Ecological and Molecular Epidemiological Studies of Japanese Encephalitis Virus and Culex Flavivirus in Toyama Prefecture

（富山県における日本脳炎ウイルスと **Culex flavivirus** の

生態学的及び分子疫学的研究）

2013

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Contents

Abbreviation used in this thesis

ADEM	acute disseminated encephalomyelitis
bp	base pairs
C	capsid
CDC	Centers for Disease Control and Prevention
cDNA	complementary deoxyribonucleic acid
CFAV	cell fusing agent virus
CO_2	carbon dioxide
CPE	cytopathic effects
CxFV	Culex flavivirus
DENV	dengue virus
DNA	deoxyribonucleic acid
E	envelope
Fig.	Figure
HSRRB	Health Science Research Resources Bank
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
km	kilometer
KRV	Kamiti River virus
min	minute
MIR	minimum infection rate
MOI	multiplicities of infection
MVE	Murray Valley encephalitis
NS	nonstructural protein
PCR	polymerase chain reaction
prM	premembrane
RNA	ribonucleic acid
RT	reverse transcription
sec	second
TBEV	tick-borne encephalitis virus
U	unit
UTR	untranslated region
V	volt

Preface

Many mosquito-borne and tick-borne pathogenic viruses, such as Japanese encephalitis virus (JEV), West Nile virus, and tick-borne encephalitis virus (TBEV), belong to the genus *Flavivirus* within the family *Flaviviridae* (Gubler et al. 2007). The virus genome is a single-stranded, positive-sense RNA molecule of about 10-11 kilobase, which comprises a single open reading frame and 5' and 3' untranslated regions (UTRs). The open reading frame encodes structural proteins (capsid (C), premembrane (prM), and envelope (E)), and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).

On the basis of nucleotide sequence information from the E gene, JEV has been divided into five genotypes (Uchil et al. 2001, Solomon et al. 2003). JEV is a mosquito-borne virus and *Culex tritaeniorhynchus* Giles is the most important vector in Japan. The virus exists in an enzootic cycle between mosquitoes and vertebrate hosts such as pigs and birds (Gubler et al. 2007). Pigs are the major amplifier and reservoir for JEV (Burke et al. 1988, Igarashi 1992).

JEV causes severe encephalitis in humans and has led to epidemics in East and South Asia. In Japan, hundreds to thousands of human cases of Japanese encephalitis (JE) were reported every year until the 1960s (Ogata 1985). Since 1992, less than 10 cases/year have been reported because of vaccinations that were introduced in Japan in 1954, and environmental changes, such as the separation of houses from pigpens. However, after the use of the JEV vaccination was discouraged from 2005 to 2009 owing to the occurrence of acute disseminated encephalomyelitis (ADEM) following the vaccination, the herd immunity of the Japanese people to the virus has decreased

(Infectious Diseases Surveillance Center 2008, Infectious Diseases Surveillance Center 2013). However, JEV is still actively circulating in Japan. Therefore, the threat of an outbreak of JEV has increased in the country. In such a situation, surveillance of JEV is quite important to know the risk of outbreak of JE in the view point of public health.

In chapter I, the author isolated JEV strains from mosquitoes and pigs in Toyama Prefecture. According to the nucleotide sequence of the E and C/prM genes, all isolates belong to genotype I. These isolates were divided into the major type and the two minor types, and isolates in 2008 and 2009 had a novel deletion in the 3'UTR. Furthermore, the author reports that the peak level of JEV circulation occurs later in the year than in the past.

Recently, novel flaviviruses which only replicate in insect cells and no relation to diseases of humans or animals have been isolated, such as cell fusing agent virus (CFAV; Stollar et al. 1975), Kamiti River virus (KRV; Sang et al. 2003, Crabtree et al. 2003), Quang Binh virus (Crabtree et al. 2009), Aedes flavivirus (Hoshino et al. 2009), and Nakiwogo virus (Cook et al. 2009). Identification of these insect-specific flaviviruses raises the basic questions about the origin of flaviviruses and the interaction between pathogenic flavivirus and insect-specific flavivirus in insect bodies.

In 2007, novel "Culex flavivirus" (CxFV) strains were reported from *Culex pipiens* L. and *Cx. tritaeniorhynchus* in Japan and *Culex quinquefasciatus* Say in Indonesia (Hoshino et al. 2007). Phylogenetic analysis showed that they were most genetically related with the other insect flaviviruses. CxFV reportedly replicates only in mosquito cells and has been isolated from both male and female mosquitoes, suggesting its possibility of vertical transmission (Hoshino et al. 2007, Farfan-Ale et al. 2009). Since CxFV strains have been isolated from many species of mosquito including *Cx. pipiens*,

Cx. tritaeniorhynchus, *Cx. quinquefasciatus*, *Culex restuans* Theobold, *Culex tarsalis* Coquillett, and *Culex interrogator* Dyar and Knab across a vast area including the United States, Latin America (Mexico, Guatemala, Trinidad, Brazil), Africa (Uganda), Indonesia, Japan, and China (Hoshino et al. 2007, Morales-Betoulle et al. 2008, Farfan-Ale et al. 2009, Kim et al. 2009, Blitvich et al. 2009, Cook et al. 2009, Saiyasombat et al. 2010, accession no. HQ678513 and HQ605702-HQ605704 in GenBank), they seem to be globally distributed within *Culex* mosquitoes. According to the country of virus isolation, CxFV strains were divided into two clades, Latin American/Caribbean and U.S./Asian (Kim et al. 2009, Blitvich et al. 2009, Saiyasombat et al. 2010). Although CxFV reportedly infects many species of *Culex* mosquito, it is not clear whether CxFV exists in nature in a mosquito-species-specific or habitat-specific manner.

In chapter II, the complete genome sequences of CxFV strains isolated from *Cx. tritaeniorhynchus* in Toyama were determined and compared with those isolated from *Cx. pipiens* group in Toyama Prefecture as well as representative isolates of strains previously reported. CxFV maintenance in *Culex* mosquitoes seemed to depend on mosquito habitat not on mosquito species.

Chapter I

Continuity and change of Japanese encephalitis virus in Toyama Prefecture, Japan

Introduction

Environmental conditions in Toyama Prefecture support the enzootic cycle for JEV because there are pigs, the amplifying host of JEV, and many rice fields where the larvae of *Cx. tritaeniorhynchus* can develop. Since the 1970s, human cases of JE have been reported in 1982 and 1997 in Toyama Prefecture (Watanabe et al. 2011). From 1965 to the present, antibodies against JEV in pig sera have been investigated in Toyama Prefecture (Infectious Diseases Surveillance Center 2013, Watanabe et al. 2011). The finding that the seroprevalence of many newly born pigs has reached above 50% almost every year suggests that JEV is still prevalent. On the other hand, since small pigpens gradually decreased in number and large ones increased, the total number of pigpens decreased from the 1960s to the 1970s (Watanabe et al. 2011, Kamimura 1998). Furthermore, pigpens have moved from locations close to rice fields and houses on the plains to hillsides and the way that insecticides are applied and the method of water control of rice fields have changed in recent years. As a result, the likelihood that pigs and people are bitten by *Cx. tritaeniorhynchus* might have decreased. Therefore, the author predicted that recent changes in the method of breeding pigs and control of rice fields affect the prevalence of mosquitoes (Watanabe et al. 2011, Kamimura 1998) and JEV.

In recent reports, researchers have discussed from where and how JEV came to Japan (Nerome et al. 2007, Nabeshima et al. 2009). It has also been considered necessary to

clarify how JEV strains were maintained in local areas after the most frequently isolated genotype changed from III to I in the 1990s (Ma et al. 2003) in Japan. A previous study of genetic change and variation in JEV genotype III in Taiwan (Jan et al. 2000) suggested that JEV isolates fall into three clusters by areas and are genetically stable in Taiwan.

In this chapter, the author isolated JEV strains from mosquitoes and pigs in Toyama Prefecture and performed genetic analysis to reveal how JEV maintains genetic continuity or undergoes genetic changes locally. Furthermore, to assess the effect of environmental changes such as the method of breeding pigs and control of rice fields, the author investigated the relationship between the prevalence of JEV and that of mosquitoes and compared this with the data described in previous reports.

Materials and Methods

Mosquitoes. To isolate viruses, mosquitoes were collected once a week using CO_2 traps from 2004 to 2009 at a total of 21 sites, which included six farms (three pigpens, two cattle sheds, and a horse stable), seven gardens of private houses, four woods, an airport, and three harbors (see Fig. 1). The traps were battery-operated light traps (Inokuchi-Tekko), CDC Miniature Light Traps (John W. Hock Company), 12 V battery-operated light traps (FHK), or 6 V battery-operated traps (Rakuno Gakuen University), which were set with dry ice or a CO_2 refill and left overnight. Some mosquitoes were collected by a net in aircraft at Toyama Airport. Mosquitoes were classified according to collection site, date of collection, species, and sex. Mosquito species were morphologically identified according to Tanaka et al. (1979). In this study, *Cx. pipiens* group contains *Culex pipiens pallens* and *Culex pipiens molestus* because these two species could not be discriminated from each other morphologically. Mosquitoes were then pooled into groups that consisted of a maximum of 50 individuals and stored at −80 ˚C.

To study the seasonal changes of the female *Cx. tritaeniorhynchus* population, mosquitoes were captured using light traps at seven farms (a pigpen, five cattle sheds, and a horse stable) from June to October every year from 2004 to 2009 (Fig. 2). The traps were set overnight once a week. Mosquitoes were classified and counted as described above. The average numbers of mosquitoes were calculated from the weekly collected numbers excluding the maximum and the minimum values among the seven farms.

