY427797
JaOArK3990	Oita Prefecture, Japan	Temperate	1990	Unknown	GIII	AB028267
JaOArK5990	Oita Prefecture, Japan	Temperate	1990	Unknown	GIII	AB028268
CH1949	Changhua, Taiwan	Temperate	1992	Unknown	GIII	AF030549
CH2195	Changhua, Taiwan	Temperate	1994	Unknown	GIII	AF030550
K94A071	South Korea	Temperate	1994	Mosquito	GIII	FJ938217
T1P1	Liu Chi Islet, Taiwan	Tropical	1997	Mosquito	GIII	AF254453
YN03-A151	Yunnan, China	Temperate	1998	Mosquito	GIII	DQ404136
14178	Lakhimpur, India	Temperate	2001	Human	GIII	EF623987
02-41	Fujian, China	Temperate	2002	Human	GIII	AY555763
02-43	Fujian, China	Temperate	2002	Human	GIII	AY555764
02-76	Fujian, China	Temperate	2002	Human	GIII	AY555765
02-84	Fujian, China	Temperate	2002	Human	GIII	AY555766
02-29	Fujian, China	Temperate	2002	Human	GIII	AY555762
02-102	Fujian, China	Temperate	2002	Human	GIII	AY555767
HLJ02-134	Heilongjiang, China	Temperate	2002	Midge	GIII	DQ404081
HLJ02-136	Heilongjiang, China	Temperate	2002	Midge	GIII	DQ404082
HLJ02-144	Heilongjiang, China	Temperate	2002	Mosquito	GIII	DQ404083
HLJ02-170	Heilongjiang, China	Temperate	2002	Mosquito	GIII	DQ404084
04940-4	Maharashtra, India	Tropical	2002	Mosquito	GIII	EF623989
TN207	Taiwan	Unknown	2002	Mosquito	GIII	EU683895
FJ03-31	Fujian, China	Temperate	2003	Human	GIII	DQ404117
FJ03-35	Fujian, China	Temperate	2003	Human	GIII	DQ404118
FJ03-39	Fujian, China	Temperate	2003	Human	GIII	DQ404119

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
FJ03-46	Fujian, China	Temperate	2003	Human	GIII	DQ404120
FJ03-56	Fujian, China	Temperate	2003	Human	GIII	DQ404121
FJ03-66	Fujian, China	Temperate	2003	Human	GIII	DQ404122
FJ03-67	Fujian, China	Temperate	2003	Human	GIII	DQ404123
FJ03-68	Fujian, China	Temperate	2003	Human	GIII	DQ404124
FJ03-69	Fujian, China	Temperate	2003	Human	GIII	DQ404125
FJ03-94	Fujian, China	Temperate	2003	Human	GIII	DQ404126
FJ03-97	Fujian, China	Temperate	2003	Human	GIII	DQ404127
GZ04-2	Guizhou, China	Temperate	2004	Mosquito	GIII	DQ404109
GZ04-4	Guizhou, China	Temperate	2004	Mosquito	GIII	DQ404110
GZ04-43	Guizhou, China	Temperate	2004	Mosquito	GIII	DQ404113
GZ04-71	Guizhou, China	Temperate	2004	Mosquito	GIII	DQ404114
GZ04-89	Guizhou, China	Temperate	2004	Mosquito	GIII	DQ404115
SH04-10	Shanghai, China	Temperate	2004	Mosquito	GIII	DQ404107
SH04-3	Shanghai, China	Temperate	2004	Mosquito	GIII	DQ404105
SH04-5	Shanghai, China	Temperate	2004	Mosquito	GIII	DQ404106
04VN75	Vietnam	Tropical	2004	Human	GIII	HQ009263
04VN79	Vietnam	Tropical	2004	Human	GIII	HQ009264
YNDL04-1	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404137
YNDL04-29	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404139
YNDL04-31	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404140
YNDL04-37	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404141
YNDL04-39	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404142
YNDL04-42	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404143
YNDL04-44	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404144

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
YNDL04-45	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404145
YNDL04-6	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404138
YNJH04-25-3	Yunnan, China	Temperate	2004	Mosquito	GIII	DQ404148
FJ05-139	Fujian, China	Temperate	2005	Human	GIII	GQ856661
FJ05-62	Fujian, China	Temperate	2005	Human	GIII	GQ856660
57434	Gorakhpur, India	Temperate	2005	Human	GIII	EF688625
HL0505a	Hualien County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260610
HL0506a	Hualien County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260615
KH0505a	Kaohsiung County, Taiwan	Tropical	2005	Mosquito	GIII	GQ260608
KH0505b	Kaohsiung County, Taiwan	Tropical	2005	Mosquito	GIII	GQ260609
TP0506a	Taipei County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260616
TC0506a	Tiachung County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260613
TC0506b	Tiachung County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260614
GP05	Uttar Pradesh, India	Temperate	2005	Human	GIII	FJ979830
YL0506a	Yilan County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260611
YL0506b	Yilan County, Taiwan	Temperate	2005	Mosquito	GIII	GQ260612
CSF-2522	Taiwan	Unknown	2006	Human	GIII	GQ260621
TC0605a	Tiachung County, Taiwan	Temperate	2006	Mosquito	GIII	GQ260618
TC0605b	Tiachung County, Taiwan	Temperate	2006	Mosquito	GIII	GQ260619
YL0605a	Yilan County, Taiwan	Temperate	2006	Mosquito	GIII	GQ260617
YL0606a	Yilan County, Taiwan	Temperate	2006	Mosquito	GIII	GQ260620
CH0706a	Changhua County, Taiwan	Temperate	2007	Swine	GIII	GQ260624
FJ07-51	Fujian, China	Temperate	2007	Human	GIII	GQ856662
HL0706a	Hualien County, Taiwan	Temperate	2007	Mosquito	GIII	GQ260625
TN0705a	Tainan County, Taiwan	Tropical	2007	Swine	GIII	GQ260623

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
TPC0706b	Taipei City, Taiwan	Temperate	2007	Mosquito	GIII	GQ260627
TY0704a	Taoyuan County, Taiwan	Temperate	2007	Swine	GIII	GQ260622
TPC0706a	Taipei City, Taiwan	Temperate	2007	Mosquito	GIII	GQ260626
Mo/Chongmingdao/12/2008	Chongmingdao, China	Temperate	2008	Mosquito	GIII	GU253955
Mo/Deqing/11/2008	Deqing, China	Temperate	2008	Mosquito	GIII	GU253961
FJ08-48	Fujian, China	Temperate	2008	Human	GIII	GQ856663
FJ08-65	Fujian, China	Temperate	2008	Human	GIII	GQ856664
HLJ08-01	Heilongjiang, China	Temperate	2008	Swine	GIII	GQ495004
HLJ08-02	Heilongjiang, China	Temperate	2008	Swine	GIII	GQ495005
HL0805a	Hualien, Taiwan	Temperate	2008	Mosquito	GIII	GQ260628
Mo/Jintan/9/2008	Jintan, China	Temperate	2008	Mosquito	GIII	GU253954
Mo/Nanjing/10/2008	Nanjing, China	Temperate	2008	Mosquito	GIII	GU253951
TC0806a	Taichung County, Taiwan	Temperate	2008	Mosquito	GIII	GQ260630
TPC0806a	Taipei City, Taiwan	Temperate	2008	Mosquito	GIII	GQ260634
YL0805a	Yilan County, Taiwan	Temperate	2008	Mosquito	GIII	GQ260629
YL0806a	Yilan County, Taiwan	Temperate	2008	Mosquito	GIII	GQ260631
YL0806e	Yilan County, Taiwan	Temperate	2008	Mosquito	GIII	GQ260632
Mo/Haimen/3/2009	Haimen, China	Temperate	2009	Mosquito	GIII	GU253947
Mo/Haimen/6/2009	Haimen, China	Temperate	2009	Mosquito	GIII	GU253950
Mo/Qidong/4/2009	Haimen, China	Temperate	2009	Mosquito	GIII	GU253948
JEV/eq/Haryana/H225/2009	Haryana, India	Temperate	2009	Equid	GIII	GQ387646
JEV/eq/India/H225/2009	Hisar, India	Temperate	2009	Equid	GIII	HQ018880
Sw(blood)/Jintan/15/2009	Jintan, China	Temperate	2009	Swine	GIII	GU253953
Sw(sperm)/Ningbo/13/2009	Ningbo, China	Temperate	2009	Swine	GIII	GU253956
Mo/Qidong/5/2009	Qidong, China	Temperate	2009	Mosquito	GIII	GU253949

Isolate	Country	Climate	Year	Host	Genotype	GenBank accession number
Sw(sperm)/Rizhao/8/2009	Rizhao, China	Temperate	2009	Swine	GIII	GU253960
Sw(blood)/Rugao/14/2009	Rugao, China	Temperate	2009	Swine	GIII	GU253957
JEV-NJ1	Sichuan, China	Temperate	2009	Mosquito	GIII	HM234674
Mo/Yixing/2/2009	Yixing, China	Temperate	2009	Mosquito	GIII	GU253959
Mo/Zhenjiang/1/2009	Zhenjiang, China	Temperate	2009	Mosquito	GIII	GU253958

Table 7.2: Descriptive analysis of the GIII and GI isolates used in this study.

		GIII dataset (n=219)		GI dataset (n=234)	
		n	% of total	n	% of total
Host	Equid	3	1.37	1	0.43
	Human	60	27.40	3	1.28
	Midge	2	0.91	1	0.43
	Mosquito	102	46.58	153	65.38
	Swine	33	15.07	65	27.78
	Unknown	19	8.68	11	4.70
Decade	1930s	2	0.91	0	0.00
	1940s	5	2.28	0	0.00
	1950s	17	7.76	0	0.00
	1960s	10	4.57	1	0.43
	1970s	26	11.87	1	0.43
	1980s	51	23.29	7	2.99
	1990s	12	5.48	16	6.84
	2000s	96	43.84	209	89.32
Origin	Australia	0	0.00	1	0.43
	Cambodia	0	0.00	1	0.43
	China	78	35.62	47	20.09
	India	8	3.65	0	0.00
	Indonesia	2	0.91	0	0.00
	Japan	61	27.85	146	62.39
	Korea	21	9.59	11	4.70
	Sri Lanka	1	0.46	0	0.00
	Taiwan	42	19.18	2	0.85
	Thailand	0	0.00	14	5.98
	Vietnam	6	2.74	12	5.13

Table 7.3: Data points utilized to plot the GIII and GI Bayesian skyline reconstructions.

Dataset	Time	Mean N_et^{-1}	N_et^{-1} upper 95% HPD	N_et^{-1} lower 95% HPD
GIII	2009.00	56.63	159.25	12.66
	2008.06	53.52	134.51	14.27
	2007.12	56.84	130.39	19.74
	2006.18	65.08	144.97	24.77
	2005.25	72.51	172.35	27.57
	2004.31	83.16	207.46	25.69
	2003.37	118.55	286.70	36.62
	2002.43	132.03	326.83	40.40
	2001.49	116.01	320.67	21.96
	2000.55	83.70	242.60	13.80
	1999.61	80.31	182.24	19.93
	1998.67	89.70	183.73	27.32
	1997.74	97.51	192.45	32.95
	1996.80	103.03	202.77	39.57
	1995.86	107.28	208.86	45.41
	1994.92	109.39	212.32	48.51
	1993.98	110.64	211.76	52.64
	1993.04	111.39	210.76	53.96
	1992.10	112.03	210.55	54.25
	1991.16	113.72	216.50	54.83
	1990.23	113.92	215.51	54.99
	1989.29	115.29	215.76	54.86
	1988.35	118.39	223.60	54.15
	1987.41	123.78	239.03	52.05
	1986.47	136.28	273.90	57.55
	1985.53	149.50	305.36	68.45
	1984.59	159.35	323.95	75.24
	1983.65	166.49	337.39	79.20
	1982.72	173.91	366.86	76.24
	1981.78	180.36	383.12	78.17
	1980.84	185.66	399.76	83.04
	1979.90	186.30	400.96	84.37
	1978.96	182.96	396.03	80.14
	1978.02	177.62	383.98	75.15
	1977.08	167.07	358.00	63.47
	1976.14	157.77	342.95	58.46
	1975.21	148.07	321.91	52.68
	1974.27	131.13	269.87	44.71
	1973.33	119.36	242.25	39.17
	1972.39	118.01	246.88	38.53
	1971.45	142.43	374.08	43.94
	1970.51	179.14	464.35	53.06
	1969.57	215.64	525.98	62.47
	1968.63	251.13	585.55	74.17

Dataset	Time	Mean N_et^1	N_et^1 upper 95% HPD	N_et^1 lower 95% HPD
	1967.70	281.76	626.55	84.99
	1966.76	308.44	652.07	97.41
	1965.82	331.01	687.56	109.66
	1964.88	353.83	731.22	121.77
	1963.94	375.28	763.56	132.78
	1963.00	398.66	818.73	146.24
	1962.06	418.42	861.98	154.73
	1961.12	435.94	895.46	165.63
	1960.19	451.41	921.65	174.73
	1959.25	466.41	939.03	182.36
	1958.31	479.37	978.30	203.61
	1957.37	487.75	986.47	211.78
	1956.43	497.23	998.16	225.20
	1955.49	502.33	1009.30	230.40
	1954.55	508.79	1032.54	235.68
	1953.61	513.93	1053.51	238.52
	1952.68	519.35	1066.94	247.91
	1951.74	519.66	1070.37	243.61
	1950.80	520.15	1070.94	238.52
	1949.86	517.19	1065.06	229.35
	1948.92	512.56	1064.85	215.32
	1947.98	505.76	1057.54	198.43
	1947.04	499.25	1054.63	178.80
	1946.10	493.37	1054.63	168.64
	1945.17	488.47	1070.94	159.74
	1944.23	484.59	1086.17	144.59
	1943.29	476.67	1087.03	125.94
	1942.35	470.04	1087.03	114.89
	1941.41	463.56	1088.21	105.39
	1940.47	452.56	1076.01	92.30
	1939.53	439.05	1054.63	79.12
	1938.59	428.13	1062.08	67.33
	1937.66	407.90	1019.52	56.36
	1936.72	387.02	1007.73	48.92
	1935.78	359.04	969.57	39.05
	1934.84	330.51	946.05	34.81
	1933.90	292.52	906.31	29.45
	1932.96	247.99	848.55	26.82
	1932.02	204.20	787.40	24.39
	1931.08	171.20	724.19	22.20
	1930.15	140.73	655.70	20.60
	1929.21	119.84	601.07	19.93
	1928.27	100.87	513.07	19.25
	1927.33	87.87	459.06	18.03
	1926.39	77.56	373.26	17.32
	1925.45	71.85	313.47	16.43
	1924.51	66.18	251.10	15.51
	1923.57	61.97	204.79	14.53
	1922.64	58.43	176.96	14.18

Dataset	Time	Mean N_et^1	N_et^1 upper 95% HPD	N_et^1 lower 95% HPD
	1921.70	56.05	167.24	13.52
	1920.76	54.36	155.45	13.05
	1919.82	52.41	144.95	12.37
	1918.88	51.03	141.19	11.83
	1917.94	49.42	136.94	10.69
	1917.00	48.06	132.28	10.43
	1916.06	47.11	125.57	9.91
GI	2009.00	21.62	71.94	5.24
	2008.47	20.63	65.00	5.51
	2007.93	18.04	52.11	4.12
	2007.40	9.48	23.86	2.30
	2006.86	14.50	31.68	4.57
	2006.33	23.88	48.54	8.37
	2005.79	27.22	55.09	11.48
	2005.26	30.20	62.33	12.60
	2004.73	43.99	108.39	15.12
	2004.19	75.02	197.83	23.90
	2003.66	132.39	342.03	37.45
	2003.12	187.69	465.47	52.99
	2002.59	233.68	553.65	75.99
	2002.05	258.47	604.73	92.25
	2001.52	276.74	660.40	96.19
	2000.98	284.35	676.52	96.48
	2000.45	288.96	723.84	97.47
	1999.92	285.38	705.80	92.26
	1999.38	274.67	670.84	79.31
	1998.85	266.31	666.37	70.85
	1998.31	262.02	661.51	70.47
	1997.78	259.20	658.15	71.73
	1997.24	258.02	647.28	77.05
	1996.71	260.86	658.82	80.77
	1996.18	266.07	681.69	82.00
	1995.64	268.44	681.69	85.37
	1995.11	271.36	685.03	93.34
	1994.57	273.04	687.17	95.98
	1994.04	273.96	684.53	99.22
	1993.50	273.55	688.77	99.00
	1992.97	272.23	677.26	98.75
	1992.44	271.09	697.77	96.78
	1991.90	267.69	681.69	94.44
	1991.37	266.50	685.04	92.63
	1990.83	262.97	681.69	90.83
	1990.30	259.31	677.22	88.15
	1989.76	254.05	664.41	84.82
	1989.23	247.62	646.79	80.77
	1988.70	240.37	611.15	77.80
	1988.16	234.99	584.49	77.10
	1987.63	226.70	552.26	73.18
	1987.09	221.30	534.55	69.32