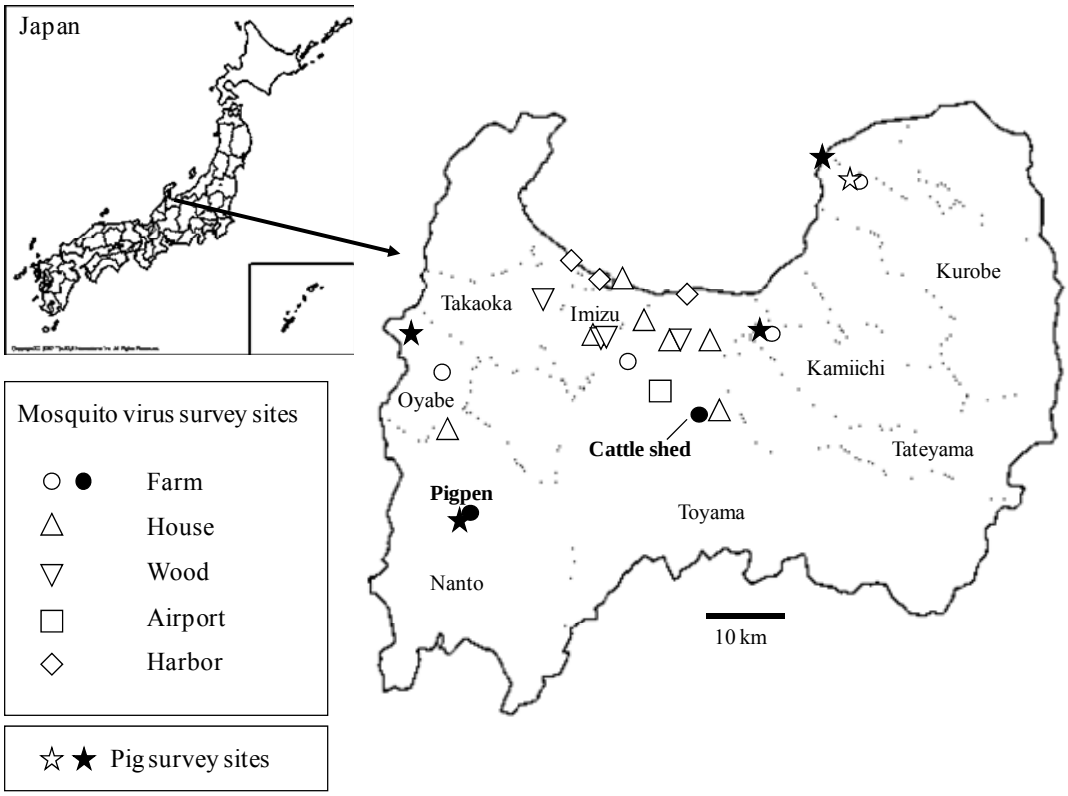

Figure 1. Map showing survey sites for virus isolation in Toyama Prefecture from 2004 to 2009. Stars indicate the sites of pigpens where pig sera were collected. Other shapes denote the corresponding sites as indicated in the box where mosquitoes were collected for virus isolation. Filled marks indicate the sites where JEV-positive specimens were collected.

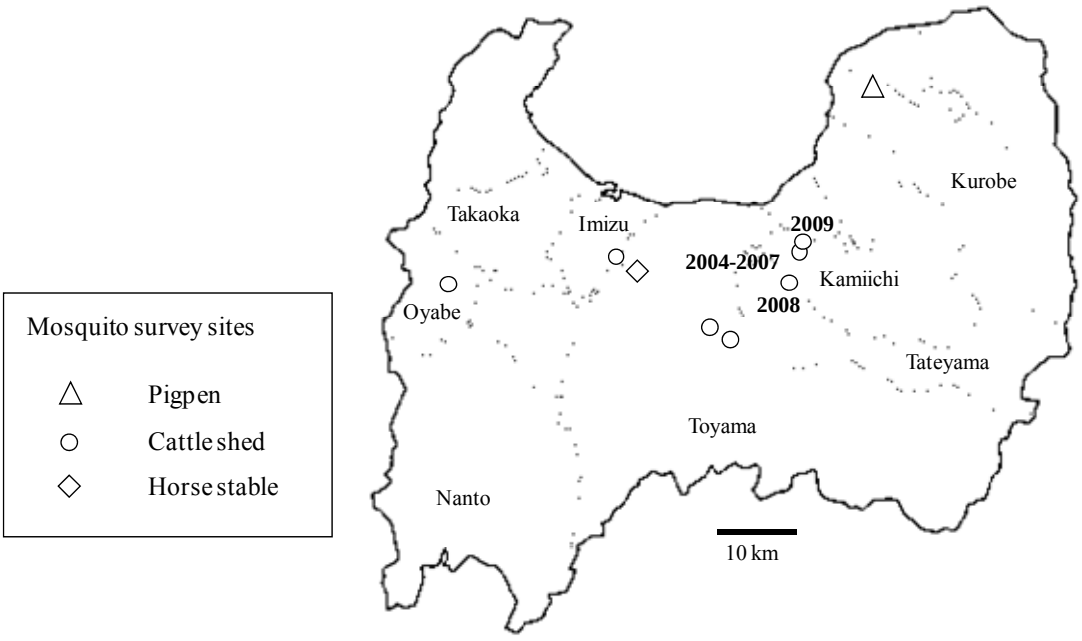

Figure 2. Map showing survey sites for detecting the seasonal changes in the number of female *Cx. tritaeniorhynchus* in Toyama Prefecture. A triangle, circles, and a diamond indicate farms where mosquitoes were collected and counted. Mosquitoes were collected at six sites from 2004 to 2009. Mosquitoes were collected at three sites only in certain years: the numbers near to the three circles indicate the years of collection. In total, mosquitoes were collected at seven sites every year.

Minimum infection rate (MIR). To estimate mosquito infection rates, the MIR was calculated. The MIR of JEV is defined as (JEV-positive pool number/number of mosquitoes tested) × 1,000.

Pig sera. A total of 1,451 sera were collected from pigs that were about six months old in four areas (Nanto city, Oyabe city, Kamiich town, Kurobe city) in Toyama Prefecture (see Fig. 1) from July to October from 2005 to 2009.

Virus isolation. Pools of mosquitoes were homogenized in a 0.5-1.0 mL maintenance medium (Eagle's Minimum Essential Medium containing 2% fetal bovine serum or 0.11% bovine serum albumin fraction V) and centrifuged at 5,867 × *g* for 5 min. The supernatants were passed through 0.45 μm filters (Ultrafree MC, Millipore Corp.). The filtrates were diluted 10-fold with the medium and inoculated onto monolayers of both C6/36 (no. IFO50010; obtained from HSRRB) and Vero (no. JCRB9013; obtained from HSRRB) cells. These cultures were incubated at either 28˚C (C6/36) or 35˚C (Vero) for 2 hr in 5% CO_2. After maintenance medium was added, these cells were incubated for 6-8 days. Pig sera were diluted 10-fold with the medium and inoculated onto the cell monolayers as described above. The cells were cultured for 2 to 3 weeks by 2 or 3 times of cell passages and culture media were collected when cytopathic effects (CPE) appeared.

RNA extraction. Viral RNA was extracted from culture supernatants with a QIAamp Viral RNA Mini Kit (Qiagen) in accordance with the manufacturer's instructions.

Reverse transcription (RT)-polymerase chain reaction (PCR). RT-PCR was carried out with either TaKaRa One Step RNA PCR Kit (AMV) (TaKaRa Bio Inc.) or Ready-To-Go RT-PCR Beads (GE Healthcare). The E gene was amplified with the primers of JE955f (5'-TGYTGGTCGCTCCGGCTTA) and JE2536r (5'-

AAGATGCCACTTCCACAYCTC) (Nerome et al. 2007). The mixture was incubated at 50˚C for 45 min, at 94˚C for 2 min, then 45 cycles at 94˚C for 1 min, 50˚C for 1 min, and 72˚C for 2 min, and finally at 72˚C for 10 min. The C/prM gene was amplified with the primers of JE-prM-FW (5'-CGYCGTGAACA AGCGGGGCARAAA) and JE-prM-RV (5'-TGCAGCGACCATAYTGSACGTAGA) (Hoshino and others, unpublished data). The 3'UTR was amplified with the primers JE10141f (5'-TGGATTGAAGAAAATGAATGGATG) and JE10965r (5'-AGATCCTGTGTTCTTC CTCTC) (Nerome et al. 2007). The mixture was incubated at 53˚C for 40 min and 40 cycles at 92˚C for 1 min, 53˚C for 1 min, and 72˚C for 1 min, and finally incubated at 72˚C for 5 min.

Sequencing analysis. After purification of the amplicons, the E, C/prM, and 3'UTR gene sequences were determined with the BigDye Terminator v1.1 or v3.1 Cycle Sequencing Kit and ABI 3100 or 3130 sequencer (Applied Biosystems). Nucleotide sequences were edited and aligned using Sequencher (version 4.7) software (Gene Codes Co.).

Phylogenetic analysis. The nucleotide sequences of the reference strains of JEV were obtained from GenBank and 1,500-base pairs (bp) sequences of the E region or 240 bp out of 299-bp sequences of the C/prM region were analyzed using MEGA 3.1 software (Kumar et al. 2004). A phylogenetic tree was constructed by the neighbor-joining method and genetic distances were calculated according to Kimura's two-parameter method (Kimura 1980). The reliability of the tree was estimated by performing 1,000 bootstrap replications and bootstrap values of 50% or higher were considered statistically significant for a grouping. A phylogenetic tree was also

constructed by maximum-likelihood method using PhyML 3.0 (http://atgc.lirmm.fr/phyml/) and NJplot (http://pbil.univ-lyon1.fr/software/njplot.html).

JEV sequences generated in this study have been submitted to GenBank under accession numbers AB538601-AB538852 and AB543738-AB543746. Accession numbers of reference strains are shown in figures.

Results

Mosquitoes and pig sera. JEV isolation was performed to investigate the species of mosquitoes and the sites where JEV is prevalent. In total, 51,265 mosquitoes (2,740 pools), representing 15 species, were used for virus isolation, which included 45,190 *Cx. tritaeniorhynchus* (88.1%), 4,590 *Cx. pipiens* group (9.0%), 1,333 *Aedes albopictus* Skuse (2.6%), and other individuals from 12 species (Table 1). Most of the *Cx. tritaeniorhynchus* were captured at farms, whereas *Cx. pipiens* group and *Ae. albopictus* were usually captured at other survey sites (Table 2).

A total of 51 out of 1,371 pools of *Cx. tritaeniorhynchus* harbored JEV through the investigation period (Table 3). All of the mosquitoes positive for JEV were female and collected at farms (Table 3). Out of these 51 pools, 35 were collected near a pigpen and 16 were collected at a cattle shed. JEV was not isolated in 2004 or 2006 from mosquitoes. Samples for virus isolation were simultaneously applied to both C6/36 and Vero cells because the viruses derived from the same sample but isolated by different culture cells often have different nucleotide sequences. In total, 77 JEV strains were isolated from mosquito samples, of which 51 and 26 strains were isolated by C6/36 and Vero cells, respectively. JEV strains were isolated from a total of nine pig sera (Table 3). Two out of these nine sera were collected in September 2005, six were from September to October in 2007, and one was in September 2008. JEV was not isolated in 2006 or 2009 from pig sera. In total, 10 JEV strains were isolated from pig sera, of which seven and three strains were isolated by C6/36 and Vero cells, respectively.

The farms where JEV-positive mosquitoes and pig sera were collected were located in rural areas and suburbs of Toyama Prefecture (filled circles and stars in Fig. 1). These

Table 1. The number of mosquitoes used for virus isolation classified by species.

Species	2004		2005		2006		2007		2008		2009		Total	
	No. sampled	No. of pools	No. sampled	No. of pools	No. sampled	No. of pools	No. sampled	No. of pools	No. sampled	No. of pools	No. sampled	No. of pools	No. sampled	No. of pools
Culex tritaeniorhynchus	2,677	139	8,233	305	3,147	192	13,370	337	13,851	304	3,912	94	45,190	1,371
Culex pipiens group	914	165	1,475	233	762	185	685	125	266	68	488	50	4,590	826
Aedes albopictus	184	82	318	115	381	127	64	33	281	45	105	34	1,333	436
Culex orientaris	1	1	5	5	19	8	13	7	1	1			39	22
Tripteroides bambusa			3	2	28	10	3	2					34	14
Aedes japonicus	5	5	10	9	5	5	3	3					23	22
Culex infantulus	4	4	5	3	5	5							14	12
Aedes flavopictus	10	8					2	2					12	10
Culex bitaeniorhynchus	1	1	4	4	1	1	2	1			2	1	10	8
Uranotaenia novobscura			6	5	1	1	1	1					8	7
Armigeres subalbatus							1	1	2	2	2	2	5	5
Anopheles sinensis	1	1	2	2	1	1							4	4
Aedes nipponicus							1	1					1	1
Lutzia vorax							1	1					1	1
Culex modestus inatomii									1	1			1	1
Total	3,797	406	10,061	683	4,350	535	14,146	514	14,402	421	4,509	181	51,265	2,740

Table 2. The number of *Cx. tritaeniorhynchus* , *Cx. pipiens group* , and *Ae. albopictus* used for virus isolation classified by sites.