Dataset	Time	Mean N_et^1	N_et^1 upper 95% HPD	N_et^1 lower 95% HPD
	1986.56	214.33	514.93	65.78
	1986.02	209.14	499.56	63.89
	1985.49	201.00	477.15	60.53
	1984.95	195.21	458.39	58.25
	1984.42	189.05	445.55	55.15
	1983.89	183.73	434.47	51.67
	1983.35	179.33	423.40	48.47
	1982.82	175.00	411.34	44.69
	1982.28	169.76	400.80	42.23
	1981.75	163.93	392.29	38.25
	1981.21	155.90	375.26	33.17
	1980.68	146.47	360.28	29.25
	1980.15	138.73	349.06	24.70
	1979.61	131.36	341.60	22.78
	1979.08	120.11	317.09	20.41
	1978.54	102.65	284.04	18.70
	1978.01	91.67	257.29	18.06
	1977.47	84.12	244.21	16.74
	1976.94	78.08	234.74	15.83
	1976.41	72.21	221.65	15.24
	1975.87	68.19	214.50	14.33
	1975.34	64.14	202.13	13.67
	1974.80	61.24	197.36	13.25
	1974.27	57.98	186.26	12.80
	1973.73	55.44	178.43	12.34
	1973.20	53.54	167.77	12.31
	1972.67	52.01	160.55	12.17
	1972.13	50.30	153.15	11.90
	1971.60	49.26	147.56	11.89
	1971.06	48.37	142.25	11.93
	1970.53	47.44	137.35	11.93
	1969.99	46.89	132.69	11.93
	1969.46	46.47	130.73	11.96
	1968.92	46.31	129.98	11.99
	1968.39	46.13	128.75	11.93
	1967.86	45.99	130.19	11.93
	1967.32	45.57	127.74	11.93
	1966.79	45.64	126.64	12.04
	1966.25	45.37	122.38	12.04
	1965.72	45.21	120.79	12.14
	1965.18	45.06	119.98	12.14
	1964.65	44.91	117.04	12.14
	1964.12	44.97	117.04	12.14
	1963.58	44.93	117.29	12.09
	1963.05	44.97	117.29	12.12
	1962.51	44.95	116.78	12.12
	1961.98	44.86	116.45	12.04
	1961.44	44.80	116.45	11.99
	1960.91	44.86	117.04	11.99

Dataset	Time	Mean N_et[1]	N_et[1] upper 95% HPD	N_et[1] lower 95% HPD
	1960.38	44.83	116.45	12.04
	1959.84	44.86	116.45	12.04
	1959.31	44.88	116.15	11.99
	1958.77	44.85	116.15	11.96
	1958.24	44.93	117.62	11.93
	1957.70	44.89	117.62	11.89
	1957.17	44.98	117.63	11.89
	1956.64	45.09	119.31	11.82
	1956.10	45.06	119.50	11.79

[1]N_et: Ne is the effective population size and t is the generation time.

Table 7.4: GI phylogeographic analysis.

Node[1]	Group	Node PP	Date of the MRCA (95% HPD)	State PP							
				Australia	Cambodia	China	Japan	Korea	Taiwan	Thailand	Vietnam
1	GI	1.00	1907 (1836, 1956)	0.09	0.15	0.06	0.01	0.03	0.03	0.31	0.32
2	GI-a	0.59	1940 (1903, 1964)	0.11	0.27	0.01	0.00	0.01	0.02	0.42	0.15
3	GI-b	0.98	1953 (1930, 1970)	0.01	0.01	0.30	0.02	0.01	0.01	0.01	0.64

[1]As indicated in the GI Bayesian MCC phylogeny.

Table 7.5: Analysis of GI phylogeographic structure.

Statistic	Observed mean (95% CI)	Null mean (95% CI)	p-value
AI	1.83 (1.44, 2.19)	14.26 (13.06, 15.48)	0.00
PS	35.48 (32.00, 39.00)	115.11 (110.87, 118.92)	0.00
UniFrac	0.60 (0.51, 0.68)	0.16 (0.11, 0.23)	1.00
NT	197.98 (150.13, 261.41)	372.02 (329.27, 417.69)	0.00
NR	72739.86 (61017.91, 88414.26)	106058.66 (100531.45, 110978.19)	0.00
PD	2145.68 (1623.44, 2962.73)	3172.13 (3029.94, 3318.33)	0.00
MC, Australia	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	1.00
MC, Cambodia	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	1.00
MC, China	11.09 (11.00, 11.00)	2.24 (1.85, 3.00)	< 0.00
MC, Japan	84.00 (84.00, 84.00)	6.17 (4.72, 9.43)	< 0.00
MC, Korea	3.17 (3.00, 5.00)	1.15 (1.00, 1.99)	0.00
MC, Taiwan	1.04 (1.00, 1.00)	1.01 (1.00, 1.00)	1.00
MC, Thailand	11.81 (12.00, 12.00)	1.23 (1.00. 2.00)	< 0.00
MC, Vietnam	3.71 (3.00, 4.00)	1.18 91.00, 2.00)	< 0.00

Table 7.6: Evolutionary parameters estimated from the GIII and GI datasets.

Evolutionary parameter	GIII	GI
Mean evolutionary rate (95% HPD)[1]	3.21 (2.66, 3.76)	5.05 (3.44, 6.58)
Mean evolutionary rate at CP_{12} (95% HPD)[1]	12.01 (8.30, 15.84)	1.57 (1.03, 2.11)
Mean evolutionary rate at CP_3 (95% HPD)[1]	6.93 (5.73, 8.21)	1.36 (1.08, 1.64)
CP_{12}/CP_3 ratio	0.20 (0.16, 0.23)	0.13 (0.11, 0.16)
Global d_N subs	294.54	170.02
Global d_S subs	823.52	852.01
Global d_N/d_S ratio (95% CI)	0.18 (0.16, 0.20)	0.10 (0.08, 0.11)

[1]Units: 10^{-4} nucleotide substitutions/site/year

Table 7.7: FEL and IFEL analyses of the GIII and GI E gene sequence alignments.

Dataset	Method	Codon	Normalized d_N/d_S	p-value
GIII	FEL	227	1.93	0.03
	FEL	261	1.86	0.05
	FEL	306	4.94	0.03
	IFEL	51	2.75	0.04
	IFEL	227	3.27	0.01
GI	FEL	15	2.06	0.03
	IFEL	126	2.02	0.04

Table 7.8: DEPS analyses of the GIII and GI E protein alignments.

Dataset	Residue	p-value	Bias	Proportion (%)	No. of sites
GIII	R	< 0.00	15.31	5.00	3
	M	< 0.00	89.30	1.00	1
	P	< 0.00	42.78	1.10	1
	G	< 0.00	14.15	2.70	1
GI	N	< 0.00	9.04	5.35	1
	T	< 0.00	19.15	1.58	1
	V	< 0.00	3.21	19.96	2
	Y	< 0.00	15.73	7.46	2

Table 7.9: Amino acid sites within the E protein of GIII and GI identified by the DEPS analyses to be under directional selection.

Dataset	Site	MRCA residue	Target residue	Inferred substitutions[1]
GIII	62	H	R	$G_4\leftrightarrow_1H$, $H_3\leftrightarrow_0R$, $H_8\leftrightarrow_{22}Y$
	76	T	M	$A_0\leftrightarrow_2T$, $M_0\leftrightarrow_5T$
	123	S	R	$N_0\leftrightarrow_1S$, $R_0\leftrightarrow_6S$
	209	K	R	$K_8\leftrightarrow_0R$
	227	S	P	$P_0\leftrightarrow_6S$
	306	E	G	$E_7\leftrightarrow_0G$, $E_1\leftrightarrow_0K$
GI	15	A	V	$A_0\leftrightarrow_1S$, $A_0\leftrightarrow_{10}V$
	87	D	Y	$D_0\leftrightarrow_6Y$
	123	S	N	$K_1\leftrightarrow_02$, $N_{16}\leftrightarrow_0S$
	129	M	T	$M_0\leftrightarrow_8T$
	141	I	V	$I_0\leftrightarrow_{15}V$
	360	F	Y	$F_0\leftrightarrow_5Y$

[1]Amino acid substitutions inferred when $A_b\leftrightarrow_cD$ indicates b substitutions from A to D and c substitutions from D to A.

Table 7.10: Spidermonkey analyses of the GIII and GI E protein alignments.

Dataset	Site 1	Site 2	PP	Supporting replicates (%)
GIII	T76M	K209R	0.99	99
	T76M	E306G	0.94	99
	I126V	V492M	0.99	99
	P132A	V143G	0.97	99
	I176T	S51V	0.94	99
	S227P	S51V	0.96	99
	S276N	S51V	0.92	99
	G318V	S229L	0.94	99
	S364P	S123R	0.98	99
	S364P	T363A	0.99	99
	S364P	S408L	0.99	99
	K398R	T363L	0.98	99
	A418V	S51V	0.95	99
	T487I	K312R	0.98	99
GI	I141V	M129T	0.98	99
	F360Y	S89N	0.93	99

Table 7.11: Molecular adaption detected in the E protein of GIII and GI using various methods.

Dataset	Site	Domain	FEL	IFEL	DEPS	Spidermonkey
GIII	51	I		X		X (176, 227, 276, 418)
	62	II			X	
	76	II			X	X (209, 306)
	123	I			X	X (364)
	126	I				X (492)
	132	I				X (143)
	143	I				X (132)
	176	I				X (51)
	209	I			X	X (76)
	227	II	X	X	X	X (51)
	229	II				X (318)
	261	II	X			
	276	I				X (51)
	306	III	X		X	X (76)
	312	III				X (487)
	318	III				X (229)
	363	III				X (364, 398)
	364	III				X (123, 363, 408)
	398	III				X (363)
	408	Stem-anchor				X (364)
	418	Stem-anchor				X (51)
	487	Stem-anchor				X (312)
	492	Stem-anchor				X (126)
GI	15	I	X		X	
	87	II			X	
	89	II				X (360)
	123	I			X	
	126	I		X		
	129	I			X	X (141)
	141	I			X	X (129)
	169	I				
	360	III			X	X (89)

Table 7.12: Details of the virus isolates used in the viral replication studies.

Isolate	Genotype	Origin	Year	Host	Passage number
KE-93-83	I-a	Thailand	1983	Human	4
JE-91	I-b	Korea	1991	Mosquito	3
JKT1724	II	Indonesia	1979	Mosquito	7
Bennett	II	Korea	1951	Human	5
Taira	III	Japan	1948	Human	5
Matsunaga	III	Japan	1939	Human	3
JKT6468	IV	Indonesia	1981	Mosquito	9
JKT7089	IV	Indonesia	1981	Mosquito	5

Table 7.13: ANOVA for C6/36 infectious titers according to HPI.

HPI	F	p-value
0	0.82	0.59
12	0.65	0.71
24	6.39	< 0.00
36	5.20	< 0.00
48	8.30	0.00
72	5.90	< 0.00
96	2.10	0.10
120	1.77	0.16
144	1.72	0.17
168	0.84	0.57
192	7.97	0.00
216	0.81	0.59

Table 7.14: Tukey's post-hoc analysis.

Comparison	HPI			
	24	36	48	72
JE-91*KE-93-83	< 0.00	< 0.00	< 0.00	< 0.00
JE-91*JKT1724	< 0.00	< 0.00	< 0.00	< 0.00
JE-91*Bennett	< 0.00	0.03	< 0.00	0.01
JE-91*Taira	< 0.00	0.02	0.01	0.14
JE-91*Matsunaga	< 0.00	0.01	< 0.00	0.02
JE-91*JKT6468	< 0.00	< 0.00	< 0.00	< 0.00
JE-91*JKT7089	< 0.00	< 0.00	< 0.00	< 0.00

Figure 7.1: Relative proportions of GIII and GI according to the decade of collection.

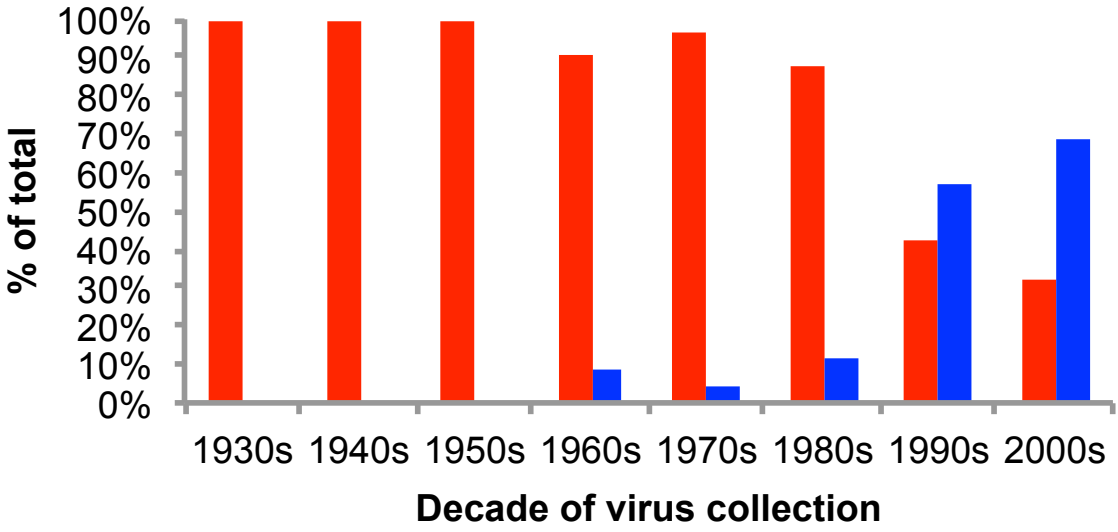


Figure 7.2: Bayesian skyline reconstructions: A) GIII, B) GI, and C) GIII versus GI. The bold lines represent the mean estimate of the effective number of infections, a measure of relative genetic diversity, through time. The dotted lines represent the 95% HPD values.

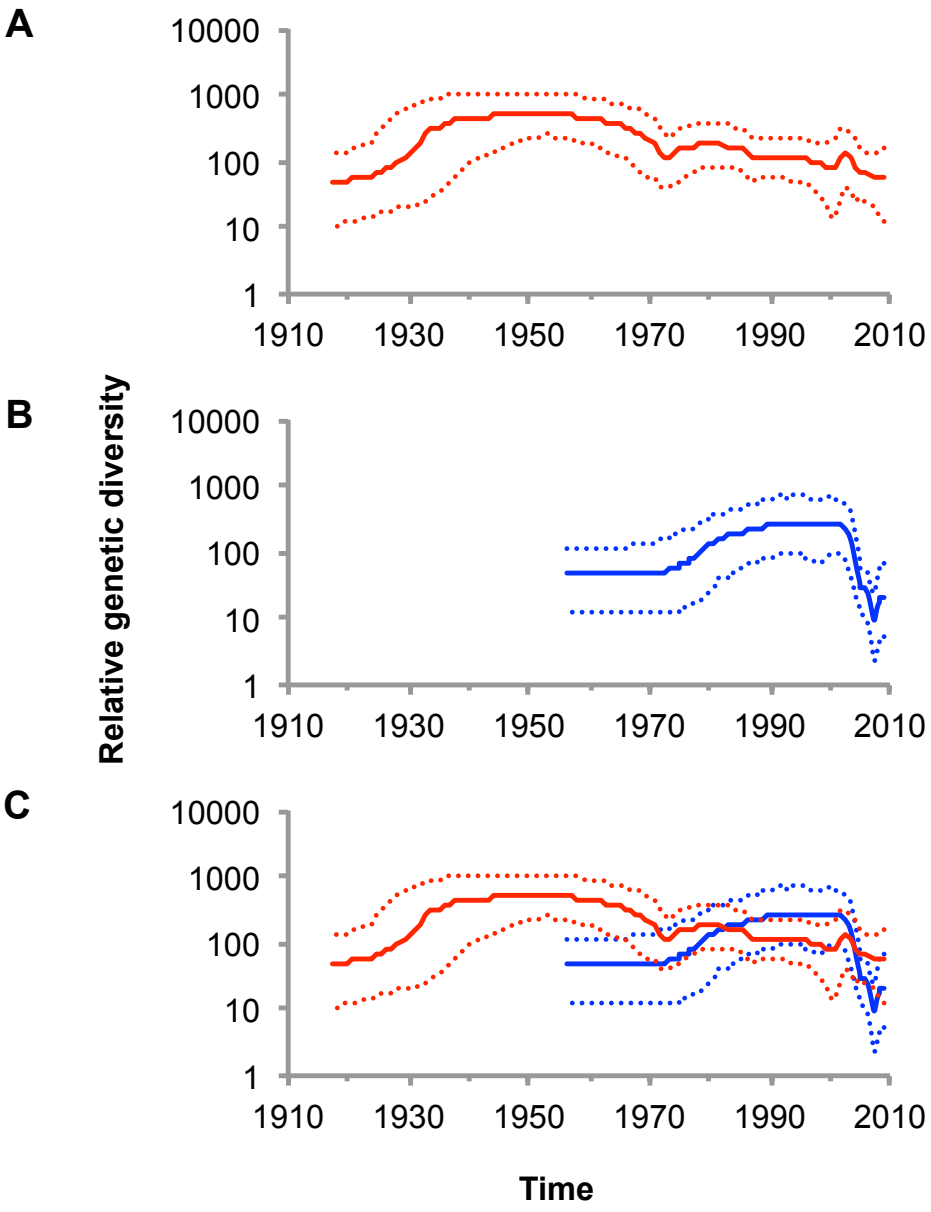


Figure 7.3: Geographical distribution of the GI sequences included in this study. For each country included in the GI phylogeographic analysis, the estimated date of the MRCA, the dates of virus collection and the number of viral sequences that were included in this study are indicated. The direction of GI dispersal is also indicated.

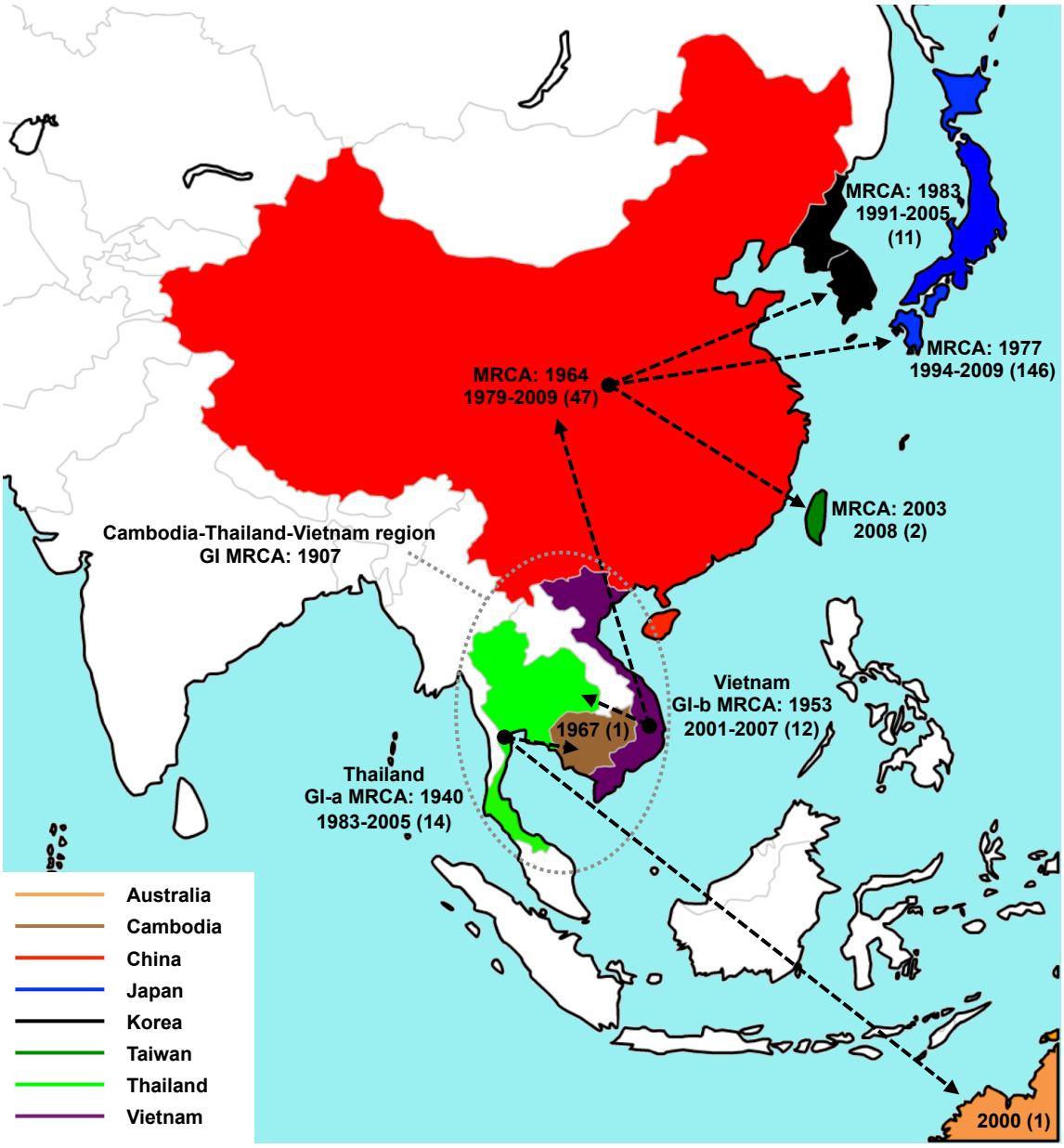


Figure 7.4: GI Bayesian MCC phylogeny. GI-a, GI-b are represented to the right of the tree. Branch tips correspond to the date of collection of each of the virus isolates. Branch lengths correspond to lengths of time, as measured by the scale located under the tree. Terminal branches are colored according to the most probable geographic location of their parental node. The numbers at the nodes correspond to the phylogeographic analysis data presented in Table 4. Asterisks (*) represent the initial divergence of the MRCA of each of the seven countries included in the GI phylogeographic analysis. Clusters of closely related viruses that were isolated in temperate Asia (Japan, Korea and China) from year-to-year are indicated to the right of the phylogeny.

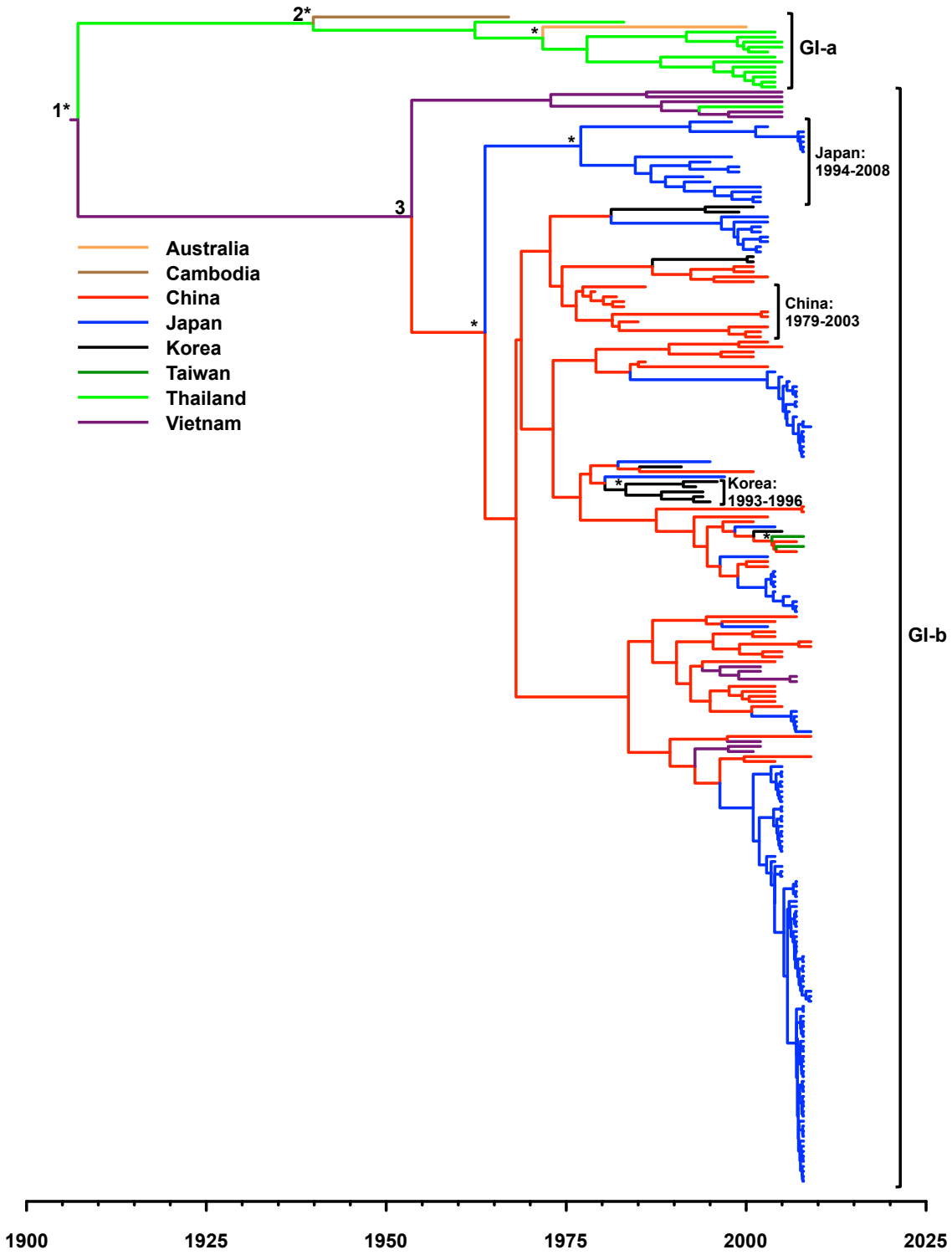

2*
*
GI-a
1*
*
Japan:
1994-2008
3
Australia
Cambodia
China
Japan
Korea
Taiwan
Thailand
Vietnam
*
China:
1979-2003
*
Korea:
1993-1996
*
GI-b
1900
1925
1950
1975
2000
2025

Figure 7.5: Sites displaying evidence of molecular adaptation mapped on the E protein dimer structure of JEV: A) GIII and B) GI. Domain I is highlighted in red, domain II is highlighted in yellow and domain III is highlighted in blue. The substitutions are indicated in gray and are only numbered on the lower monomer.

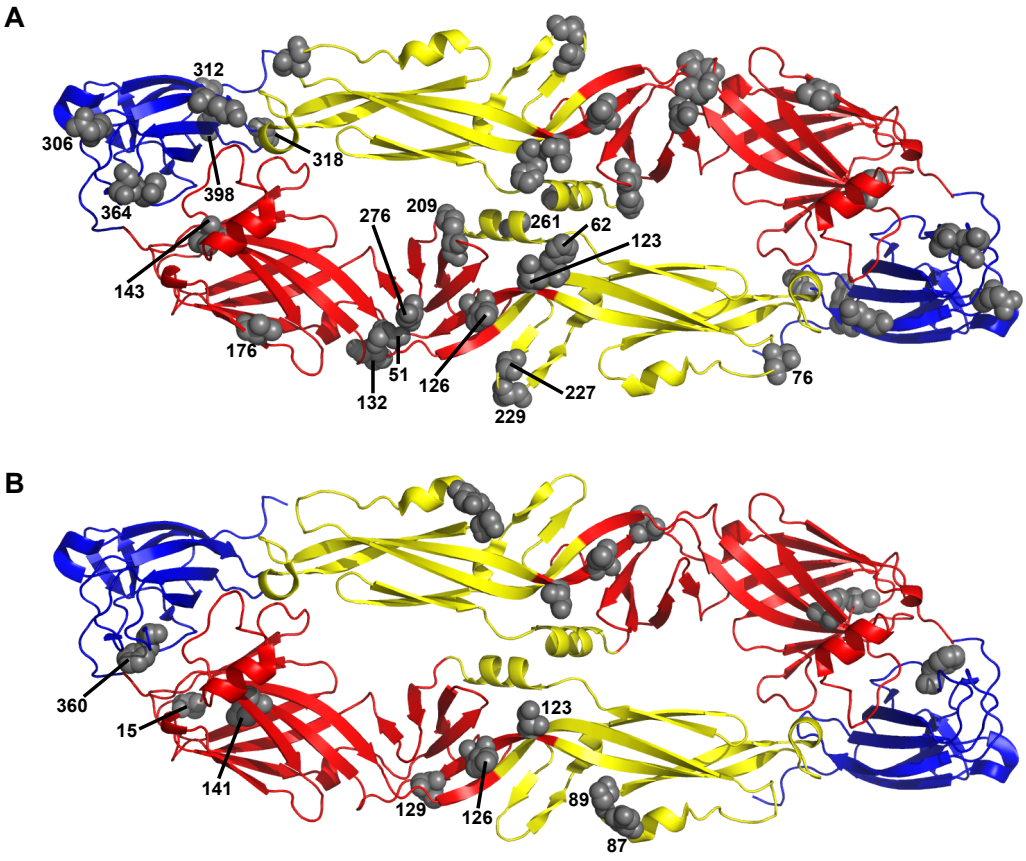


Figure 7.6: GI Bayesian MCC phylogenies with branches colored according to the amino acid residue present at the following sites of the E protein: A) 15, B) 87, C) 89, D) 123, E) 126, F) 129, G) 141, and H) 360.

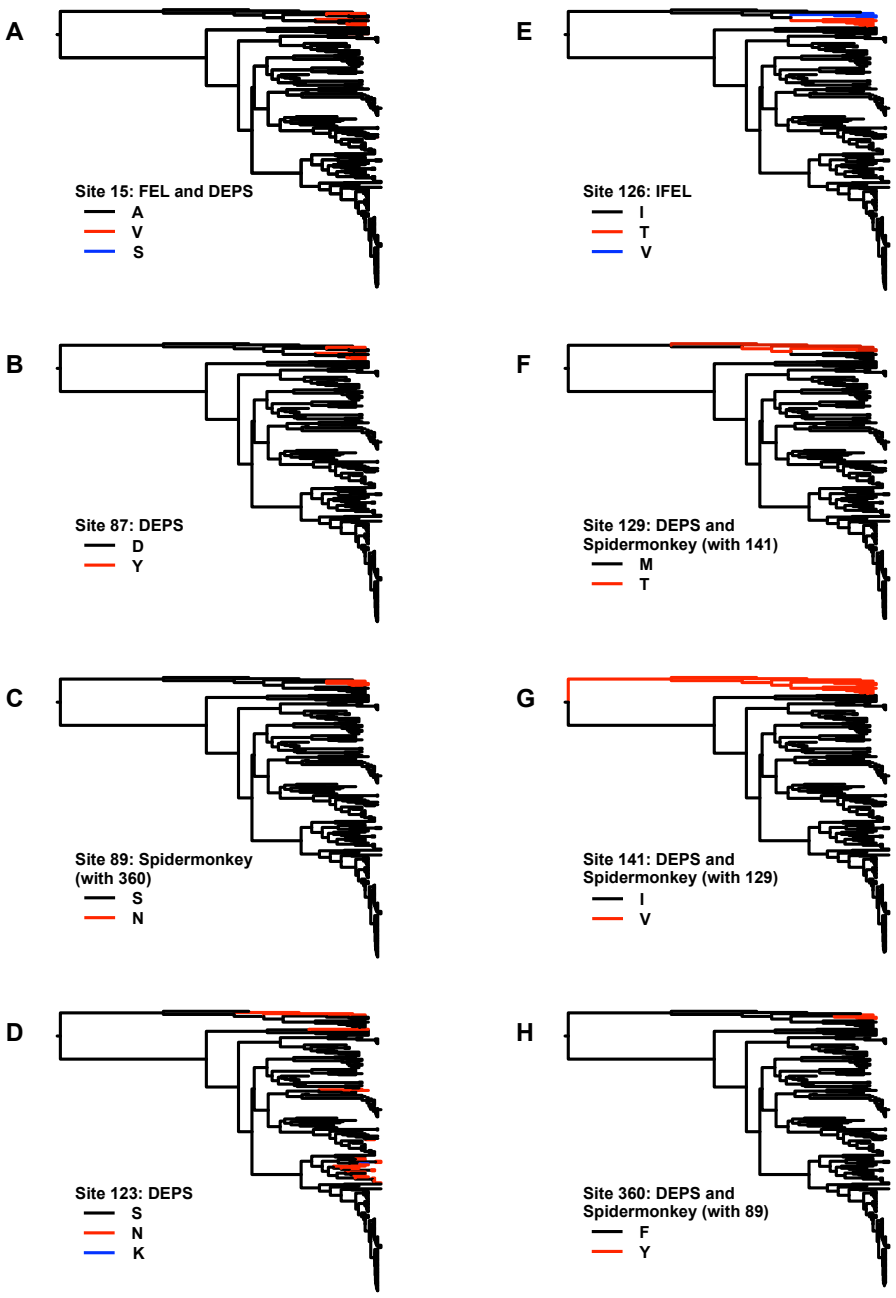


Figure 7.7: Multiplication kinetics of isolates representative of GI-IV in DEF cells infected at an MOI of 0.1 and then incubated at: A) 37°C and B) 41°C. The lower limit of detection was 1 $\log_{10}$PFU/mL for samples taken 0 to 36 HPI, and 2 $\log_{10}$PFU/mL for samples taken 48 HPI and onwards.