Sites	*Cx. tritaeniorhynchus*		*Cx. pipiens* group		*Ae. albopictus*	
	No. sampled	No. of pools	No. sampled	No. of pools	No. sampled	No. of pools
Farm	42,381	979	1,427	129	13	8
House	2,326	233	1,835	320	755	251
Wood	407	109	936	260	182	101
Airport	53	41	102	63	81	23
Harbor	23	9	290	54	302	53
Total	45,190	1,371	4,590	826	1,333	436

Table 3. The numbers of pools of *Cx. tritaeniorhynchus* or pig sera from which JEV strains were isolated.

	Sites	2004	2005	2006	2007	2008	2009	Total
Cx. tritaeniorhynchus (No. of pools)	Farm	0 / 41	11 / 154	0 / 121	10 / 292	27 / 286	3 / 85	51 / 979
	House	0 / 75	0 / 99	0 / 59	NT	NT	NT	0 / 233
	Wood	0 / 23	0 / 44	0 / 8	0 / 34	NT	NT	0 / 109
	Airport	NT	0 / 8	0 / 4	0 / 11	0 / 11	0 / 7	0 / 41
	Harbor	NT	NT	NT	NT	0 / 7	0 / 2	0 / 9
	Total	0 / 139	11 / 305	0 / 192	10 / 337	27 / 304	3 / 94	51 / 1,371
Pig serum sample	Nanto	NT	0 / 93	0 / 124	1 / 178	0 / 90	0 / 60	1 / 545
	Oyabe	NT	2 / 80	0 / 101	2 / 170	0 / 85	0 / 60	4 / 496
	Kamiichi	NT	NT	0 / 45	3 / 80	0 / 75	0 / 60	3 / 260
	Kurobe	NT	NT	NT	NT	1 / 90	0 / 60	1 / 150
	Total	NT	2 / 173	0 / 270	6 / 428	1 / 340	0 / 240	9 / 1,451

NT: not tested

JEV-positive number/tested number

sites were distributed in both western and eastern areas of Toyama Prefecture and were not concentrated in any particular place. JEV strains were simultaneously isolated from mosquitoes and pig sera at a pigpen in Nanto city (filled circle and star in Fig. 1).

To clarify the correlation between the seasonal change in mosquito numbers and the prevalence of JEV, the average numbers of female *Cx. tritaeniorhynchus* collected at seven farms (Fig. 2) were counted from 2004 to 2009. Their seasonal changes were compared with the MIR of JEV at two survey sites, the pigpen in Nanto city and the cattle shed in Toyama city (Fig. 3A, Fig. 1).

At the pigpen, the MIR of JEV peaked from September to October while the number of female *Cx. tritaeniorhynchus* at the seven farms showed two peaks in August and September (Fig. 3A), indicating that the MIR of JEV increased after the peak in the number of female mosquitoes. JEV isolation from mosquitoes in this pigpen was not performed in 2004. On the other hand, the MIR at the cattle shed peaked from August to early September in 2005, 2007, and 2008, when most of the mosquitoes were captured (Fig. 3A). In 2009, the MIR of JEV at the cattle shed followed the peak in the number of mosquitoes and peaked in early October.

In 2006, only a few mosquitoes were captured at the two survey sites (Fig. 3A), and thus JEV was not isolated from either mosquitoes or pigs (Fig. 3A, Table 3).

The number of female *Cx. tritaeniorhynchus* and the MIR of JEV among them from 1966 to 1972 (Katori et al. 1975) are shown in Fig. 3B. JEV strains were isolated from the end of July to early September when both the MIR and the number of mosquitoes peaked, with the exception of 1968. The data suggests that the JEV isolation period has been delayed in recent years compared with that in 1966-1972.

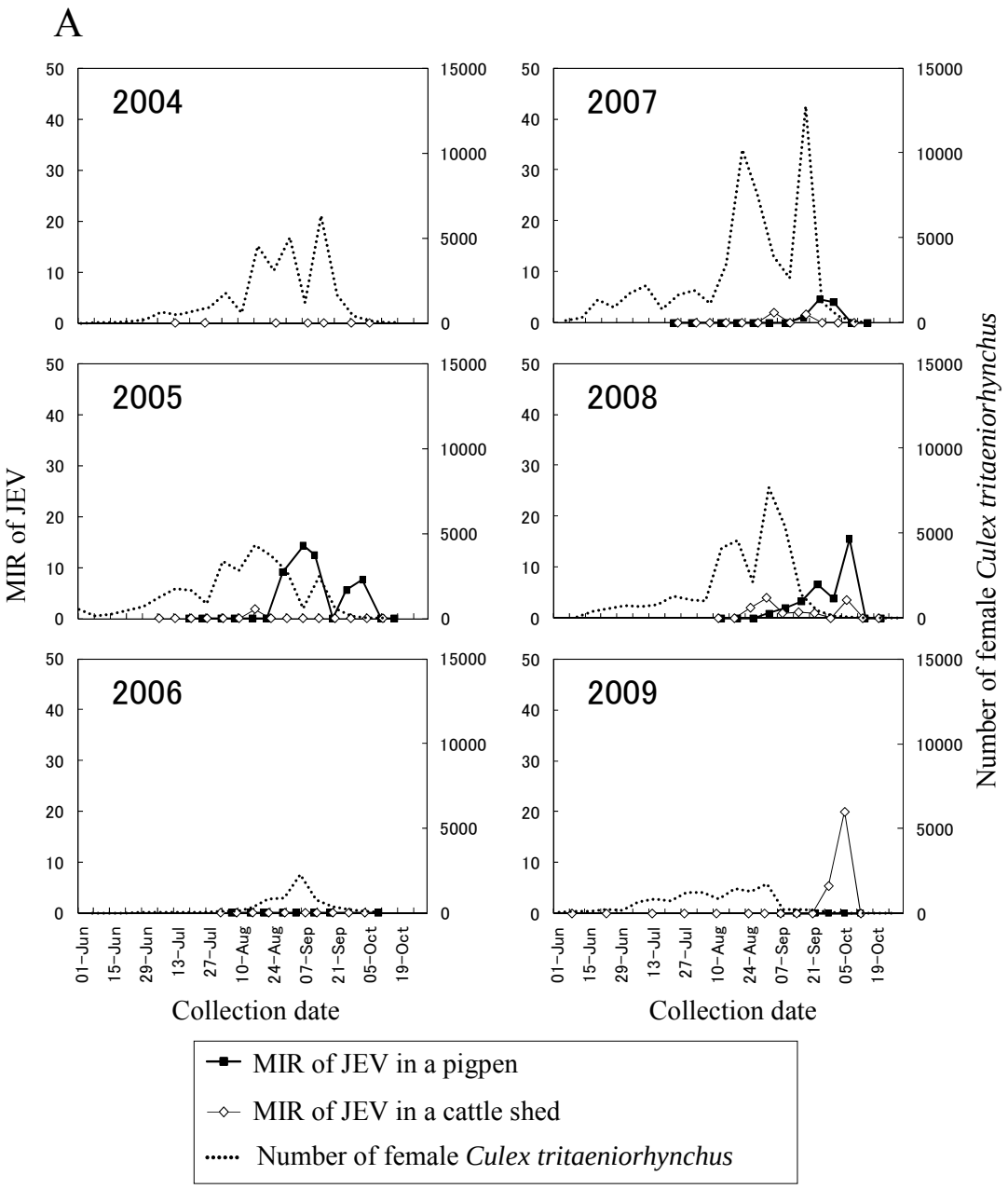

Figure 3. The MIR of JEV and number of female *Cx. tritaeniorhynchus*. The MIRs were calculated using the following formula: (JEV-positive pool number/number of mosquitoes tested) × 1,000. The numbers at the top left of each graph indicate years. (A) The MIR of JEV of female *Cx. tritaeniorhynchus* at the pigpen in Nanto city (Fig. 1) and at the cattle shed in Toyama city (Fig. 1), and the number of female *Cx. tritaeniorhynchus* at seven farms (Fig. 2). The numbers of female *Cx. tritaeniorhynchus* are shown as averages. The average numbers of mosquitoes were calculated from the weekly collected numbers excluding the maximum and the minimum values among the seven farms, to remove anomalous data. Virus isolation was not performed at the pigpen in Nanto city in 2004. (B) The MIR of JEV of female *Cx. tritaeniorhynchus* in pigpens and cattle sheds and the average number of female *Cx. tritaeniorhynchus* from 1966 to 1972. These data were obtained from reports of previous studies conducted in Toyama Prefecture (Katori et al. 1975, Watanabe et al. 2011). The average numbers of female *Cx. tritaeniorhynchus* were calculated as described in (A) among four to 10 farms. For the MIR, the first and last dates of investigation and the dates when JEV was detected from mosquitoes are plotted. The first date in 1970 was May 25th and is not plotted in this graph.