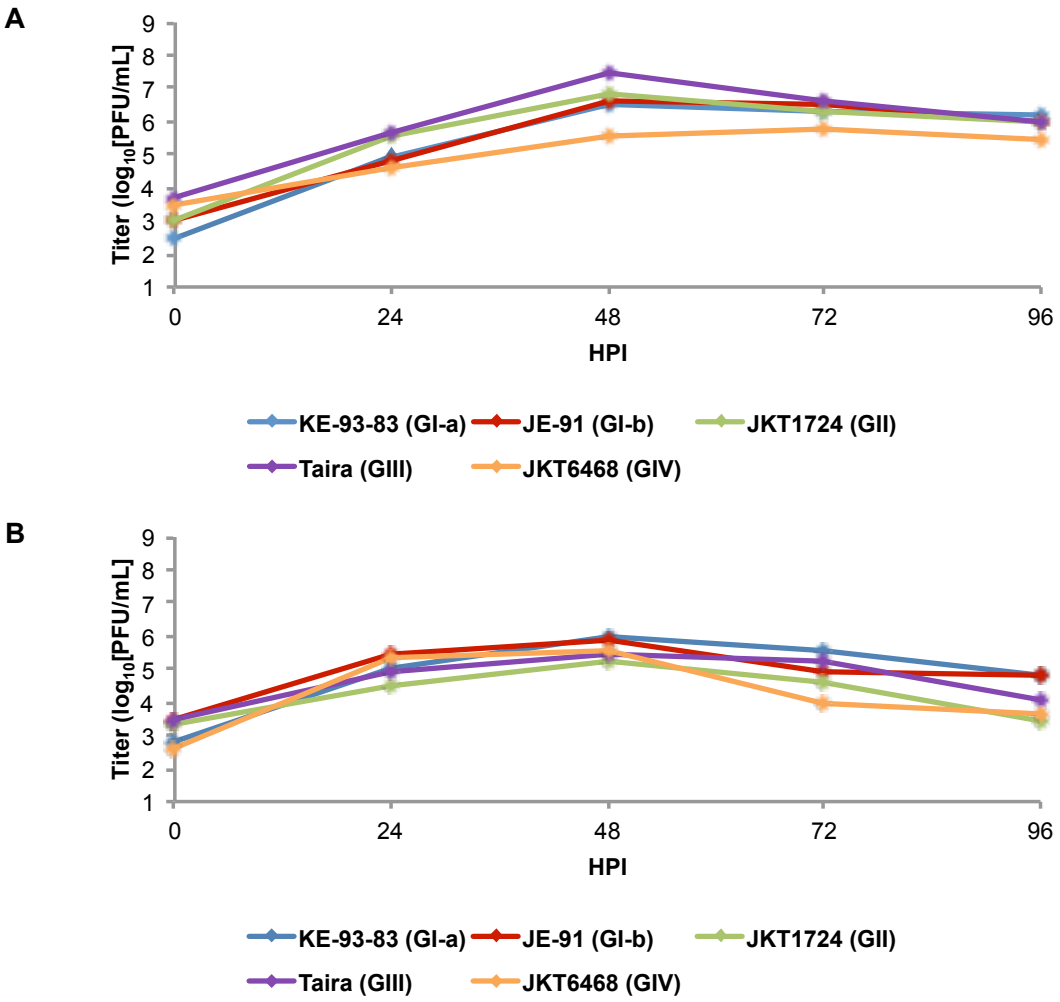
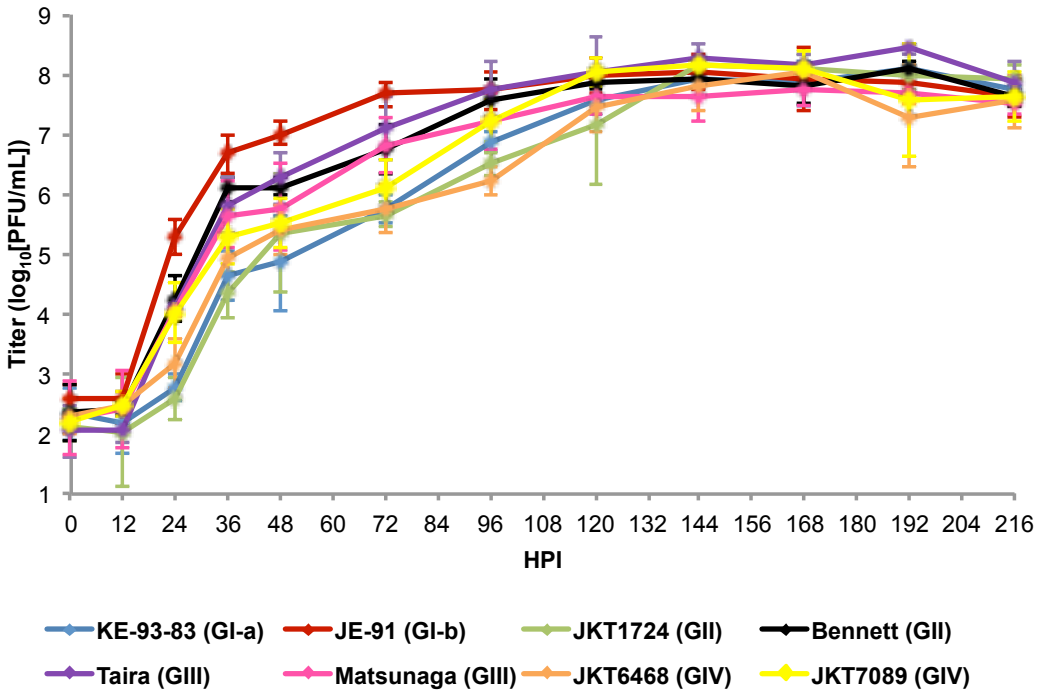


Figure 7.8: Multiplication kinetics of isolates representative of GI-IV in C6/36 cells infected at an MOI of 0.1 and then incubated at 28°C. Error bars represent SD values based on triplicate experiments. The lower limit of detection was 1 $\log_{10}$PFU/mL for samples taken 0 to 36 HPI, and 2 $\log_{10}$PFU/mL for samples taken 48 HPI and onwards.



Chapter 8: Phenotypic characterization of isolates representative of genotypes I-IV of Japanese encephalitis virus

8.1 ABSTRACT

Japanese encephalitis virus (JEV) is comprised of five epidemiologically and geographically distinct genotypes (GI-V); however, there is a lack of information regarding the phenotypic properties of the JEV genotypes. Therefore, a series of studies were performed to elucidate the phenotypic properties of GI-IV of JEV. No differences in viral multiplication were observed among isolates representative of GI-IV in Vero or A549 cells incubated at 37°C. Further, temperature-sensitivity assays in Vero cells revealed that isolates representative of GI-IV exhibited similar levels of plaquing at 37 and 41°C. In terms of the magnitude and duration of viremia, the blackbird model of JEV viremia used in this study agreed with two older experimental studies that utilized ardeids. Moreover, 100.0% of blackbirds developed detectable viremias following inoculation with the GI-a and GI-b isolates, while only 66.7% of birds developed viremias following inoculation with the GII and GIV isolates. Further, the average duration of viremia in blackbirds with detectable viremias was 1.8 days for the GI-a and GIV isolates, 3.0 days for the GII isolate, and 3.8 days for the GI-b isolate. These results suggest that GI-b may have emerged and established itself throughout Asia due to an increased multiplicative ability in avians. In the young mouse model of neuroinvasiveness, genotype II was found to be potentially less neuroinvasive compared to the other genotypes.

8.2 INTRODUCTION

Japanese encephalitis virus is comprised of five epidemiologically and geographically distinct genotypes. Prior to the initiation of the work presented in this chapter, little was known about the phenotypic properties of the JEV genotypes. Only the mouse neurovirulence phenotypes of the JEV genotypes had previously been determined,

and no large differences in either the 50% lethal dose (LD_{50}) or average survival time (AST) following the intracranial inoculation of 6-8 week old C57BL/6 mice with isolates representative of GI-IV of JEV were shown (Beasley *et al*., 2004). Therefore, the objective of this portion of the dissertation research was to delineate phenotypic properties of GI-IV of JEV, and then determine if a relationship exists between the epidemiological characteristics and geographical distributions of the JEV genotypes and the their phenotypes. In this chapter, isolates representative of GI-IV of JEV were phenotypically described in terms of their *in vitro* multiplication efficiency and temperature-sensitivity in mammalian cells, viremia profiles in blackbirds, and neuroinvasiveness in young mice.

8.3 RESULTS

Details of the JEV isolates that were utilized for the studies presented in this chapter are shown in Table 8.1.

8.3.1 *In vitro* multiplication kinetics of GI-IV of JEV in mammalian cells

The *in vitro* multiplication efficiencies of isolates representative of GI-IV of JEV were compared in duck embryo fibroblast (DEF), *Aedes albopictus* C6/36, African green monkey kidney Vero, and adenocarcinomic human alveolar basal epithelial A549 cells inoculated at an multiplicity of infection (MOI) of 0.1. The results of the multiplication kinetics experiments in DEF and C6/36 cells were presented in Chapter 7 (Figures 7. 7 and 7.8).

Vero cells have been used extensively in virological studies to compare the multiplication kinetics of virus isolates. No statistically significant differences in viral multiplication were observed among isolates representative of GI-IV of JEV in Vero cells incubated at 37°C (Figure 8.1).

The interferon (IFN)-α/β-competent A549 cell line has previously been used to differentiate between emergent, pathogenic West Nile Virus (WNV) lineage I isolates (IFN-α/β resistant) and non-emergent, avirulent WNV lineage II isolates (IFN-α/β sensitive) (Keller *et al*., 2006). As such, GIV of JEV may be confined to Indonesia due to the sensitivity of this genotype to the IFN-α/β produced by the reservoir/amplifying host of the virus. Additionally, GI-b of the virus may have emerged and established itself as the dominant Asian JEV genotype due to the resistance of this genotype to the IFN-α/β produced by the reservoir of the virus. Therefore, the A549 cell line was used to test two hypotheses: 1) A549 cells infected with a GIV isolate and incubated at 37°C will exhibit decreased viral multiplication compared to cells infected with GI-III of JEV and 2) A549 cells infected with a GI-b isolate and incubated at 37°C will exhibit increased viral multiplication compared to cells infected with a GI-a isolate, as well as isolates representative of GII-IV of the virus. In disagreement with the hypotheses, no large differences in viral multiplication were observed among isolates representative of GI-IV of JEV in A549 cells (Figure 8.2).

8.3.2 Temperature-sensitivity phenotypes of GI-IV of JEV in Vero cells

Vero cells have been used extensively in virological studies to detect the temperature-sensitive phenotype. Therefore, isolates representative of GI-IV were assayed by plaque titration in Vero cells incubated at 37 and 41°C to determine if any of the viruses exhibited a temperature-sensitive phenotype. All of the viral isolates exhibited similar levels of plaquing efficiency at both temperatures (Table 8.2).

8.3.3 Avian viremia profiles following inoculation with GI-IV of JEV

Many species of waterbirds (ardeids) are protected in the United States; therefore in collaboration with Dr. Richard Bowen of Colorado State University red-winged

blackbirds (*Agelaius phoeniceus*) (Nemeth *et al*., 2009) were used to study the induction and duration of viremias following subcutaneous inoculation of the blackbirds with GI-IV of JEV. No studies have determined whether WNV-seronegative avians inoculated subcutaneously with the mouse-brain adapted, highly mouse neuroinvasive P3 isolate of JEV will exhibit a neuroinvasive phenotype. Nor have any studies compared the magnitude and duration of viremias in avians inoculated subcutaneously with GI-IV of JEV. The failure of GIV of the virus to spread beyond the Indonesian archipelago may due to the decreased multiplicative ability of this genotype in avians compared to GI-III. Additionally, GI-b may have emerged and established itself as the dominant Asian genotype due to the increased multiplicative ability of this genotype in avian compared to GI-a, as well as GII-IV. Therefore, red-winged blackbirds (*Agelaius phoeniceus*) inoculated subcutaneously with 3,000 PFU of isolates representative of GI-IV of JEV were used to investigate three predictions: 1) WNV-seronegative blackbirds inoculated with the P3 virus isolate will exhibit a neuroinvasive phenotype (death due to encephalitis), whereas blackbirds inoculated with the other JEV isolates will not exhibit a neuroinvasive phenotype, 2) fewer blackbirds inoculated with the GIV isolate of JEV will exhibit detectable viremias and the duration of the viremias will be decreased compared to isolates representative of GI-III of the virus, and 3) more blackbirds inoculated with the GI-b isolate of JEV will exhibit detectable viremias and the duration of the viremias will be increased compared to the GI-a virus isolate, as well as isolates representative of GII-IV of JEV. Similar to the results of older studies that experimentally infected various species of herons and egrets (Buescher *et al*., 1959a; Soman *et al*., 1977), by three days post infection (DPI) 70.8% of the blackbirds inoculated with the JEV isolates developed detectable viremias and 37.5% of the blackbirds maintained their viremias for at least three days (Table 8.3). Contrary to hypothesis one, none of the blackbirds died as a result

of inoculation with the P3 isolate (Table 8.3). In fact, none of the WNV field seropositive blackbirds developed detectable viremias following challenge with the P3 isolate of JEV, while 66.7% of the WNV-seronegative blackbirds developed detectable viremias following inoculation with the P3 isolate of the virus (Table 8.3). In partial agreement with hypotheses two and three, 100.0% of blackbirds developed detectable viremias following inoculation with the GI-a (KE-93-83) and GI-b (JE-91) isolates, while only 66.7% of birds developed viremias following inoculation with the GII (JKT1724) and GIV (JKT6468) isolates (Table 8.3). Further, the average duration of viremia in blackbirds with detectable viremias was 1.8 days for the GI-a (JE-91) and GIV (JKT6468) isolates, 3.0 days for the GII isolate (JKT1724), and 3.8 days for the GI-b isolate (JE-91) (Table 8.3). The P3 isolate (GIII) received multiple mouse brain passages, and was therefore was not compared to the wild-type isolates.