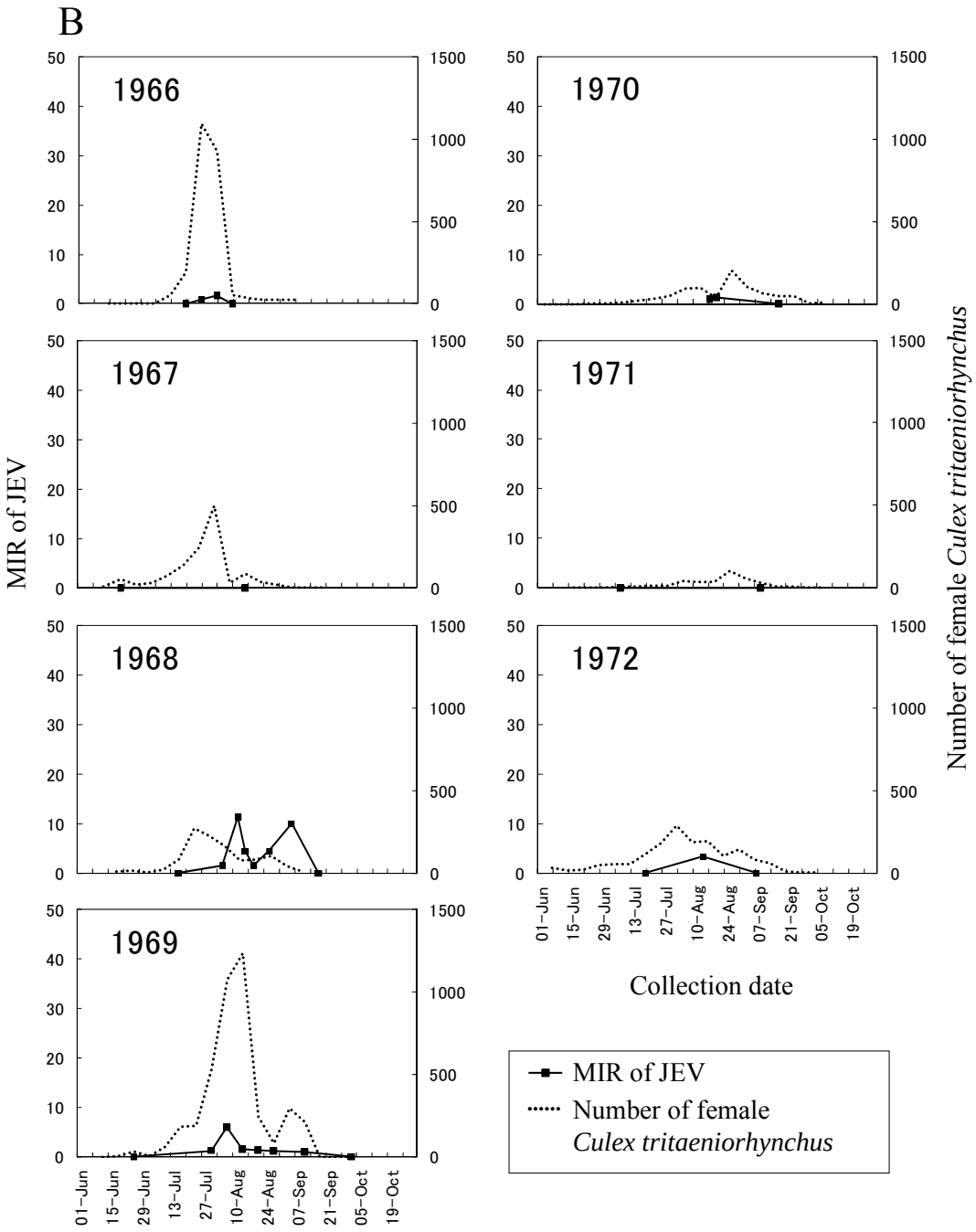

Figure 3. (Continued)

Phylogenetic analysis. To estimate how JEV undergoes genetic change or continuity in Toyama Prefecture over several years, phylogenetic analysis was performed for 87 isolates (77 from mosquitoes and 10 from pig sera) in Toyama.

All JEV isolates were classified into genotype I according to the sequencing analyses of both E (Fig. 4A) and C/prM genes (Fig. 4B). Genotype I of JEV became the dominantly isolated genotype in Japan in the 1990s, before that time genotype III was the most frequently isolated genotype in the country (Ma et al. 2003).

Using the E gene sequences, the isolates in this study were further divided into three clusters, tentatively named as A, B, and C (Fig. 4A). There were 19 to 35 nucleotide differences among clusters A, B, and C (Table 4). With the exception of 16 isolates that are colored in grey in cluster A, isolates in each cluster had the same amino acid sequences (Table 5). Cluster A was further divided into three subclusters, tentatively named as A-1, A-2, and A-3 (Table 4). These subclusters were different from each other by two to 13 nucleotides. Subcluster A-1 was composed of 21 isolates in 2005. Fifteen isolates in 2007, seven isolates in 2008, and three isolates in 2009 belonged to subcluster A-2. Subcluster A-3 was composed of 35 isolates in 2008. The phylogenetic tree of the JEV isolates in this study and the reference strains is shown in Fig. 4A. The isolates that belonged to cluster A were similar to the reference strains isolated in Hyogo, Japan, 2008 (accession no. AB481224), Sw/Mie/40/2004 (isolated in Mie, Japan, 2004), 01VN88 (isolated in HaTay, Vietnam, 2001), SC04-16 (isolated in Sichuan, China, 2004), and 02VN105 (isolated in HaNam, Vietnam, 2002). Three isolates in 2007 belonged to cluster B (Table 4) and their nucleotide sequences matched 100% with strain JaNAr07-04 (isolated in Nagasaki, Japan, 2004) and were similar to

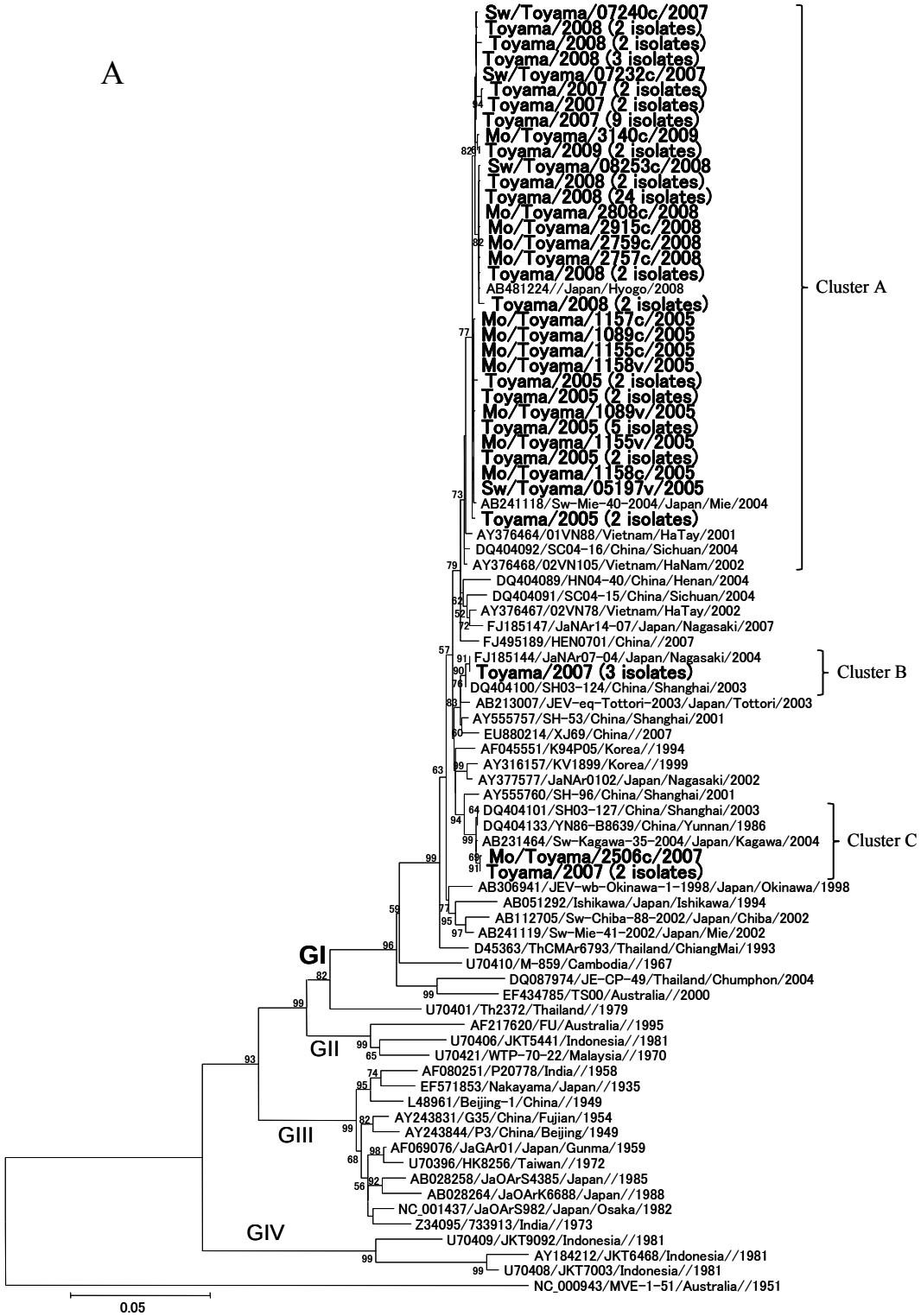

Figure 4. Phylogenetic tree of E (A) and C/prM (B) genes of JEV isolates. JEV isolates in Toyama Prefecture are shown as Toyama/year (isolate numbers) or isolate name. Isolate names are given to the JEVs isolated in this study as indicated by Mo (mosquitoes) or Sw (swine)/Toyama (prefecture)/sample no. and inoculated cell (c:C6/36, v:Vero)/year. GI-GIV indicates JEV genotypes. Reference strains are shown by "accession no./strain name/country/prefecture/year". The sequence of Murray Valley encephalitis (MVE) virus was used as an outgroup. The scale shows the genetic distance in nucleotide substitutions per site. Numbers at branches indicate bootstrap values (%) greater than 50%. Bootstrap replications were performed 1,000 times.

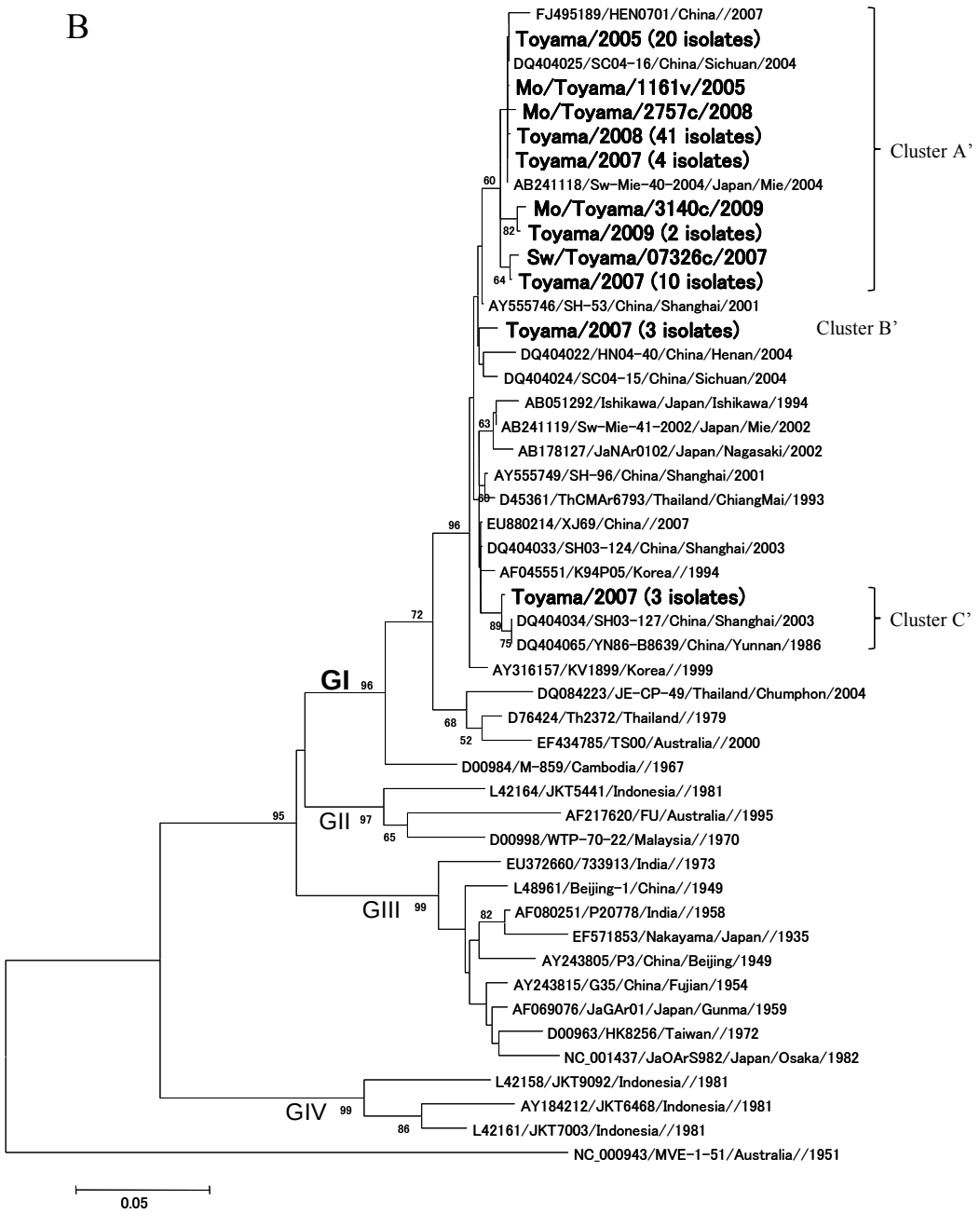

Figure 4. (Continued)

Table 4. Nucleotide sequence differences within the E gene among strains isolated in this study.

No. of isolates				Cluster	Subcluster	Nucleotide no. in the E region	3	6	15	30	54	87	105	120	156	201	204	207	228	240	261	291	294	435	438	481	510	522	541	570	594	642	654	690	692	753	765	795	841	849	865	894	934
2005	2007	2008	2009			Consensus	T	T	A	T	C	C	A	A	A	C	T	A	A	C	C	C	T	A	T	G	T	T	G	C	C	T	C	C	C	T	T	G	A	T	C	C	A
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5													T																	T											T	
	1														G															T				C								R
	1														G															T				C								
	2														G		C													T				C				G				
	2														G		C													T				C				G				
	9				A	A-2									G															T				C								
		2													G															T				T	C							
		2													G														A	T					C								
		3													G															T					C								
			1												G															T					C								
			2												G															T					C								
		1													G	T														T			T		C								
		1													G															T			T		C								
		1													G															T			T		C								
		1											T		G															T			T		C								
		1			A	A-3									G															T			T		C								
		2													G															T			T		C								
		2													G															T			T		C		C						
		2													G										A					T			T		C								
		24													G															T			T		C								
	3				B				C	G	C	T							G	T		T			C			C		T	T										T	
	1				C			C	C					G			T	G			T	C	G	C		A	C		T	T							A				
	2							C	C					G			T	G			T	C	G	C		A	C		T	T							A				

Table 4. (Continued)

No. of isolates				Cluster	Subcluster	Nucleotide no. in the E region	957	967	969	984	993	996	1011	1028	1035	1055	1059	1068	1071	1077	1004	1006	1116	1120	1134	1149	1152	1164	1176	1185	1194	1212	1218	1233	1263	1323	1341	1389	1398	1425	1429	1434	1480
2005	2007	2008	2009			Consensus	T	G	C	T	T	T	T	C	A	G	G	A	A	C	G	T	C	A	C	C	G	G	T	C	A	C	T	G	T	T	T	A	C	A	G	G	C
1																	A					Y																					
1							C										A									R																	
1																	R										R															
1																											R															
1																	A																										
1																	A						T																				
1				A	A-1												R						Y																				
1																																										
2																																										
2											Y																															
2																								G			A															
2																	A																										
5																																										
	1																										A															
	1																										A								C							
	2													T													A										G					
	2													T													A															
	9			A	A-2																						A															
		2																									A															
		2																G									A						A									
		3																									A															
			1							C										T							A															
			2																	T							A															
		1																									A												G			
		1																									A											T	G			
		1					R																				A												G			
		1																									A												G	R		
		1		A	A-3								C														A												G			
		2																							T		A												G			
		2																									A												G			
		2														A					A						A												G			
		24																									A												G			
	3			B						C												T		T							T	C		C							T
	1			C					T	C		C			G				G			T					C	T	G	T	C				A				A	T
	2									C		C			G				G			T					C	T	G	T	C				A				A	T

Y: C or T, W: A or T, R: A or G

Isolates which had superimposed signals in the nucleotide sequence are colored in grey.

Table 5. Amino acid sequence differences within the E gene among strains isolated in this study.

No. of isolates				Cluster	Subcluster	Amino acid no. in the E region	161	181	231	281	312	323	343	365	366	374	477
2005	2007	2008	2009			Consensus	A	G	T	T	K	V	A	S	S	M	D
1															S or P		
1																	
1																	
1																	
1																	
1																	
1				A	A-1												
1																	
2																	
2																	
2																V	
2																	
5																	
	1										K or E						
	1																
	2									A			V				
	2									A			V				
	9			A	A-2												
		2							M								
		2						S									
		3															
			1														
			2														
		1															
		1															
		1										V or I					
		1															D or N
		1		A	A-3												
		2															
		2															
		2					T							N			
		24															
	3			B													
	1			C													
	2																

Isolates which have amino acid differences from another isolate(s) are colored in grey.

SH03-124 (isolated in China, 2003) (Fig. 4A). The other three isolates that belonged to cluster C (Table 4) were similar to strains Sw/Kagawa/35/2004 (isolated in Kagawa, Japan, 2004), YN86-B8639 (isolated in Yunnan, China, 1986), and SH03-127 (isolated in Shanghai, China, 2003) (Fig. 4A).

As for the C/prM gene, isolates in this study were further divided into three clusters, tentatively named as A', B', and C' (Fig. 4B). There were four to nine nucleotide differences among these three clusters (Table 6). Cluster B' had one amino acid difference from clusters A' and C'. All the isolates classified into clusters A, B, and C according to the E gene corresponded with those classified into clusters A', B', and C' according to the C/prM gene, respectively. Cluster A' was further divided into three subclusters, tentatively named as A'-1, A'-2, and A'-3 (Table 6). These subclusters were different from each other by one to five nucleotides. Cluster A'-3 had one amino acid difference from clusters A'-1 and A'-2. All 21 isolates in 2005, four isolates in 2007, and all 42 isolates in 2008 belonged to subcluster A'-1 (Table 6). Subclusters A'-2 and A'-3 were composed of 11 isolates in 2007 and three isolates in 2009, respectively. The isolates that belonged to cluster A' were similar to strains SC04-16 and Sw/Mie/40/2004 (Fig. 4B) and were the same isolates that fell into cluster A in the E gene phylogeny. Clusters B' and C' were each composed of three isolates in 2007 (Table 6). The three isolates in cluster B' were not similar to existing strains (Fig. 4B). The three isolates in cluster C' were similar to strains YN86-B8639 and SH03-127 and were the same isolates that fell into cluster C in the E gene phylogeny.

The author also generated a phylogenetic tree using maximum-likelihood (data not shown) and found that the isolates were divided into clusters A, B, and C (E gene) or A', B', and C' (C/prM) as observed using Kimura's two-parameter method (Fig. 4A, 4B).

Table 6. Nucleotide sequence differences within the C/prM gene among strains isolated in this study.

No. of isolates				Cluster	Subcluster	Nucleotide no.	C region				prM region										
							324	345	365	372	12	31	81	93	102	126	153	169	210	213	216
2005	2007	2008	2009			Consensus	C	T	C	T	A	C	C	A	C	T	T	G	C	T	G
20	4	41																			
1				A'	A'-1			C													
		1																		C	
	10			A'	A'-2							T									
	1											T				G					
			2	A'	A'-3					C								T			
			1							C				G				T			
	3			B'			T		T				T		T		C				A
	3			C'			T				G				T		C		T		A

Among 21 JEV strains isolated in 2007, 11 isolates that were collected from mosquitoes on September 25 or October 1 in the pigpen in Nanto city belonged to clusters A'/A, B'/B, and C'/C. Therefore, JEV strains of three types (clusters A'/A, B'/B, and C'/C) co-circulated from the end of September to early October 2007 in the pigpen in Nanto city. Furthermore, superimposed signals in the nucleotide sequence were observed for the E gene in 10 isolates (colored in grey in Table 4). This indicates that these isolates contained at least two different strains.

All the isolates were divided into either eight or three types according to the nucleotide sequences or deletions, respectively, in the 3' UTR (Fig. 5). All the isolates in 2005 and 18 isolates in 2007 were revealed to have the same deletion (nucleotide no. 5-6, 14-26, 35, 46, and 58-59) as the strains of Ishikawa (Takegami et al. 2000) and Sw/Mie/40/2004 (Nerome et al. 2007). The other three isolates in 2007 had an additional nine-nucleotide deletion (nucleotide no. 34-43) similar to Sw/Kagawa/35/2004. These isolates were found to constitute the clusters C and C' by phylogenetic analysis according to E and C/prM genes, as indicated in Fig. 4A and 4B. All the isolates in 2008 and 2009 had a novel additional deletion of seven nucleotides (nucleotide no. 44-51), although they are divided into two different subclusters A-2 and A-3 according to the E gene (Table 4) and subclusters A'-1 and A'-3 according to the C/prM gene (Table 6).

From the results stated above, the changes in JEV in Toyama Prefecture from 2005 to 2009 are summarized in Fig. 6. In 2005, strains of "A'-1 (C/prM)/A-1 (E)/Ishikawa type (3'UTR)" predominated. In 2007, strains of "A'-1/A-2/Ishikawa type" and "A'-2/A-2/Ishikawa type" were isolated. Minor strains of "B'/B/Ishikawa type" and