8.3.4 Young mouse neuroinvasion phenotypes of GI-IV of JEV

A previous study was able to show differences in mortality in young A2G mice intraperitoneally inoculated with various yellow fever virus (YFV) isolates (Fitzgeorge & Bradish, 1980), suggesting that a young mouse model of neuroinvasiveness could be used to show differences in mortality in young mice inoculated intraperitoneally with isolates representative of GI-IV of JEV. Therefore, litters of eight-day-old Swiss Webster mice were inoculated intraperitoneally with isolates representative of GI-IV of JEV at 10-fold serial dilutions ranging in infectious titer from 1 to 10,000 PFU. The details of the study are shown in Table 8.4. The LD_{50} ranged from 0.6 PFU for GIII (Taira) and GIV (JKT6468) isolates to 1.2 PFU for the GII isolate (JKT1724) (Table 8.4). With the exception of one animal (KE-93-83 [GI-a]), all mice inoculated with ≥ 10 PFU of JEV died of infection prior to the end of the study at 25 DPI (Table 8.4). Therefore, the

following comparisons will only be between litters inoculated with 1 PFU of isolates representative of GI-IV of JEV. Figure 8.3 demonstrates that 100.0% of mice inoculated with the GIII isolate (Taira) died by five DPI and 100.0% of mice inoculated with the GIV isolate (JKT6468) died by 7 DPI, while 22.2% of mice inoculated with the GI-a isolate (KE-93-83) survived until the end of the study, 28.6% of mice inoculated with the GI-b isolate (JE-91) survived until the end of the study, and 71.4% of mice inoculated with the GII isolate (JKT1724) survived until the end of the study. Accordingly, the average survival time (AST) (includes mice that survived until the end of the study at 25 DPI) ranged from 5.0 ± 0.0 DPI for the GIII isolate (Taira) to 19.7 ± 9.0 DPI for the GII isolate (JKT1724) (Table 8.4). Table 8.5 shows that the AST of mice inoculated with the GII isolate (JKT1724) was significantly longer than mice inoculated with the GI-a isolate (KE-93-83) ($p = 0.03$), the GIII isolate (Taira) ($p = 0.00$), and the GIV isolate (JKT6468) ($p = 0.00$). Further, the AST of mice inoculated with the GIII isolate (Taira) was significantly shorter than mice inoculated with the GI-a isolate (KE-93-83) ($p = 0.03$), the GI-b isolate (JE-91) ($p = 0.01$), the GII isolate (JKT174) ($p = 0.00$), and the GIV isolate (JKT6468) ($p = 0.01$) (Table 8.5). The average day of death (ADD: excludes mice that survived until the end of the study at 25 DPI) was between four and seven DPI for mice inoculated with isolates representative of GI-IV of JEV (Table 8.4). There were no statistically significant differences in the ADD between mice inoculated with isolates representative of GI-IV of the virus (Table 8.6).

8.4 Discussion

Prior to the initiation of the work presented in this chapter, only the mouse neurovirulence phenotypes of the genotypes of JEV had been described, and no large differences in either the LD_{50} or AST following the intracranial inoculation of six to

eight-week old C57BL/6 mice with isolates representative of GI-IV of the virus were demonstrated (Beasley *et al*., 2004). Therefore, isolates representative of GI-IV of JEV were phenotypically characterized in terms of their *in vitro* multiplication kinetics and temperature-sensitivity in mammalian cells, viremia profiles in blackbirds, and neuroinvasiveness in young mice.

8.4.1 Isolates representative of GI-IV of JEV exhibit no difference *in vitro* multiplication efficiency or temperature-sensitivity in mammalian cells

No differences in viral multiplication were observed among isolates representative of GI-IV of JEV in either Vero or A549 cells. Further, all of the viral isolates exhibited similar levels of plaquing efficiency at both 37 and 41°C. It is possible that if more than one isolate representative of each genotype was used, differences in viral multiplication or temperature sensitivity between the genotypes may have been recognized in mammalian cell cultures. However, it was decided prior to the initiation of the work presented in this chapter that only one isolate representative of each genotype would be utilized in the phenotypic studies. If a phenotypic difference between the isolates was recognized, the study would be repeated with additional isolates representative of each genotype. Although no differences in viral multiplication between the isolates were demonstrated in mammalian cells; in Chapter 7, GI-b (JE-91 isolate) had significantly higher infectious titers in C6/36 cells compared to the other genotypes at 24, 26 and 48 hours post infection (HPI). As discussed in Chapter 7, it is possible that an increased multiplication kinetics of GI-b viruses compared to GIII viruses at early time points in *Culex tritaeniorhynchus* may have resulted in mosquitoes that became infected more rapidly thereby resulting in a shortened extrinsic incubation period. This would have led to an increased number of GI-b enzootic transmission cycles and would

explain how GI-b emerged and established throughout Asia eventually displacing GIII as the dominant JEV genotype.

8.4.2 GI-b of JEV may have emerged and established itself throughout Asia due to an increased multiplicative ability in avians

The induction and duration of viremias were compared among red-winged blackbirds (*Agelaius phoeniceus*) following subcutaneous inoculation with GI-IV of JEV. The geographical range of *Agelaius phoeniceus* spans most of North America, extending north to southern Alaska, south to the Yucatan peninsula, east to the Pacific Coast of California and Canada, and east to the eastern seaboard (Rosenthal, 2004). By 3 DPI, 70.8% of the blackbirds subcutaneously inoculated with 3,000 PFU of JEV developed detectable viremias, and 37.5% of the blackbirds maintained their viremias for at least three days. This is in agreement with an earlier study conducted in California that found that following subcutaneous inoculation of 0.2 mL of a 300 mouse LD_{50} dilution of the Okinawa strain of JEV (7^{th} to 8^{th} mouse brain passage), tricolor redwing blackbirds (*Agelaius tricolor*) developed high viral titers that persisted several days (Hammon *et al*., 1951). Interestingly, following an outbreak of JE in Guam sera collected from a blackbird (species unknown) was positive for JEV antibody (unknown if neutralizing antibody) (Hammon *et al*., 1951), suggesting that blackbirds could frequently be involved in the enzootic transmission cycle of JEV. Nonetheless, the results of the blackbird JEV viremia model presented here are in agreement with two older experimental studies involving ardeids (Buescher *et al*., 1959a; Soman *et al*., 1977). The first study performed in Japan found that following subcutaneous inoculation with 1-320 mouse LD_{50} of the M1/311 isolate of JEV (infected mouse-brain suspension), 50.0% of the blue-crowned night herons (*Nycticorax nycticorax*), 33.3% of the little egrets (*Egretta garzetta*), and 75.0% of the plumed egrets (*Egretta intermedia*) developed detectable viremias (log_{10}2.5-3.0

mouse LD_{50}/0.03 mL whole blood) that were obtained between two and four DPI and lasted at least two days (Buescher *et al*., 1959a). A second study conducted in India found that following the subcutaneous inoculation with 2.7-3.5 dex LD_{50} of JEV (Indian mosquito isolate that had received three mouse brain passages), 100% of the cattle egrets (*Bubulcus ibis*) and 100% of the Indian pond herons (*Ardeola grayii*) developed detectable viremias (trace-2.5 dex LD_{50}) that were obtained at two DPI and lasted at least three days (Soman *et al*., 1977). The agreement between the blackbird and ardeid models of JEV avian viremia suggest that red-winged blackbirds are a good model for studying JEV viremia in avians.

The mouse-brain adapted, highly mouse neuroinvasive P3 isolate of JEV did not exhibit a neuroinvasive phenotype in blackbirds. This is in agreement with an older study that found that chicks subcutaneously inoculated with either the 19th mouse-brain passage of the M1/311 JEV isolate or the Nakayama JEV isolate that had received 70-80 mouse-brain passages did not exhibit a neuroinvasive phenotype (Buescher *et al*., 1959a). With the 7th mouse-brain passage of the M1/311 isolate, viremia was detected in all eight chicks between two and four DPI and lasted at least four days; while, with the 19th mouse-brain passage of the M1/311 isolate, viremia was detected in only two of the seven chicks between four and seven DPI and lasted only two days (Buescher *et al*., 1959a).

West Nile virus field seropositive blackbirds were protected against challenge with the P3 isolate of JEV. This is congruent with a previous study demonstrating that WNV field seropositive blackbirds were protected against challenge with wild-type GIII and GI JEV isolates (Nemeth *et al*., 2009). If JEV was introduced into North America, the presence of WNV seropositive avians would likely decrease the transmission rate of

JEV to swine, equine, and humans. This may perhaps even prevent the establishment of JEV in North America.

Interestingly, 100.0% of blackbirds developed detectable viremias following inoculation with the GI-a and GI-b isolates, while only 66.7% of birds developed viremias following inoculation with the GII and GIV isolates. Further, the average duration of viremia in blackbirds with detectable viremias was 1.8 days for the GI-a and GIV isolates, 3.0 days for the GII isolate, and 3.8 days for the GI-b isolate. These results suggest that GI-b may have emerged and established itself throughout Asia due to an increased multiplicative ability in avians. Moreover, GIV may be confined to Indonesia and GII may be limited to tropical Asia due to a decreased multiplicative ability in avians. Although interesting, these findings need to be statistically confirmed using an increased number of blackbirds. It would also be of value to include a wild-type GIII isolate in the study, as this genotype was the dominant Asian JEV genotype for at least 50 years.

8.4.3 Genotype II of JEV may be less neuroinvasive in a young mouse model of JEV neuroinvasion compared to the other genotypes

The LD_{50} of 8-day old Swiss Webster mice intraperitoneally inoculated with 10-fold serial dilutions of JEV ranging in infectious titer from 1-10,000 PFU, ranged from 0.6 PFU for GIII and GIV isolates to 1.2 PFU for the GII isolate. However, none of the mice inoculated with the lowest dose (1 PFU) of the GIII and GI isolates survived until the end of the study at 25 DPI. Therefore, the actual LD_{50} could be significantly lower for the GIII and GIV isolates. Accordingly, the AST of mice inoculated with 1 PFU of the GIII isolate (5.0 ± 0.0) and the GIV isolate (6.0 ± 1.0) was lower compared to mice inoculated with isolates representative of GI-a (9.7 ± 8.7), GI-b (11.0 ± 9.6) and GII (19.7 ± 9.0). Most interestingly, the AST of mice inoculated with 1 PFU of the GII isolate was

significantly longer than mice inoculated with the corresponding doses of the GI-a, GIII, and GIV isolates. Further, the AST of mice inoculated with 1 PFU of the GIII isolate was significantly shorter than mice inoculated with corresponding doses of the GI-a, GI-b, GII, and GIV isolates. Excluding mice that survived until the end of the study, there was no significant difference in the ADD of mice inoculated with 1 PFU of JEV. Importantly, these results suggest that GI-b (newly dominant JEV genotype) is no more neuroinvasive, and GIV (never documented to cause human disease) is just as neuroinvasive (or potentially more neuroinvasive), as the other JEV genotypes. However, this study should be repeated with lower virus inocula to provide better LD_{50} estimates for each genotype.

8.4.4 Conclusions

This chapter elucidated phenotypic properties of isolates representative of GI-IV of JEV through the use of mammalian cell culture systems (Vero and A549 cells), a blackbird model of virulence, and a young mouse model of neuroinvasiveness. Although no differences in viral multiplication or temperature sensitivity were observed among the four JEV genotypes, isolates representative of GI-IV of the virus displayed potentially different phenotypes in the blackbird model of JEV viremia and the young mouse model of JEV neuroinvasiveness. Notably, 100.0% of blackbirds developed detectable viremias following inoculation with the GI-a and GI-b isolates, while only 66.7% of birds developed viremias following inoculation with the GII and GIV isolates. Further, the average duration of viremia in blackbirds with detectable viremias was 1.8 days for the GI-a and GIV isolates, 3.0 days for the GII isolate, and 3.8 days for the GI-b isolate. However, this study needs to be repeated with a greater number of blackbirds. Genotype II was found to be potentially less neuroinvasive in young mice compared to the other genotypes, however this study should be repeated with lower virus inocula to obtain more

accurate LD_{50} estimates. Overall, this study demonstrated that the blackbird model JEV virulence and the young mouse model of neuroinvasiveness can be used to show differences among the genotypes of JEV.

Table 8.1: Details of the JEV isolates used in this study.

Isolate (genotype)	Origin	Year	Host	Passage no.
KE-93-83 (GI-a)	Thailand	1983	Human	4
JE-91 (GI-b)	Korea	1991	Mosquito	3
Miller Vietnam (GI)	Vietnam	2003	Mosquito	Unknown
JKT1724 (GII)	Indonesia	1979	Mosquito	7
Bennett (GII)	Korea	Circa 1951	Human	5
Taira (GIII)	Japan	1948	Human	5
Matsunaga (GIII)	Japan	1939	Human	3
P3 (GIII)	China	1954	Mosquito	Multiple mouse brain passages
JKT6468 (GIV)	Indonesia	1981	Mosquito	9
JKT7089 (GIV)	Indonesia	1981	Mosquito	5

Table 8.2: Plaque efficiency of GI-IV of JEV in Vero cells at 37°C versus 41°C.

Isolate (genotype)	Temperature (°C)	Titer (log_{10}[PFU/mL])	Plaque efficiency
KE-93-83 (GI-a)	37	7.3	-0.13
	41	7.43	
JE-91 (GI-b)	37	8.2	0.73
	41	7.48	
JKT1724 (GII)	37	7.9	0.43
	41	7.48	
Taira (GIII)	37	6.34	0.52
	41	5.82	
JKT6468 (GIV)	37	7.3	0.05
	41	7.26	

Table 8.3: Viremia profiles of blackbirds following subcutaneous inoculation with isolates representative of GI-IV of JEV.

			Titer (log_{10}[PFU/mL])					
Blackbird	WNV serostatus	JEV isolate (genotype)	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	7 DPI
125	Negative	KE-93-83 (GI-b)	<2.0	2.3	4.3	5.1	<2.0	<2.0
134	Negative	KE-93-83 (GI-b)	<2.0	3.3	<2.0	<2.0	<2.0	<2.0
135	Negative	KE-93-83 (GI-b)	2.0	2.0	<2.0	<2.0	<2.0	<2.0
140	Negative	KE-93-83 (GI-b)	<2.0	<2.0	<2.0	<2.0	5.0	<2.0
877	Negative	KE-93-83 (GI-b)	<2.0	<2.0	4.5	5.6	<2.0	<2.0
879	Negative	KE-93-83 (GI-b)	<2.0	<2.0	3.1	3.3	<2.0	<2.0
111	Negative	Miller Vietnam (GI)	2.0	3.4	4.4	4.0	<2.0	<2.0
116	Negative	Miller Vietnam (GI)	2.6	3.3	2.5	<2.0	<2.0	<2.0
148	Negative	Miller Vietnam (GI)	<2.0	2.3	<2.0	3.0	3.0	<2.0
867	Negative	Miller Vietnam (GI)	<2.0	2.6	3.7	3.0	4.3	<2.0
881	Negative	Miller Vietnam (GI)	2.0	2.0	2.3	3.0	2.3	2.5
882	Negative	Miller Vietnam (GI)	<2.0	2.8	4.7	3.3	<2.0	<2.0
158	Negative	JKT1724 (GII)	<2.0	<2.0	<2.0	3.3	3.3	<2.0
863	Negative	JKT1724 (GII)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
864	Negative	JKT1724 (GII)	<2.0	3.0	<2.0	<2.0	<2.0	<2.0
875	Negative	JKT1724 (GII)	<2.0	3.2	4.0	5.1	3.6	<2.0
880	Negative	JKT1724 (GII)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
890	Negative	JKT1724 (GII)	3.0	2.7	4.5	5.6	3.3	[1]<2.0
124	Positive[5]	P3 (GIII)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
139	Positive[5]	P3 (GIII)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
866	Positive[5]	P3 (GIII)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
868	Positive[5]	P3 (GIII)	<2.0	<2.0	<2.0	<2.0	<2.0	[2]ND
871	Positive[5]	P3 (GIII)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
114	Negative	P3 (GIII)	<2.0	<2.0	3.5	<2.0	<2.0	<2.0
117	Negative	P3 (GIII)	<2.0	2.6	2.5	<2.0	<2.0	<2.0
130	Negative	P3 (GIII)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
150	Negative	P3 (GIII)	2.0	2.0	2.6	<2.0	<2.0	<2.0
154	Negative	P3 (GIII)	2.0	3.0	<2.0	<2.0	<2.0	[3]<2
885	Negative	P3 (GIII)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
133	Negative	JKT6468 (GIV)	2.3	2.0	<2.0	<2.0	<2.0	<2.0
143	Negative	JKT6468 (GIV)	<2.0	2.0	<2.0	3.3	<2.0	<2.0
870	Negative	JKT6468 (GIV)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
872	Negative	JKT6468 (GIV)	<2.0	<2.0	3.7	<2.0	<2.0	<2.0
873	Negative	JKT6468 (GIV)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
886	Negative	JKT6468 (GIV)	<2.0	<2.0	<2.0	3.3	4.8	[4]ND

[1]Died 7 DPI for unknown reasons. [2]Died 13 DPI for unknown reasons. [3]Died 6 DPI likely due to old age. [4]Not done.[5]WNV field seropositive.

Table 8.4: Young mouse neuroinvasion phenotypes of GI-IV of JEV.