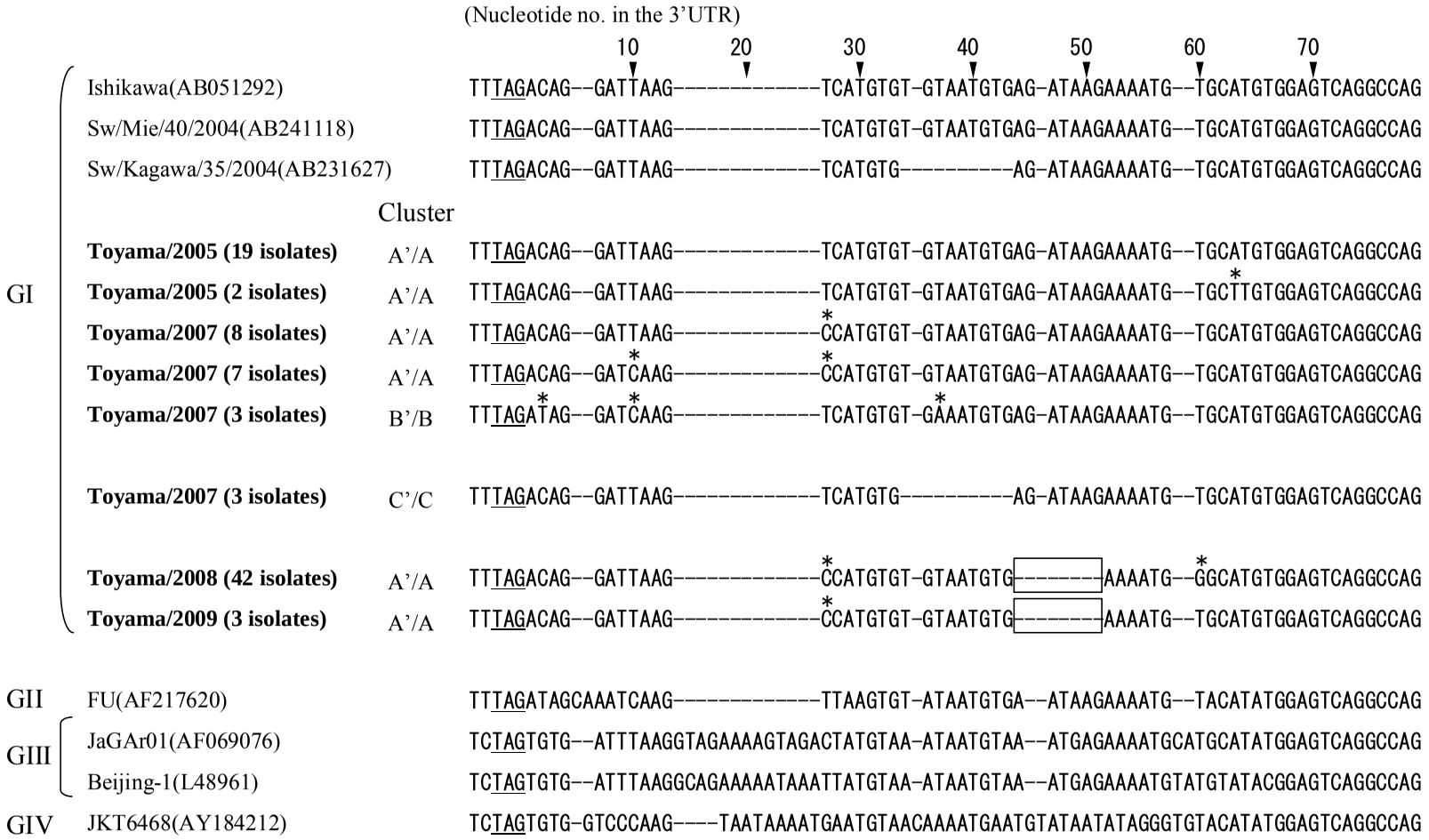

Figure 5. Alignment of nucleotide sequences of the 3'UTRs of Toyama isolates and the reference strains of JEV. JEV isolates in Toyama Prefecture are shown as Toyama/year (number of isolates) with bold letters. Reference strains are shown by "strain name (accession no.)". GI-GIV indicates JEV genotypes. Deletions are indicated by hyphens. The stop codons are underlined. Novel deletion sites are boxed. Nucleotides of the strains isolated in this study that were different from "Toyama/2005 (19 isolates)" are indicated by asterisks.

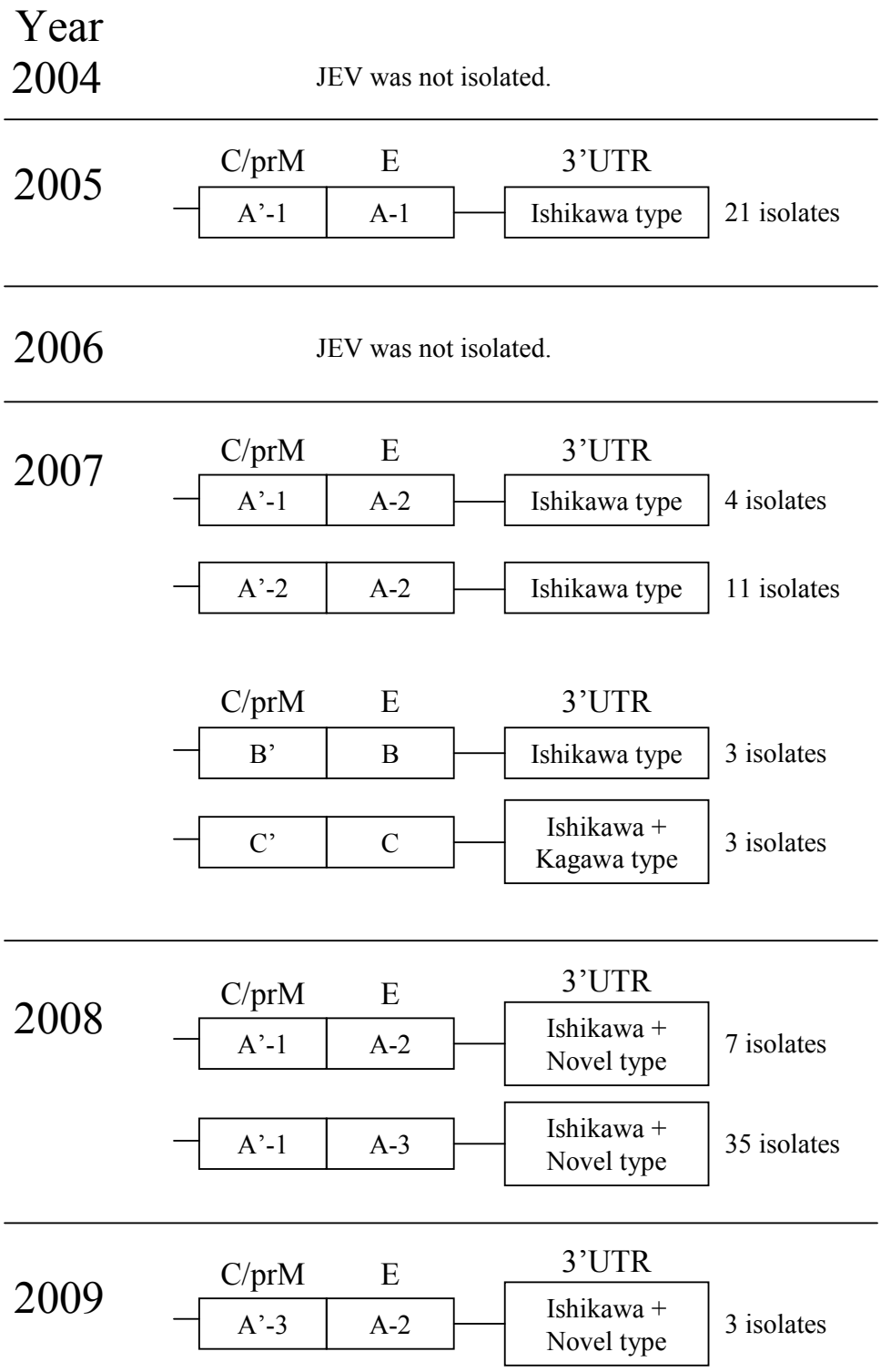

Figure 6. The changes of JEV strains in Toyama Prefecture from 2005 to 2009. The C/prM and E genes are shown by cluster or subcluster names. The 3'UTRs are shown by the deletion types indicated in Fig. 5. "Ishikawa type" indicates the same deletion as Ishikawa (accession no. AB051292) and Sw/Mie/40/2004 (accession no. AB241118). "Ishikawa + Kagawa type" indicates the same deletion as Sw/Kagawa/35/2004 (accession no. AB231627). "Ishikawa + Novel type" indicates the novel deletion type observed in 2008 and 2009.

"C'/C/Ishikawa + Kagawa type" were isolated but these types were not isolated in 2008. In 2008, strains of "A'-1 (C/prM)/A-2 (E)" remained circulating although the 3'UTR of these strains had a novel deletion. Major strains were "A'-1/A-3/Ishikawa + Novel type" in 2008. In 2009, strains were "A'-3/A-2/Ishikawa + Novel type". The results show that predominant strains in cluster "A'/A" changed from year to year but certain subcluster strains "A'-1/A-2" remained circulating from 2007 to 2008. Minor strains "B'/B" and "C'/C" were present only in one year (2007) and disappeared in later years.

Virus replication characteristics in tissue culture of Vero and C6/36 cells were examined among these isolates belonging to different clusters and having different deletions in the 3'UTR. Culture supernatants were collected one, two, three, and six days after infection of Vero (multiplicities of infection (MOI) were 0.01 and 0.001) and C6/36 cells (0.001 and 0.0001) and virus titers in culture fluids were determined. Virus titers peaked from two to three days in Vero and at six days in C6/36 cells. The ranges of the peak virus titers were about 5×10^7-5×10^8 FFU/mL (MOI 0.01 in Vero cell), 10^8-10^9 FFU/mL (MOI 0.001 in Vero cell), 10^9-10^{10} FFU/mL (MOI 0.001 in C6/36 cell) and 10^8-10^9 FFU/mL (MOI 0.0001 in C6/36 cell). Among several isolates belonging to different clusters and having different deletions in the 3'UTR, virus replication did not correlate with the different clusters or deletion status (data not shown).

Discussion

There has been much discussion concerning how JEV appears every summer in Japan. One possible explanation is that the virus is introduced from tropical or subtropical zones of other Asian countries every year. Another explanation is that JEV overwinters in Japan and re-emerges in early summer.

This study was performed to investigate how JEV maintains genetic continuity or undergoes genetic change locally for several years. JEV isolation and genetic characterization were performed in Toyama Prefecture, Japan, from 2005 to 2009.

Overall, strain "A'/A" seems to have remained in Toyama Prefecture and changed gradually. This fact may indicate that this type of JEV is a predominant strain that is endemic locally. The novel deletion in the 3'UTR might be an additional change. On the other hand, strains "B'/B" and "C'/C" might be sporadically introduced to Japan and did not become predominant strains. Overwintering might be one of the factors for maintenance of predominant strain. JEV in Japan is considered to be a mixture of the overwintering type and a type from overseas because one subcluster was isolated only in Japan, while another type of JEV was also isolated elsewhere, such as in China and Vietnam (Nabeshima et al. 2009). JEV has not been isolated yet from overwintering mosquitoes in Japan, while JEV was shown to overwinter locally in Hokkaido, Japan because outbreaks of abortion in pigs caused by JEV were observed in early June, the interepidemic period of JEV (Takashima et al. 1988). In another report, JEV was maintained during winter in lizards experimentally, although it was not isolated from wild ones (Doi et al. 1983). Therefore, the overwintering mechanism of JEV in Japan is still unclear. On the other hand, JEV was isolated from overwintering mosquitoes in

Korea (Rosen 1986). In Taiwan, one of the subtypes was shown to have been present for at least 11 years (Jan et al. 2000). Because Japan, like Taiwan, consists of islands, some subtypes of JEV may also be maintained for several years in Japan. Although many isolates of JEV in Japan are considered to be originated from Southeast Asia, not all the viruses migrated from outside of Japan might have been sustained. Our results support this hypothesis.

All of the isolates in Toyama Prefecture were similar to strains in China and Vietnam and these reference strains had already been isolated before the time when strains in this study were isolated. This result also supports the theory that JEV was introduced from Southeast Asia and continental East Asia to Japan (Nabeshima et al. 2009). Furthermore, strains of genotype III isolated before 1990 in Japan were similar to those in Korea and Taiwan (data not shown). All of the strains isolated in Korea after 1991 belonged to genotype I, as did those in Japan. However, in Taiwan, strains of genotype III were isolated until 2002. These results indicate that the movement of JEV may be linked between Japan and Korea, which are geographically close. It will be clear whether recent isolates in Korea are similar to isolates in Toyama if nucleotide sequences of recent isolates in Korea become available.

The strains in 2005 and 2007 mainly had the same deletions in the 3'UTR as Ishikawa strain, while three strains in cluster C in 2007 had the same deletions as Sw/Kagawa-35/2004. JEV strains in 2008 and 2009 had an additional novel deletion in the 3'UTR, even though they belonged to the same cluster as strains in 2005 and 2007 according to both E and C/prM genes. This novel deletion might have occurred in the JEV maintained in Toyama Prefecture over winter or originated in another area and spread to Toyama Prefecture. Since JEV strains with this novel deletion have so far only

been found in Toyama Prefecture, this novel deletion might have occurred in the JEV maintained in Toyama Prefecture. However, further analysis of isolates in other areas may clarify this issue.

On the other hand, strains of "B'/B/Ishikawa type" and "C'/C/Ishikawa + Kagawa type" most likely migrated from other regions and then became extinct. They were detected in 2007 but were not isolated in 2008 and 2009. They were not detected because of rarity compared with the level of prevalent strains or they might have disappeared in 2008 because they did not fit the local environmental conditions or competed with other types of strains.

All JEVs isolated from female *Cx. tritaeniorhynchus* were collected at farms and they belonged to genotype I. This result confirms that *Cx. tritaeniorhynchus* is the major vector of genotype I of JEV in Toyama Prefecture. JEV strains were not only isolated at pigpens but also at a cattle shed. Since JEV is not known to cause viremia in cattle (Horimoto et al. 1987, Ilkal et al. 1988), mosquitoes harboring JEV might have flown from other places, such as a pigpen 2 km away, to this shed. The fact that neither an antibody nor JEV was detected from seven cattle under one year old in 2009 (data not shown) supports the above hypothesis. In a previous report, cattle acquired antibody after experimental infection with JEV (Ilkal et al. 1988). Because *Cx. tritaeniorhynchus* were few in number in 2009 (Fig. 3A), there might have been little opportunity for the infection of cattle.

Although JEV strains were isolated from August to October in 2005-2009 in this study, they were mainly isolated from the end of July to early September in Toyama Prefecture in 1966-1972 (Fig. 3B) (Katori et al. 1975), from July to August in Nagasaki prefecture in 1964-1973 (Hayashi et al. 1973, Fukumi et al. 1976), and from July to

early September in Osaka prefecture in 1968-1997 (Nakamura et al. 2002). On the other hand, the number of *Cx. tritaeniorhynchus* peaked at the end of July in the 1970s, and from the end of August to the beginning of September after the 1990s in Toyama Prefecture (Watanabe et al. 2011). Thus, the late isolation of JEV in this study seems to correlate with the late increase in the number of *Cx. tritaeniorhynchus*. Although the reason for the late peak in the number of mosquitoes is not clear, it might be due to the way that insecticides are applied and/or the method of water control of rice fields. From the late 1960s to the 1970s, rice fields were filled with water in May and *Cx. tritaeniorhynchus* developed from June to July. The growth of *Cx. tritaeniorhynchus* was suppressed by the first application of insecticide from a helicopter at the end of July, the second application in early August, and the drying of rice fields at the end of August before harvest time (Watanabe et al. 2011, Kamimura 1998). The application of insecticide using a helicopter was stopped in 1995 because it disturbed ecological systems around rice fields. Recently, because rice fields have been filled with water relatively late in the season and insecticide has been applied onto rice seedlings and the drying of rice fields in June, *Cx. tritaeniorhynchus* has not developed in June. However, insecticides are not used frequently, except on seedlings. After the refilling of rice fields with water, *Cx. tritaeniorhynchus* may develop and peak from August to September. These factors may also affect the late prevalence of JE, which has recently mainly occurred in September (Infectious Diseases Surveillance Center 2008), despite occurring in August in the past (Ogata 1985).

Furthermore, JEV strains were isolated from mosquitoes in a pigpen after the peak in the number of female *Cx. tritaeniorhynchus*. This is in contrast to a previous report showing that JEV infections of mosquitoes occurred before or at the peak in the number

of mosquitoes (Fig. 3B) (Katori et al. 1975). At temperatures higher than 24˚C, JEV reproduction in *Cx. tritaeniorhynchus* was found to be faster and virus titer was higher and peaked earlier after infection than at lower temperatures (Shichijo et al. 1972). In the 1960s and 1970s, the number of *Cx. tritaeniorhynchus* peaked in July when the temperature was high. JEV might effectively reproduce in mosquitoes and peak in number at the time of the peak in the number of mosquitoes. Recently, the number of *Cx. tritaeniorhynchus* peaked in August and September, a period with a lower temperature. As a result, JEV might not have effectively reproduced in mosquitoes and thus the peak in the MIR of JEV followed the peak in the number of *Cx. tritaeniorhynchus*. This also means that, recently, the MIR for JEV in mosquitoes has not been high in August to September when the number of mosquitoes peaks; therefore, the risk of infection in humans that are bitten by mosquitoes may now be lower in late summer and autumn than in summer. Furthermore, mosquitoes have more difficulty biting people in autumn than in summer because people wear long sleeves. These factors might be related to the recent decrease in the prevalence of JE in Japan, in addition to the effects of human vaccinations.

In conclusion, JEV still circulates between mosquitoes and pigs in Toyama Prefecture and is correlated with the prevalence of mosquitoes; however, the peak level of JEV circulation occurs later in the year than in the past. According to the nucleotide sequence information derived from the E and C/prM genes, all isolates belong to genotype I. The major type of JEV might have remained in Toyama Prefecture and gradually changed over five years, while two minor types of JEV might have migrated from other countries and then become extinct. JEV isolates in 2008 and 2009 had a novel deletion in the 3'UTR.

Summary

To determine the mechanisms of maintenance and evolution of JEV in a temperate zone, the author attempted to isolate JEV from mosquitoes and pigs in Toyama Prefecture, Japan. A total of 87 JEV strains were isolated from female *Cx. tritaeniorhynchus* mosquitoes and pigs during 2005-2009. The prevalence of JEV in Toyama Prefecture was seasonally late in comparison with that of the virus during 1966-1972. Furthermore, JEV strains were isolated after the peak in the number of female *Cx. tritaeniorhynchus*. Among JEV strains isolated in this study, two distinct groups were observed within genotype I of the phylogeny generated from nucleotide sequence information derived from the E and C/prM genes: strains belonging to the major type were isolated during 2005-2009, and strains from the minor type were isolated only in 2007. The major type has exhibited gradual change in its E and C/prM genes, and all isolates obtained in 2008 and 2009 had a novel deletion of seven nucleotides in the variable region of the 3'UTR.

Chapter II

Ecological and genetic analyses of the complete genomes of Culex flavivirus strains isolated from *Culex tritaeniorhynchus* and *Culex pipiens* (Diptera: Culicidae) group mosquitoes

Introduction

CxFV were reported in 2007 first (Hoshino et al. 2007). Although CxFV reportedly infects many species of *Culex* mosquito, it is not clear whether CxFV exists in nature in a mosquito-species-specific or habitat-specific manner.

The complete genome sequences were determined in CxFV strains from *Cx. pipiens* such as strains NIID-21-2 (Hoshino et al. 2007), Tokyo (Hoshino et al. 2007), Iowa07 (Blitvich et al. 2009), and H0901 (unpublished data, accession no. HQ678513) as well as *Cx. quinquefasciatus*, strains CxFV-Mex07 (Farfan-Ale et al. 2009) and HOU24518 (Kim et al. 2009). These CxFV strains were found to be similar to each other. In comparison with other flavivirus strains (CFAV, KRV, JEV, dengue virus (DENV), Yokose virus, TBEV, and Apoi virus), CxFV was most similar to CFAV, in particular, in the prM and E genes (69.0-69.5% nucleotide identities) (Hoshino et al. 2007). According to the E and NS5 genes, the strains isolated from *Cx. tritaeniorhynchus* are similar to those from *Cx. pipiens* (Hoshino et al. 2007, Hoshino et al. 2009); however, the nucleotide sequences in other regions were not determined and might be different.

Cx. tritaeniorhynchus is the major vector of JEV, the causative agent of severe encephalitis in humans. Since both CxFV and JEV are harbored by *Culex* species of mosquitoes in Japan, two viruses may either affect or share host mosquitoes. For

example, they may interfere or help each other to increase. Therefore, clarification of the mechanism of maintenance of CxFV is important not only ecologically but also from a public health perspective.

Surveillances of mosquito and JEV have been conducted continuously from 1969 in Toyama Prefecture, Japan, and habitat partitioning of *Cx. tritaeniorhynchus*, *Cx. pipiens* group mosquitoes (containing *Cx. pipiens pallens* and *Cx. pipiens molestus*), and *Ae. albopictus* has been precisely characterized (Watanabe et al. 2011, Chapter I of this thesis). Whereas *Cx. tritaeniorhynchus* was mainly distributed on farms, *Cx. pipiens* group and *Ae. albopictus* were distributed at other sites such as the gardens of private houses. In this study, the complete genome sequences of CxFV strains isolated from *Cx. tritaeniorhynchus* were determined and compared with those isolated from *Cx. pipiens* group in Toyama Prefecture as well as representative isolates of strains previously reported. From these data, CxFV maintenance in *Culex* mosquitoes and their habitats are discussed.

Materials and Methods

Mosquitoes. To isolate viruses, mosquitoes were collected once a week using CO_2 traps from 2004 to 2009 at the same 21 sites as described in Chapter I (Fig. 1).

Virus isolation. CxFV isolation was performed at the same time as JEV isolation described in Chapter I. Pools of mosquitoes homogenized and filterd were inoculated onto monolayers of C6/36 cells. Two or three cell passages were performed and culture media were collected when CPE appeared.

Detection of CxFV. Viral RNA was extracted from culture supernatants that exhibited CPE with a QIAamp Viral RNA Mini Kit (Qiagen) in accordance with the manufacturer's instructions. RT-PCR was carried out with either QIAGEN OneStep RT-PCR Kit (Qiagen) or TaKaRa One Step RNA PCR Kit (AMV) (TaKaRa Bio Inc.). The 258 bp of the NS5 gene were amplified with primers MA and cFD2 (Kuno 1998, Table 7). For PCR, the mixture was incubated at 53˚C for 30 min, and then 40 cycles of 94˚C for 1 min, 53˚C for 1 min, and 72˚C for 1 min.