Isolate (genotype)	Dose (PFU)	No. of mice	Number of right-censored mice at 25 DPI	LD_{50} (PFU)	AST (DPI ± SD)[1]	ADD (DPI ± SD)[2]
KE-93-83 (GI-a)	1	9	2	0.9	9.7 ± 8.7	5.3 ± 0.5
	10	7	1		7.9 ± 7.6	5.0 ± 0.0
	100	5	0		5.0 ± 0.0	5.0 ± 0.0
	1,000	9	0		5.0 ± 0.0	5.0 ± 0.0
	10,000	10	0		5.0 ± 0.0	5.0 ± 0.0
JE-91 (GI-b)	1	7	2	0.8	11.0 ± 9.6	5.4 ± 0.5
	10	9	0		5.0 ± 0.0	5.0 ± 0.0
	100	8	0		5.0 ± 0.0	5.0 ± 0.0
	1,000	7	0		5.3 ± 0.5	5.2 ± 0.5
	10,000	10	0		4.6 ± 0.5	4.6 ± 0.5
JKT1724 (GII)	1	7	5	1.2	19.7 ± 9.0	6.5 ± 0.7
	10	7	0		5.4 ± 0.5	5.4 ± 0.5
	100	11	0		5.3 ± 0.5	5.3 ± 0.5
	1,000	9	0		5.0 ± 0.0	5.0 ± 0.0
	10,000	11	0		4.7 ± 0.5	4.7 ± 0.5
Taira (GIII)	1	9	0	0.6	5.0 ± 0.0	5.0 ± 0.0
	10	10	0		5.1 ± 0.3	5.1 ± 0.3
	100	9	0		4.7 ± 0.5	4.7 ± 0.5
	1,000	11	0		4.0 ± 0.0	4.0 ± 0.0
	10,000	8	0		4.0 ± 0.0	4.0 ± 0.0
JKT6468 (GIV)	1	9	0	0.6	6.0 ± 1.0	6.0 ± 1.0
	10	7	0		5.6 ± 0.5	5.6 ± 0.5
	100	10	0		5.0 ± 0.0	5.0 ± 0.0
	1,000	6	0		5.2 ± 0.4	5.2 ± 0.4
	10,000	9	0		5.0 ± 0.0	5.0 ± 0.0
Control	-	9	9	-	25.0 ± 0.0	-

[1]Estimates are limited to the largest survival time if censored. [2]Estimates exclude mice that were euthanized at 25 DPI.

Table 8.5: Test of equality of survival distributions in mice inoculated with 1 PFU of GI-IV of JEV.

		Log rank (Mantel-Cox)		
Virus (Genotype)	Virus (Genotype)	Chi-square	Degrees of freedom	p-value
KE-93-83 (GI-a)	Control	11.21	1	0.00
KE-93-83 (GI-a)	JE-91 (GI-b)	0.15	1	0.70
KE-93-83 (GI-a)	JKT1724 (GII)	4.93	1	0.03
KE-93-83 (GI-a)	Taira (GIII)	4.86	1	0.03
KE-93-83 (GI-a)	JKT6468 (GIV)	0.00	1	0.97
JE-91 (GI-b)	Control	9.16	1	0.00
JE-91 (GI-b)	JKT1724 (GII)	3.38	1	0.07
JE-91 (GI-b)	Taira (GIII)	6.43	1	0.01
JE-91 (GI-b)	JKT6468 (GIV)	0.23	1	0.64
JKT1724 (GII)	Control	2.78	1	0.10
JKT1724 (GII)	Taira (GIII)	15.00	1	0.00
JKT1724 (GII)	JKT6468 (GIV)	8.65	1	0.00
Taira (GIII)	Control	17.00	1	0.00
Taira (GIII)	JKT6468 (GIV)	7.67	1	0.01
JKT6468 (GIV)	Control	17.50	1	0.00

Table 8.6: Test of equality of death distributions in mice inoculated with 1 PFU of GI-IV of JEV.

I Isolate (genotype)	J Isolate (genotype)	Mean difference (I-J)	SE	p-value
KE-93-83 (GI-a)	JKT1724 (GII)	-1.20	0.50	0.91
	Taira (GIII)	0.30	0.20	0.85
	JKT6468 (GIV)	-0.70	0.40	0.59
	JE-91 (GI-b)	-0.10	0.30	1.00
JE-91 (GI-b)	JKT1724 (GII)	-1.10	0.60	0.92
	Taira (GIII)	0.40	0.20	0.86
	JKT6468 (GIV)	-0.60	0.40	0.85
JKT1724 (GII)	Taira (GIII)	1.50	0.50	0.90
	JKT6468 (GIV)	0.50	0.60	1.00
Taira (GIII)	JKT6468 (GIV)	-1.00	0.30	0.16

Figure 8.1: Multiplication profiles of GI-IV of JEV in Vero cells infected at an MOI of 0.1 and then incubated at 37°C. Error bars represent SD values based on triplicate experiments. The lower limit of detection was 1 $\log_{10}$PFU/mL for samples taken 0 to 36 HPI, and 2 $\log_{10}$PFU/mL for samples taken 48 HPI and onwards.

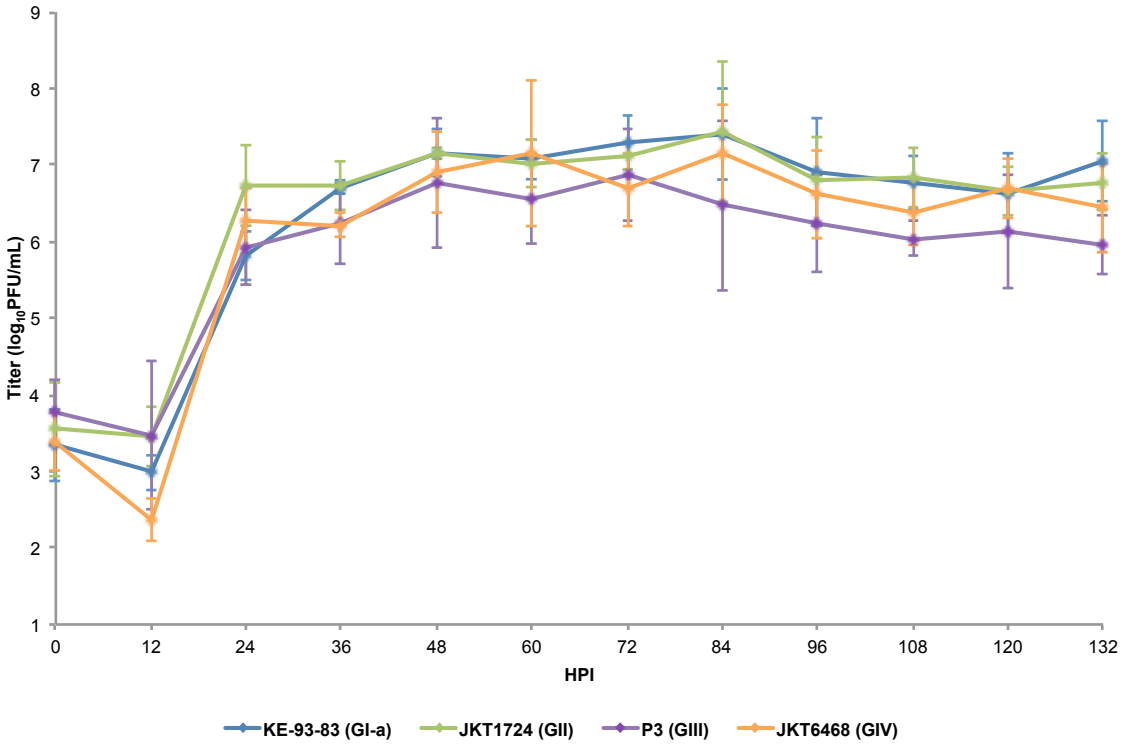


Figure 8.2: Multiplication profiles of GI-IV of JEV in A549 cells infected at an MOI of 0.1 and then incubated at 37°C. The lower limit of detection was 1 $\log_{10}$PFU/mL for samples taken 0 to 36 HPI, and 2 $\log_{10}$PFU/mL for samples taken 48 HPI and onwards.

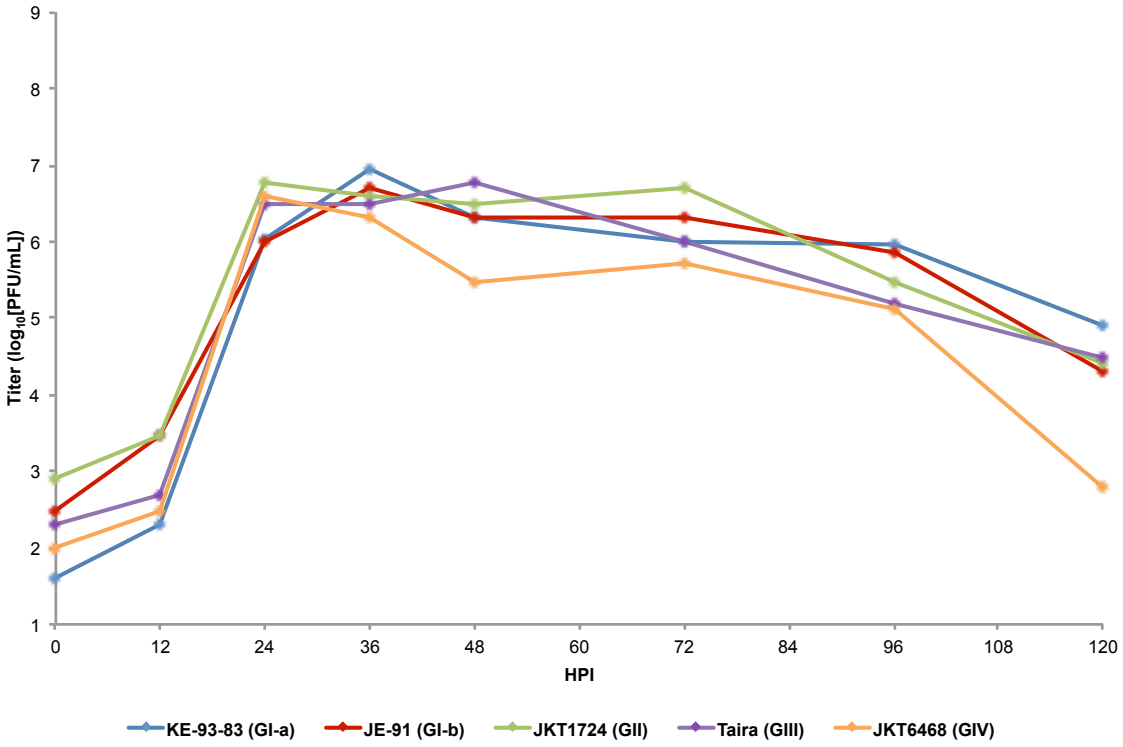
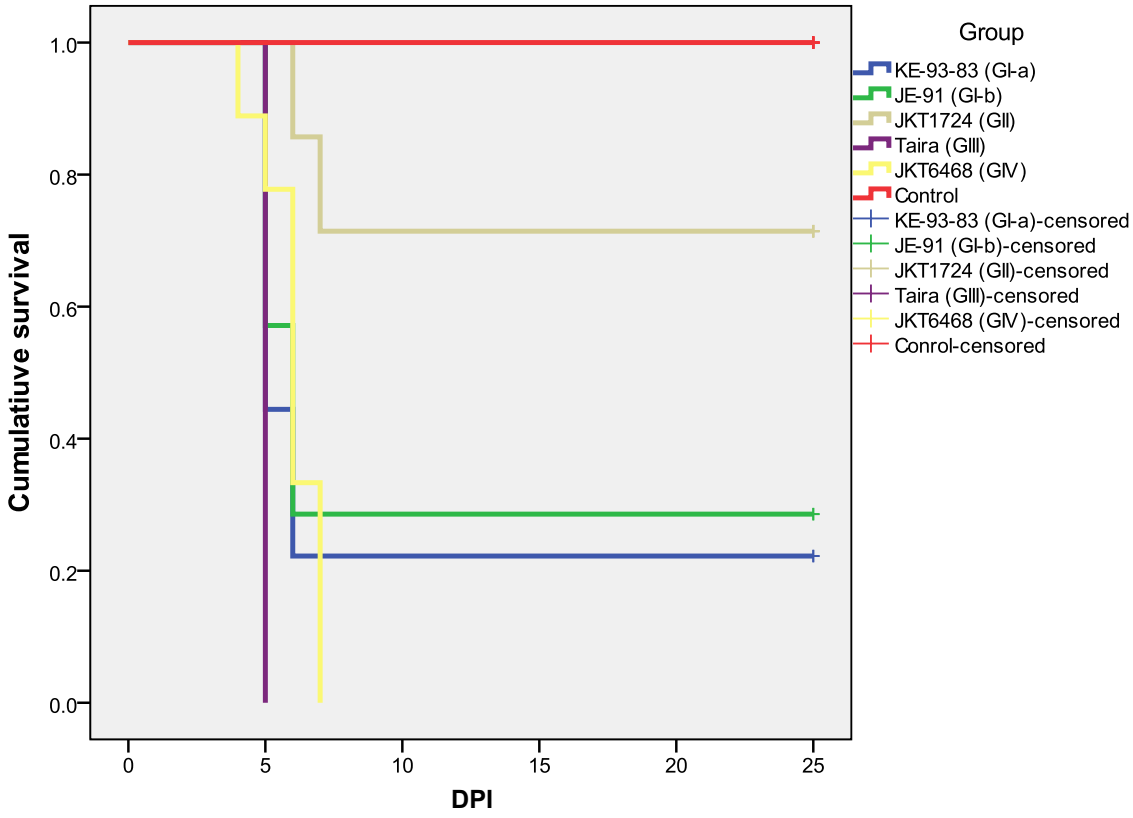


Figure 8.3: Kaplan-Meir survival plot for comparison of five litters of 8-day old Swiss Webster mice intraperitoneally inoculated with 1 PFU of isolates representative of GI-IV of JEV. The "+" symbol shown at 25 DPI for the control, JKT1724 (GII), JE-91 (GI-b), and KE-93-83 (GI-b) groups indicates that mice from these groups survived until the end of the study and were sacrificed.



Chapter 9: Discussion

9.1 UNANSWERED QUESTIONS

Prior to the initiation of the work presented in this dissertation, Japanese encephalitis virus (JEV) was comprised of at least four geographically distinct genotypes. Genotype I (GI) included isolates collected in northern Australia, northern Cambodia, China, Japan, Korea, Malaysia, Taiwan, Thailand, and Vietnam between 1967 and present. Genotype II (GII) included isolates collected sporadically in northern Australia, Indonesia, Malaysia, Papua New Guinea, and southern Thailand between 1970 and 1999. Genotype III (GIII) included isolates collected in China, India, Japan, Korea, Malaysia, Nepal, the Philippines, Sri Lanka, Taiwan, Thailand, and Vietnam between 1935 and present. Genotype IV (GIV) included isolates collected in Indonesia between 1980 and 1981. A single viral isolate collected in Singapore from a patient living in Malaysia was thought to represent a fifth genotype of JEV on the basis of limited cross-neutralization and phylogenetic studies (Solomon *et al*., 2003); however this isolate was not fully sequenced and confirmed to be JEV until 2011 (Mohammed *et al*., 2011). Therefore with the exception of the JEV phylogeography study presented in Chapter 6, this dissertation research focused on GI-IV of the virus.

In addition to exhibiting distinct geographical distributions, the genotypes of JEV displayed unique epidemiological histories. Although a limited quantity of nucleotide sequence data were available for JEV isolates collected prior to the 1970s, GIII appeared to have been the dominant JEV genotype throughout Asia for at least 50 years (from the collection of the prototype Nakayama strain of JEV, a GIII virus, in 1935). However, in recent years, multiple reports indicated that GI had displaced GIII as the most frequently isolated JEV genotype in a number of Asia countries including China (Wang *et al*., 2007), Thailand (Nitatpattana *et al*., 2008), South Korea (Nam *et al*., 1996), Japan (Ma *et al*., 2003), Malaysia (Tsuchie *et al*., 1997), Vietnam (Nga *et al*., 2004), India (Fulmali *et*

al., 2011), and Taiwan (Chen *et al*., 2011). In contrast to the wide geographical distribution of genotypes I and III, genotypes II and IV had only been collected in tropical Asia (south of the Tropic of Capricorn [23.5°N]. Even more intriguing, GIV had been isolated from mosquitoes only on three islands encompassing the Indonesian archipelago during a two-year time frame (Chen *et al*., 1992), and it was unclear if this viral genotype had been the etiological agent of human disease or has the potential to cause human disease.

Although it was clear from previous studies that JEV exhibited four geographically and epidemiologically distinct genotypes, many questions regarding the molecular epidemiology of JEV remained unanswered. First, a lack of genetic information on JEV isolates collected prior to the 1970s raised specific questions regarding the geographical distribution and time frame of circulation of the JEV genotypes (e.g., Was there evidence of GI circulation prior to 1967? Was there evidence of GII circulation prior to 1970 and had this genotype circulate outside of tropical Asia? Was GIII truly the dominant JEV genotype for over 50 years? Was there evidence of GIV circulation prior to 1981 and did this genotype circulate outside of Indonesia?) Second, the open reading frame (ORF) of only 29 JEV isolates had been sequenced and only one of these isolates was a GII virus (FU isolate, Australia, 1995). As such, the phylogenetic and evolutionary relationships among GII isolates, and between GII and the other JEV genotypes were poorly understood. Further, selective pressures and codon usage patterns had never been examined using a dataset consisting of nucleotide sequence data derived from the ORF of JEV isolates. Third, although it was previously proposed that JEV originated in the Indonesia-Malaysia region, only 13 Indonesian JEV isolates collected between 1978 and 1981 had been genetically characterized, consequently leading to a limited understanding of the genetic variation and evolution of the virus. Fourth, the

phylogeography of JEV and its genotypes had never been examined using a comprehensive dataset, resulting in many unaddressed questions (e.g., When and where did JEV and its genotypes originate, and what is their geographical range? Is there an association between genotype and climate? What amino acid substitutions were involved in the phylogenetic divergence of the viral genotypes?) Finally, no studies had examined the epidemiological and evolutionary dynamics underlying the geographically expansive displacement of GIII by GI of JEV throughout Asia.

When the work presented in this dissertation was commenced, little was known about the phenotypic properties of the JEV genotypes. Only the mouse neurovirulence phenotypes of the JEV genotypes had been determined, and no large differences in either the 50% lethal dose (LD_{50}) or average survival time (AST) following intracranial inoculation of 6-8 week-old C57BL/6 mice with isolates representative of GI-IV of JEV were shown (Beasley *et al*., 2004). The JEV genotypes had yet to be compared in terms of their *in vitro* multiplication kinetics in mammalian, avian, and mosquito cell cultures, temperature-sensitivity in avian and mammalian cell cultures, viremia in avians, and neurovirulence in young mice.

The **overall objective** of this dissertation research was to utilize experimental approaches to better understand the genotypic and phenotypic determinants of JEV geographical expansion. The **central hypothesis** of this research was that GIV of JEV remained confined to Indonesia, whereas genotypes I and III spread throughout Asia due to viral molecular determinants that relate to differences in mosquito vector and/or avian host preference. This hypothesis was tested by relating differences in the molecular epidemiology of JEV isolates to the phenotypic properties of the isolates through *in vitro* and *in vivo* investigations.

9.2 MAIN FINDINGS AND FUTURE DIRECTIONS

In Chapter 3, the non-structural five (NS5) gene/3' untranslated region (UTR) and envelope (E) gene nucleotide sequences of 26 JEV isolates obtained from specimens collected in Japan, Korea, and the "USSR" between 1935 and 1991 were determined and phylogenetically characterized. The results of this study confirmed that GIII was the predominant genotype of JEV in Japan and Korea between 1935 (isolation of the prototype isolate, a GIII virus) and 1991 (collection of the GI JE-91 isolate of JEV in Korea). Sequencing of the Autumn 4 isolate ("USSR", 1943) revealed that GIII existed as far north as the "USSR". Genetic characterization of the Bennett isolate (Korea, circa 1951) demonstrated that GII has been circulating for at least 19 years longer than previously thought, and represents the only collection of a GII virus outside tropical Asia.

In Chapter 4, the nucleotide sequence of the ORF of three JEV GII isolates collected between 1951 and 1978 in Korea, Malaysia and Indonesia were determined and then compared with the previously sequenced GII FU isolate (Australia, 1995), as well as with 27 virus isolates representative of the other three genotypes. Based on nucleotide and amino acid composition, GII isolates were the most similar to GI isolates. Compared to other flaviviruses such as YFV, the nucleotide and amino acid divergence among and within the four genotypes of JEV is low (von Lindern *et al*., 2006). Selection analyses revealed that all genotypes and genes of JEV are predominantly under purifying selection. Purifying selection acts to maintain phenotypic traits and results in virus-encoded proteins being conserved over time due to selective pressures against deleterious variants. In contrast to RNA viruses with one vertebrate host only, strong purifying selection dominates in arboviruses due to the constraints imposed by the alternation of vertebrate and arthropod host infections (Weaver, 2006). Evidence of positive selection was detected at amino acid 24 of the NS4B gene-protein. This signifies that the non-

conservative, serine to proline/leucine substitution that occurred at this site in four of the eight GI isolates, all four of the GII isolates, and two of the 18 GIII isolates tended to be beneficial, rather than neutral or deleterious. The NS4B protein of flaviviruses has been found to inhibit the interferon (IFN)-α/β signaling cascade at the level of signal transducer and activator of transcription (STAT) phosphorylation, suggesting that amino acid mutations at site 24 of this protein may be important in the antagonization of the host IFN response to the virus (Munoz-Jordan *et al*., 2003; Munoz-Jordan *et al*., 2005). No differences in viral multiplication were observed among isolates representative of GI-IV of JEV in the IFN-α/β-competent A549 cell line (Chapter 8), however the amino acid sequences of the NS4B protein of the virus isolates used in this experiment are unknown (except for the GIV JKT6468, which possesses a serine residue at amino acid 24 of the NS4B protein). Therefore, the NS4B gene of the remaining four JEV isolates used in the A549 cell line viral multiplication experiment should be sequenced to determine whether any of the isolates possess a positively selected proline or leucine residue at amino acid 24 of the NS4B protein. If none of the isolates possess a proline or leucine residue at this site, it may be interesting to repeat the A549 cell line viral multiplication experiment using either the GII Bennett, WTP-70-22, or JKT654 GII isolates (all of these isolates possess a proline at amino acid 24 of the NS4B protein) to determine what effect a proline at amino acid 24 of the NS4B protein would have on viral multiplication.

In Chapter 5, the nucleotide sequence of the E gene of 24 JEV isolates that were made from mosquitoes collected throughout the Indonesian archipelago from 1974 to 1987 were determined, and a series of phylogenetic and evolutionary adaptation analyses were performed. Phylogenetic analysis indicated that over a 14-year time span three genotypes of JEV circulated throughout Indonesia and a statistically significant association between the year of virus collection and genotype was revealed: isolates

collected between 1974 and 1980 belonged to GII, isolates collected between 1980 and 1981 belonged to GIV, and isolates collected in 1987 belonged to GIII. Prior to this study, a GIII virus had never been collected in Indonesia. Three of the GII Indonesian isolates grouped with an isolate that was collected during the JE outbreak that occurred in Australia in 1995, two of the GIII Indonesian isolates were closely related to a Japanese isolate collected 40 years previously, and two Javanese GIV isolates possessed six amino acid substitutions within the E protein when compared to a previously sequenced GIV isolate collected in Flores. Several amino acids within the E protein of the Indonesian isolates were found to be under directional evolution and/or co-evolution. Conceivably, the tropical climate of the Indonesia-Malaysia region, together with its plethora of distinct fauna and flora, may have driven the emergence and evolution of JEV. This is consistent with the extensive genetic diversity among JEV isolates observed in this study, and further corroborates the hypothesis that JEV originated in the Indonesia-Malaysia region (Solomon *et al*., 2003).

In Chapter 6, the largest JEV sequence dataset compiled to date, representing the E gene of 487 isolates collected from 12 countries over 75 years, was used to elucidate the evolutionary history of the virus on a spatiotemporal scale and determine if an association existed between genotype and climate. Utilization of this large collection of sequence data led to the division of GI into two clusters, GI-a and GI-b, where GI-a included isolates collected in tropical Asia only and GI-b included isolates collected primarily in temperate Asia. Categorical data analysis established that GIII and the recently emerged GI-b are temperate genotypes that are likely maintained year-round in northern latitudes by either hibernating mosquitoes, transovarial transmission in mosquitoes, poikilothermic vertebrates and/or bats, while GI-a and GII are tropical genotypes likely maintained primarily through mosquito-bird and/or mosquito-swine

transmission cycles. This suggests that the spread and establishment of GI-b throughout Asia may have been due to its ability to efficiently overwinter in temperate Asia. Finally, this study identified genotype-defining amino acid substitutions within the E protein that may have played a critical role in the adaptation of these viral genotypes to their respective environments. The phylogenetic divergence of GI-b from GI-a was defined by a conservative isoleucine to valine substitution at amino acid 141 of the E protein. This substitution, and/or other substitutions found elsewhere in the genome may have provided a phenotypic advantage to GI-b viruses that led to the emergence and establishment of this genotype throughout Asia, and the subsequent displacement of GIII as the dominant JEV genotype. However, no genomic sequences of GI-a viruses are available to compare with published GI-b and GIII sequences. Therefore, to gain a better understanding of the molecular determinants underlying the geographically expansive JEV genotype displacement, the genomic sequences of the two GI-a isolates (M859/Cambodia/1967/Mosquito and KE-93-83) available in the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) should be determined and compared with previously published GI-b and GIII sequences.

In Chapter 7, the largest collection of GIII and GI E gene-derived viral sequences assembled to date were utilized to reconstruct the spatiotemporal chronology of the JEV genotype displacement, and to determine the evolutionary and epidemiological dynamics underlying this significant event. Bayesian skyline reconstructions indicated that a decade prior to the genotype displacement, the relative genetic diversity of GI had surpassed that of GIII, thereby conferring a selective advantage to GI. Phylogeographic analysis revealed that GI-a diverged in Thailand and has remained confined to tropical Asia, whereas GI-b diverged in Vietnam and was then dispersed northwards to China, where it was subsequently dispersed to Japan, Korea and Taiwan. To determine what phenotypic

advantages may have facilitated the genotype displacement, the *in vitro* multiplication kinetics of GIII and GI isolates were compared in duck embryo fibroblasts (DEF) and *Aedes albopictus* C6/36 cells. No differences in viral multiplication were observed among genotype III and I viruses in DEF cells; however the GI-b isolate (JE-91) had significantly higher infectious titers in C6/36 cells from 24-48 hours post infection (HPI) compared to the GI-a and GIII isolates. Unfortunately, the WRCEVA at UTMB does not possess any other GI-b isolates to ensure that this observed difference in early viral multiplication represents a genotypic property specific to GI-b rather than a strain property specific to the JE-91 isolate. In nature, an increased viral multiplication efficiency of GI-b viruses compared to GIII viruses at early time points in mosquitoes may have resulted in a shortened extrinsic incubation period (EIP) that led to an increased number of GI enzootic transmission cycles and the subsequent displacement of GIII. However, it should be kept in mind that *Aedes albopictus* C6/36 cells were established from mosquito larvae homogenates, which may not accurately represent the cell types and expression profiles of the mosquito midgut epithelium and other cell types that are associated with infection, dissemination, and infection of JEV. Further, a the results of a recent study indicated that C6/36 cells lack a functional RNA interference (RNAi) response, suggesting that these cells may not accurately model mosquito-arbovirus interactions at the molecular level (Brackney *et al*., 2010). On another note, two previous studies demonstrated that following peroral infection of *Culex pipiens* and *Culex tarsalis* the EIP of the WN02 genotype was up to four days shorter compared to the NY99 genotype (Ebel *et al*., 2004; Moudy *et al*., 2007), however a recent study using different virus isolates and mosquitoes strains did not confirm a shorter EIP for the WN02 genotype compared to the NY99 genotype in *Culex tarsalis* (Anderson *et al*., 2012). Nevertheless, the results of these studies suggest a model whereby earlier transmission of

WN02 genotype viruses in at least some strains of *Culex pipiens* and *Culex tarsalis* led to higher WN02 infection rates in avian hosts and the subsequent establishment of the WN02 genotype throughout North America. A similar model may also explain how GI-b displaced GIII as the dominant JEV genotype throughout Asia. Clearly, future work should focus on elucidating the mechanistic basis for the geographically expansive JEV genotype displacement. First, *Cx. tritaeniorhynchus* should be fed blood meals containing either a GI-b isolate or a GIII isolate to test the hypothesis that the EIP of GI-b is significantly shorter compared to the EIP of GIII. A shorter EIP for GI-b compared to GIII may have led to earlier transmission of GI-b, resulting in higher GI-b infection rates in avian hosts and the subsequent displacement of GIII throughout Asia. It should be noted that a shorter EIP would only result in more efficient viral transmission if the gonotrophic cycle were at least as short. A previous study used a time series analysis to analyze data derived from female *Cx. tritaeniorhynchus* (n = 17,482, 15% parous) collected at a buffalo shed near suburban Bangkok, Thailand throughout 30 consecutive days (Malainual *et al*., 1998). The time series analyses yielded a gonotrophic cycle length of five days and a survival rate per cycle of 20%. This closely agrees with two previous Japanese studies that estimated a gonotrophic cycle length of three days by the mark-recapture method (Buei *et al*., 1980) and through laboratory observations (Kawai, 1969). Second, to evaluate the mechanistic basis of the geographically expansive genotype displacement, C6/36 cells infected with either a GI-b isolate or a GIII isolate and incubated at the lower temperatures observed in temperate Asia should be used to test the hypothesis that GI-b multiplies to significantly higher infectious titers compared to GIII. It is possible that the genotype displacement may have been facilitated by the ability of GI-b to multiply to higher infectious titers at the lower temperatures found in temperate Asia (e.g. Northern China and Japan) compared to GIII. On another note, GIV may be

confined to the Indonesian archipelago due to the inability of this genotype to efficiently replicate in mosquitoes at the lower temperatures found in temperate Asia. Again, C6/36 cells infected with isolates representative of GI-IV and incubated at the lower temperatures observed in temperate Asia could be used to test the hypothesis that GIV multiplies to significantly lower infectious titers compared to GI-III. Finally, C6/36 cells should be co-infected with equal concentrations of a GI isolate and a GIII isolate to investigate the relative fitness of the two genotypes and test the hypothesis that GI-b is able to multiply more rapidly during the initial period of infection and competitively exclude a GIII isolate. As GI and GIII co-circulated in temperate Asia (and in some areas continue to co-circulate), it is likely that competitive fitness played a role in the JEV genotype displacement.

In Chapter 8, a blackbird model of JEV avian viremia and a young mouse model of JEV neuroinvasiveness were utilized to compare the GI-IV of JEV. Overall, 70.8% of the blackbirds inoculated with JEV developed detectable viremias by 3 days post infection (DPI) and 37.5% of the blackbirds maintained their viremias for at least three days. These findings are in agreement with the results of two older experimental studies involving ardeids (Buescher *et al*., 1959a; Soman *et al*., 1977), suggesting not only that blackbirds are a good model of avian JEV viremia, but also supporting the previous notion that blackbirds may occasionally be involved in the enzootic transmission cycle of JEV (Hammon *et al*., 1951). It was also revealed that WNV field seropositive blackbirds were protected against challenge with the P3 isolate of JEV. This is in agreement with a previous study demonstrating that WNV field seropositive blackbirds were protected against challenge with wild-type GIII and GI JEV isolates (Nemeth *et al*., 2009). If JEV was introduced into North America, the presence of WNV seropositive avians would theoretically decrease the transmission rate of JEV to swine, equine, and humans. This

may perhaps even prevent the establishment of JEV in North America. Interestingly, 100.0% of blackbirds developed detectable viremias following inoculation with the GI-a and GI-b isolates, while only 66.7% of blackbirds developed viremias following inoculation with the GII and GIV isolates. Further, the average duration of viremia in blackbirds with detectable viremias was 1.8 days for the GI-a and GIV isolates, 3.0 days for the GII isolate, and 3.8 days for the GI-b isolate. These results suggest that GI-b may have emerged and established itself throughout Asia due to an increased multiplicative ability in avians. Moreover, GIV may be confined to Indonesia and GII may be limited to tropical Asia due to a decreased multiplicative ability in avians. Although interesting, these findings need to be statistically confirmed using an increased number of blackbirds and different doses of virus as inocula. It would also be of value to include a wild-type GIII isolate in the study, as this genotype was the dominant Asian JEV genotype for at least 50 years. An older study was able to show differences in mortality in young A2G mice intraperitoneally inoculated with various yellow fever virus isolates (Fitzgeorge & Bradish, 1980), suggesting that a young mouse model of neuroinvasiveness could also be used to show differences in mortality in young mice inoculated intraperitoneally with isolates representative of GI-IV of JEV. The results of the young mouse model of JEV neuroinvasiveness suggest that GII may be less neuroinvasive in young mice compared to the other genotypes. However, the study should be repeated with lower virus inocula to obtain more accurate 50% lethal dose (LD_{50}) estimates for all viral genotypes (all mice inoculated with the lowest dose [1 PFU] of GIII and GIV died before the end of the study).

9.3 STRENGTHS AND WEAKNESSES OF THE COALESCENT ANALYSES, AND EXPERIMENTAL MODELS FOR NATURAL HOST AND VECTOR JEV INFECTION

Although valuable information was obtained from the experiments outlined in this dissertation, the data should be interpreted in light of the experimental limitations.

9.3.1 Coalescent analyses

The evolutionary analyses described in Chapters 6 and 7 were reliant on the availability of sampled JEV sequences. Isolations of JEV are biased towards Japan and China, where surveillance has been performed with greater intensity, while relatively few isolates have been sampled from tropical Asia. Further, unlike other flaviviruses, such as WNV and the dengue viruses, there is a very limited number of ORF sequences of wild-type JEV isolates. Therefore, in an effort to limit the bias that would have resulted from using small ORF JEV sequence datasets, large, spatiotemporally distributed datasets of E gene sequences were utilized in the evolutionary analyses. While the E gene of JEV was found to be a good evolutionary proxy of the ORF, it was not possible to perform evolutionary adaptation analyses to assess if amino acids in other regions of the genome may have increased the fitness of GI-b, leading to viruses with an increased multiplicative ability in avians or mosquitoes.

Selection analyses performed in Chapters 4, 5, and 7 detected the presence of strong purifying selection. A recent study found that strong purifying selection could lead to distortions in both the topology of phylogenies and the branch lengths within phylogenies, hence altering patterns of sampled genetic variation (Nicolaisen & Desai, 2012). This phenomenon may have resulted in slightly skewed evolutionary rate and time of the most recent common ancestor (MRCA) estimates in Chapters 6 and 7.

Two recent studies have described the presence of flavivirus-related RNA viruses in the *Aedes spp*. mosquito host genome (Crochu *et al*., 2004; Roiz *et al*., 2009). The existence of flaviviral-like sequences in mosquito genomes could have resulted from the

integration of the viral sequences into the mosquito genome following infection with the virus (Crochu *et al*., 2004; Roiz *et al*., 2009). Alternatively, flaviviruses may have originated from the genome of *Aedes spp*. (Crochu *et al*., 2004; Roiz *et al*., 2009). Although evidence supports the former explanation, if flaviviruses did indeed originate from the genome of mosquitoes, flaviviruses would likely be much older than previously thought. Further, the estimated rates of molecular evolution and time of the MRCA presented in this dissertation (Chapters 6 and 7) would likely be invalid.

9.3.2 *In vitro* models of natural JEV host infection

Although *in vitro* models of natural host infection provide a controlled environment to test hypotheses and generate new hypotheses, they are subject to several notable limitations.

In Chapter 7, DEF cells were used as a model of natural avian host JEV infection. Although it is unknown what particular cells are associated with avian JEV infection, it is likely that fibroblasts are not the target cell. This may explain why the preliminary blackbird model of JEV viremia presented in Chapter 8 suggests there could be a difference in the induction and duration of viremias between blackbirds inoculated with GI-a and GI-b compared to blackbirds inoculated with GII and GIV; while no difference was observed in viral multiplication in DEF cells inoculated with GI-IV of JEV. However, the DEF model allows the effects of high temperatures on JEV multiplication to be investigated, which cannot be achieved with Vero cells.

In Chapter 7, *Aedes albopictus* C6/36 cells were used as a model of natural mosquito JEV infection. Although the GI-b isolate had significantly higher infectivity titers in C6/36 cells at early time points compared to isolates representative of the other viral genotypes, as aforementioned, it should be noted that C6/36 cells were established

from mosquito larvae homogenates and the results of previous studies suggest these cells lack a functional RNAi response following viral infection (Brackney *et al*., 2010). Further, a previous study showed that replication in C6/36 cells was not useful for predicting the effect of chikungunya virus (a mosquito-borne alphavirus) mutations on infection of *Ae. albopictus* mosquitoes *in vivo* (Tsetsarkin *et al*., 2007).

In Chapter 8, the IFN-α/β-competent adenocarcinomic human alveolar basal epithelial A549 cell line was used, in addition to the DEF model of JEV infection, to explore whether the distinct epidemiologies and geographical distributions of the JEV genotypes could be explained by differences in viral genotype sensitivity to IFN- α/β. Although most elements of the mammalian type I IFN system have been conserved in avians (Staeheli *et al*., 2001), A549 cells may not represent an accurate model of natural host JEV infection due to undescribed differences between avian and human cell signaling pathways. However, A549 cells have been used to study the related WNV, which makes comparison with JEV in the same cell type of interest.

9.3.3 Blackbird model of JEV viremia

In Chapter 8, a blackbird model of JEV viremia was used to compare the induction and duration of viremias following inoculation with GI-IV of JEV. Not only did the blackbird model of JEV viremia agree with the results of older experimental studies of ardeids (Buescher *et al*., 1959a; Soman *et al*., 1977), it revealed that 100.0% of the blackbirds developed detectable viremias following inoculation with GI-b of the virus and the viremias lasted 3.8 days (longer compared to the other genotypes). However, only one viral inoculum (3,000 PFU) was used and there were only five to six blackbirds per group. Therefore, the study should be validated using an increased number of viral inocula and an increased number of blackbirds per group.

9.4 Evolutionary patterns of JEV and other arboviruses

Similar to that of other arboviruses, JEV was found to be under strong purifying selection (Chapters 4, 5, and 7) and estimated rates of molecular evolution (Chapters 6 and 7) were lower than those of single host vertebrate RNA viruses. This is consistent with constraints imposed by alternating replication cycles in vertebrate and arthropod hosts (Weaver, 2006). Like other arboviruses with avian vertebrate hosts (Eastern equine encephalitis virus, Highlands J virus, Western equine encephalitis virus, Barmah Forest virus, and Sindbis virus) it was revealed that genotypes I and III of JEV have expansive geographic distributions, which could be reflective of efficient viral dispersal by avians (Weaver, 2006). Unlike other members of the JEV serocomplex, JEV uses both avian maintenance and domestic swine amplifying hosts. This allows JEV to undergo secondary amplification, leading to increased levels of enzootic viral circulation and an elevated probability of spillover to humans (Weaver, 2006). Interestingly, unlike WNV and USUV, JEV has never been reported to cause severe disease in avians. This could either be due to selection for resistance by ardeid populations exposed for long time periods to infection or selection for attenuation in ardeids (Weaver, 2006).

9.5 Potential for the emergence and establishment of JEV in the United States

Japanese encephalitis virus is maintained in an enzootic transmission cycle involving primarily ardeids and *Culex* mosquitoes, and is amplified in peridomestic areas by transmission between domestic swine and *Culex* mosquitoes. As highlighted by the recent emergence of WNV into the western hemisphere and Usutu virus (USUV) into the European continent, the invasion of JEV into previously unoccupied regions is a real threat. Increasing trade and tourism between the United States and Asia could increase the risk of introduction of JEV into the United States via an infected adult female

mosquito aboard an airplane or ship (Nett *et al*., 2009). Other potential modes of introduction include a migrating, viremic avian, an imported viremic avian or reptile, or an act of bioterrorism (Nett *et al*., 2009).

If JEV were successfully introduced into the United States, the lack of active JEV surveillance, the cross-reactivity between JEV and other endemic flaviviruses (WNV, Saint Louis encephalitis virus, and dengue virus), the nonspecific clinical presentation, and the likelihood that health care providers would not consider JEV in the differential diagnosis would likely delay detection of the virus (Nett *et al*., 2009). This delay in detection could provide JEV the opportunity to spread beyond its initial point of introduction and establish itself throughout the United States (Nett *et al*., 2009).

Many factors are important in predicting whether JEV would continue to spread and subsequently establish itself throughout the United States. Several mosquito species, including Cx. tarsalis, *Cx. pipiens quinquefasciatus* Say, and *Cx. p. pipiens*, found throughout the United States have experimentally demonstrated the ability to transmit JEV and should be considered potential vector hosts (Reeves & Hammon, 1946).

Unlike WNV and USUV, JEV also utilizes domestic swine as amplifying hosts, which can drive epidemics by producing an abundance of infected mosquitoes. Unlike what has been observed in many areas of Asia, domestic swine in the United States typically reside in large, centralized farms that are located away from residential areas (Nett *et al*., 2009). Therefore, domestic swine may play a minimal role in JEV amplification if the virus were imported into the United States (Nett *et al*., 2009).

The ability of JEV to be maintained, and then subsequently spread and establish in the United States will likely depend on several factors including, but not limited to: 1) the availability, abundance, and location of avians, and 2) contact rates between avians and mosquito vectors (Nemeth *et al*., 2012). Previous studies suggest that some ardeid

and passerine species found in the United States are competent avian hosts for JEV (Banerjee & Deshmukh, 1987; Boyle *et al*., 1983; Buescher *et al*., 1959a; Nemeth *et al*., 2012; Soman *et al*., 1977). As aforementioned, if JEV was introduced into North America, the presence of WNV seropositive avians would theoretically decrease the transmission rate of JEV to swine, equine, and humans. This may perhaps even prevent the establishment of JEV in North America. Nonetheless, both JEV and WNV have co-circulated in India for over 60 years.

The ability of the newly emerged GI-b virus to be maintained year-round in temperate climates suggests that if JEV were introduced into JEV-naïve regions it may become established. Therefore, to predict what geographic areas are at the greatest risk of JEV incursion it is imperative to obtain a further understanding of the host and vector ranges of GI-b.

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Vita

Amy Josephine Schuh was born in Tampa, Florida, on November 5, 1978, to June Miller and Layton Thomas Schuh. She graduated from Hayesville High School, Hayesville, NC, in 1997 and from the University of North Carolina, Chapel Hill, NC in 2001 with a Bachelor of Science in Clinical Laboratory Science. She worked at Angel Medical Center, Franklin, NC and Murphy Medical Center, Murphy, NC as a Clinical Laboratory Scientist prior to earning her Master of Public Health in Epidemiology from the University of Alabama, Birmingham, AL in 2006. She completed both her internship and directed research in the laboratory of Dr. Thomas Unnasch. For her internship, she modified an existing dynamic transmission model of eastern equine encephalitis virus to test the hypothesis that *Culex erraticus* is the major vector of the virus in the southeastern United States, and for her directed research she performed statistical analyses on several large datasets to determine the host choice and West Nile virus infection rates in blood-fed mosquitoes her directed research. These experiences, in addition to her participation in a field-based epidemiology course and dengue virus surveillance project in Kingston, Jamaica, led her to develop a strong interest in arbovirology and commence a Doctor of Philosophy in Experimental Pathology in 2006 at the University of Texas Medical Branch, Galveston, TX. She completed her dissertation research in the laboratory of Dr. Alan Barrett, investigating the genotypic and phenotypic determinants of Japanese encephalitis virus geographical expansion through a combination of *in vitro* and *in vivo* studies.

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EDUCATION:

08/06-present
PhD Candidate, Experimental Pathology, University of Texas Medical Branch, Galveston, TX

08/04-present
MPH, Epidemiology, University of Alabama, Birmingham, AL

08/97-05/01
BS, Clinical Laboratory Science, University of North Carolina, Chapel Hill, NC

CERTIFICATION:

08/01-present
Medical Laboratory Scientist, American Society of Clinical Pathologists

APPLIED EPIDEMIOLOGY TRAINING:

06/10-07/10
Course in Field Epidemiology, Naval Medical Research Unit-6, Tumbes, Peru

- Provided hands-on training in the design, conduct, and publication of epidemiological field investigations

08/04
Course in Infectious Disease Surveillance and Control, University of the West Indies, Mona, Jamaica

- Provided didactic and hands-on training in vector ecology and biology, infectious disease surveillance and control, and water and sanitation in a developing country

PROFESSIONAL EXPERIENCE:

05/06-08/06
Medical Technologist, Clinical Laboratory, Murphy Medical Center, Murphy, NC
Supervisor: Mary Hughes

- Worked in all departments of the laboratory

11/05-05/06
Research Assistant, Department of Health Care Organization and Policy, University of Alabama, Birmingham, AL
Supervisor: Dr. Bisakha Sen

- Performed tasks related to a project on, "Childhood predictors of juvenile delinquency"
- Conducted literature reviews, as well as performed data management and analysis tasks using SAS software

02/03-06/04
Medical Technologist, Clinical Laboratory, Murphy Medical Center, Murphy, NC
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Clinical Laboratory Scientist, McClendon Clinical Laboratories, University of North Carolina Health Care System, Chapel Hill, NC
Supervisor: David Weaver

- Worked in the hematology and urinalysis departments of the core laboratory

PREDOCTORAL RESEARCH ACTIVITIES:

08/07-present
Doctoral dissertation research, University of Texas Medical Branch, Galveston, TX
Advisor: Dr. Alan Barrett

- Elucidation of genotypic and phenotypic determinants of Japanese encephalitis virus geographical expansion

01/10-12/11
Doctoral-level research, University of Texas Medical Branch, Galveston, TX
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- Characterization of the envelope protein domain III of wild-type JEV isolates using monoclonal antibodies

03/07-05/07
Doctoral-level research rotation, University of Texas Medical Branch, Galveston, TX
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- Characterization of a naturally occurring mutation in the envelope protein domain III of a dengue-3 virus

02/07-04/07
Doctoral-level research rotation, University of Texas Medical Branch, Galveston, TX
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- Phylogenetic characterization of chikungunya virus isolates

10/06-02/07
Doctoral-level research rotation, University of Texas Medical Branch, Galveston, TX
Advisor: Dr. Stephen Higgs

- Determined susceptibility of three strains of *Aedes aegypti* to the New Guinea-C strain of dengue-2 virus

01/06-05/06
Master-level directed research, University of Alabama, Birmingham, AL
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- Performed statistical analyses on several large datasets to determine the host choice and West Nile virus infection rates in blood-fed mosquitoes

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Master-level internship, University of Alabama, Birmingham, AL
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- Modified an existing dynamic transmission model of eastern equine encephalitis virus to test the hypothesis that *Culex erraticus* is the major vector of the virus in the southeastern USA

BIBLIOGRAPHY:

Peer-reviewed publications:

Schuh AJ, Guzman H, Tesh RB, Barrett AD. Genetic diversity of Japanese encephalitis virus isolates obtained from the Indonesian archipelago between 1974 and 1987. Vector Borne Zoonotic Dis. 2012. In Press.

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Matsui K, Gromowski GD, Li L, Schuh AJ, Lee JC, Barrett AD. Characterization of dengue complex-reactive epitopes on dengue 3 virus envelope protein domain III. Virology 2009. Feb 5;384(1):16-20.

Manuscripts in preparation:

Schuh AJ, Ward MJ, Leigh Brown AJ, Barrett AD. Phylogeography of Japanese encephalitis virus: Genotype is associated with climate.

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Schuh AJ, Tesh RB, Barrett AD. Genetic characterization of Japanese encephalitis virus genotype II strains isolated from 1951 to 1978. Poster presentation. Annual Meeting of the American Society of Tropical Medicine and Hygiene, Atlanta, GA. November 3-7, 2010.

Schuh AJ, Tesh RB, Barrett AD. Analysis of JEV genomes reveals evidence of the forces involved in shaping the evolution of the virus. Poster presentation. Symposium in "Controlling Emerging Infectious Diseases in the 21st Century", Galveston, TX. February 26-28, 2010.

Schuh AJ, Tesh RB, Innis BL, Barrett AD. The evolutionary analysis of Japanese encephalitis virus and the molecular characterization of genotype II of the virus. Oral and poster presentations. Duke/National University of Singapore Emerging Infectious Diseases Inauguration Symposium, Singapore, Malaysia. December 8-11, 2009.

Schuh AJ, Tesh RB, Innis BL, Barrett AD. The evolutionary analysis of Japanese encephalitis virus and the molecular characterization of genotype II of the virus. Poster presentation. Annual Meeting of the American Society of Tropical Medicine and Hygiene, Washington, DC. November 18-22, 2009.

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Schuh AJ, Li L, Innis BL, Barrett AD. Molecular epidemiology of Japanese encephalitis virus. Poster presentation. Keystone Symposium in "Molecular Evolution as a Driving Force in Infectious Diseases", Breckenridge, CO. April 8-13, 2008.

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