After purification of the amplicons, the nucleotide sequences were determined with the BigDye Terminator v1.1 or v3.1 Cycle Sequencing Kit and ABI 3100 or 3130 sequencer (Applied Biosystems). The nucleotide sequences were aligned using Sequencher software version 4.7 (Gene Codes Co.) and compared with those of CxFV in the GenBank database. The MIR was defined in terms of CxFV-positive pool number per 1,000 mosquitoes tested.

Analysis of the complete genomes of CxFV isolates. Viral RNA was extracted from culture supernatants at passage four with a QIAamp Viral RNA Mini Kit to obtain a sufficient virus titer for analysis. RT-PCR was performed with the primers shown in

Table 7. Primers used to amplify and sequence CxFV cDNA.

Primer name	Position[a]	Gene	Polarity	Sequence (5'-3')	Reference
MA	8842 - 8863	NS5	forward	CATGATGGGRAARAGRGARRAG	Kuno 1998
cFD2	9099 - 9074	NS5	reverse	GTGTCCCAGCCGGCGGTGTCATCAGC	Kuno 1998
CXFV-E-399FW	1282 - 1301	E	forward	TTGCGGAGAGGGRTATAACG	
NS3-1	4924 - 4899	NS3	reverse	AAATCCATAACCGTACAGTCCAACGA	Hoshino et al. 2007
NS3-2	5245 - 5270	NS3	forward	TCTGATGGAGAAAGGCATTGACAGCA	Hoshino et al. 2007
NS5-1	8908 - 8883	NS5	reverse	CCAGATAATCCTTGATCCTCGTGCCT	Hoshino et al. 2007
CXFV-E1	850 - 870	prM	forward	CGTCTTGTTGTGCGTGGCTRT	Hoshino et al. 2007, modified
CXFV-E-399R1	1659 - 1640	E	reverse	ATGGCATTATCCCAKACGAG	
NS3-1F1	4614 - 4633	NS3	forward	TTTGGCATGTGACATCTGGT	
NS3-2R1	5569 - 5550	NS3	reverse	TGAAACTCCCCCAAGTTCAC	
CXFV-NS5-FW	8889 - 8913	NS5	forward	GGGGATCAAGGATYATCTGGTACA	Hoshino et al. 2009
CXFV-NS5-RV	9956 - 9933	NS5	reverse	CTTTCACTGKCATCCAYGGGTTGT	Hoshino et al. 2009, modified
CXFV-NS5-F581	9526 - 9545	NS5	forward	GTCAAAAACGCGCAAGAACT	
CXFV-3'-F1	9900 - 9881	NS5	forward	TGGATGACGACACAGGACAT	
CXFV-E-R2	1161 - 1142	E	reverse	AGCTGAGATCCTCCAGGACA	
CXFV-E-R3	1113 - 1094	E	reverse	TGACAATCAGCGAGCAATTC	
CXFV-E-R4	983 - 964	E	reverse	TGGTCATCTTTTCCGCTTTC	

[a] Numbers of the first and last nucleotide positions in CxFV genome, reference strain NIID-21-2 (accession no. AB377213).

Table 7. The mixture with primers CXFV-NS5-FW and CXFV-NS5-RV was incubated at 50˚C for 45 min, at 94˚C for 2 min, and then 45 cycles of 94˚C for 1 min, 50˚C for 1 min, and 72˚C for 2 min, and finally at 72˚C for 10 min. PCR with primers CXFV-E-399FW and NS3-1, NS3-2, and NS5-1 was performed as follows. Viral RNA (12 µl) was treated with 3 µl of DNase mixture (containing 1 U of DNase I) at 37˚C for 30 min and then at 75˚C for 5 min, followed by the addition of 15 µl of a mixture containing Super Script II RNase H^- transcriptase XL (Invitrogen) or Super Script III RNase H^- transcriptase XL (Invitrogen). RT was performed at 42˚C for 60 min, and the enzyme was inactivated at 99˚C for 5 min. A 50 µl PCR reaction mixture contained 2 µl of cDNA, 2.5 U of *TaKaRa LA Taq* (TaKaRa Bio Inc.), 10x LA PCR buffer II 5 µl, 2.5 mM $MgCl_2$, and 0.4 mM of each dNTP. Amplification was performed under the following conditions: 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 6 min. RT-PCR with primers CXFV-E1 and CXFV-E-399R1, NS3-1F1 and NS3-2R1, CXFV-NS5-FW and CXFV-NS5-RV was performed as described in “Detection of CxFV”. The sequences were determined by primer walking.

The extreme 3’ ends of CxFV isolates were determined using poly(A) Polymerase Tailing Kit (EPICENTRE Biotechnologies) and 3’-Full RACE Core Set (TaKaRa Bio Inc.) with primer CXFV-NS5-F581 (Table 7). Nested PCR was performed by TaKaRa Ex Taq (TaKaRa Bio Inc.) with primers CXFV-3'-F1 (Table 7) and 3 sites Adaptor Primer in 3’-Full RACE Core Set. The extreme 5’ ends of CxFV isolates were determined by 5’ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen), with primers CXFV-E-R2, CXFV-E-R3, and CXFV-E-R4 (Table 7).

CxFV isolated in this study have been submitted to GenBank under accession numbers AB701766-AB701776 and AB772323-AB772405.

Phylogenetic analysis. The nucleotide and amino acid sequences of the reference strains were obtained from GenBank. Alignment and phylogenetic analyses were performed using MEGA 3.1 software (Kumar et al. 2004). A phylogenetic tree was constructed by the neighbor-joining method and genetic distances were calculated according to Kimura's two-parameter method (Kimura 1980). The reliability of the tree was estimated by performing 1,000 bootstrap replications. The homologies among CxFV strains were analyzed by GENETYX software (version 10, Genetyx Corp. 2009). The similarities versus position with the reference strains were plotted using SimPlot program version 3.5.1 (Lole et al. 1999) (distributed by the author Ray at http://www.welch.jhu.edu/).

Results

Infection rate of CxFV. A total of 94 CxFV strains were isolated: 12 pools from 45,190 *Cx. tritaeniorhynchus* (45,184 females and six males) and 82 pools from 4,590 *Cx. pipiens* group (4,564 females and 26 males) (Table 8). The MIRs of *Cx. tritaeniorhynchus* and *Cx. pipiens* group were calculated as 0.3 and 17.9, respectively, with the latter being significantly higher than the former ($P < 0.001$). They were exclusively isolated from female mosquitoes. Three CxFV strains were isolated from three pools in which JEV were isolated in Chapter I.

CxFV-positive mosquitoes were collected in the sites indicated in Fig. 7, which included three farms, seven gardens of private houses, three woods, and Toyama Airport. These sites were widely distributed in Toyama Prefecture.

CxFV strains were isolated from *Cx. tritaeniorhynchus* and *Cx. pipiens* group from May to October and from April to November, respectively (Table 8). The strains were most frequently isolated in July (39 strains, 41.5%). The MIR and the number of *Cx. pipiens* group peaked at almost the same time in July to August (Fig. 8), when they were compared at one site (A in Fig. 7) in 2005. The number of CxFV-positive pools in *Cx. tritaeniorhynchus* increased in July (4 isolates, 33.3%) at the same time as that in *Cx. pipiens* group, although only a small number of CxFV were isolated from *Cx. tritaeniorhynchus* and the number of *Cx. tritaeniorhynchus* in July was less than those in August and September in Chapter I.

The complete genomes of CxFV isolates. The complete genomes of 11 Toyama isolates were sequenced and the characteristics of the Toyama and reference strains were summarized (Table 9). Three isolates from *Cx. tritaeniorhynchus* and four

Table 8. The numbers of *Cx. tritaeniorhynchus* or *Cx. pipiens* group from which CxFV were isolated.

Species	Year	No. of pools			No. of mosquitoes tested	MIR[a]	Date of CxFV isolation	
		Positive	/	Tested			Earliest	Latest
Cx. tritaeniorhynchus	2004	1	/	139	2,677	0.4	13-July	13-July
	2005	1	/	305	8,233	0.1	6-July	6-July
	2006	2	/	192	3,147	0.6	12-July	13-Sept.
	2007	3	/	337	13,370	0.2	16-May	29-Aug.
	2008	2	/	304	13,851	0.1	25-July	25-July
	2009	3[b]	/	94	3,912	0.8	9-Sept.	6-Oct.
	Total	12	/	1,371	45,190	0.3	16-May	6-Oct.
Cx. pipiens group	2004	8	/	165	914	8.8	7-July	15-Sept.
	2005	34	/	233	1,475	23.1	15-June	12-Oct.
	2006	17	/	185	762	22.3	7-June	5-Sept.
	2007	14	/	125	685	20.4	20-June	10-Oct.
	2008	8	/	68	266	30.1	26-April	17-Nov.
	2009	1	/	50	488	2.0	12-Nov.	12-Nov.
	Total	82	/	826	4,590	17.9	26-April	17-Nov.

[a] MIR, Minimum infection rate of CxFV

[b] These three pools were also JEV positive.

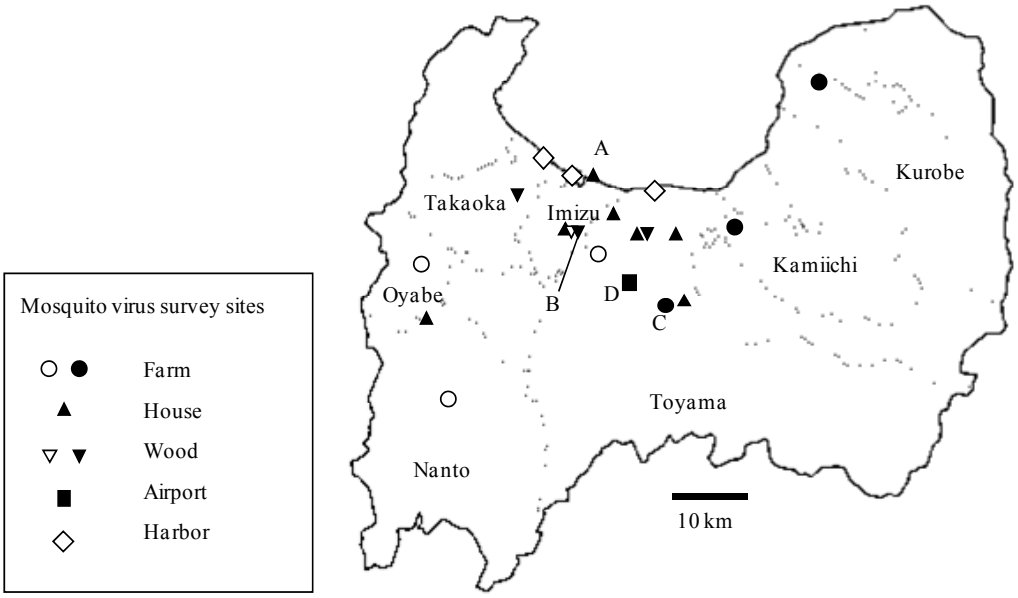

Figure 7. Map showing survey sites for virus isolation. Marks denote the corresponding sites as indicated in the box where mosquitoes were collected for virus isolation. Filled marks indicate the sites where CxFV-positive mosquitoes were collected. A, B, C, and D in the map are survey sites where CxFV were isolated and the nucleotide sequences of the isolates were determined.

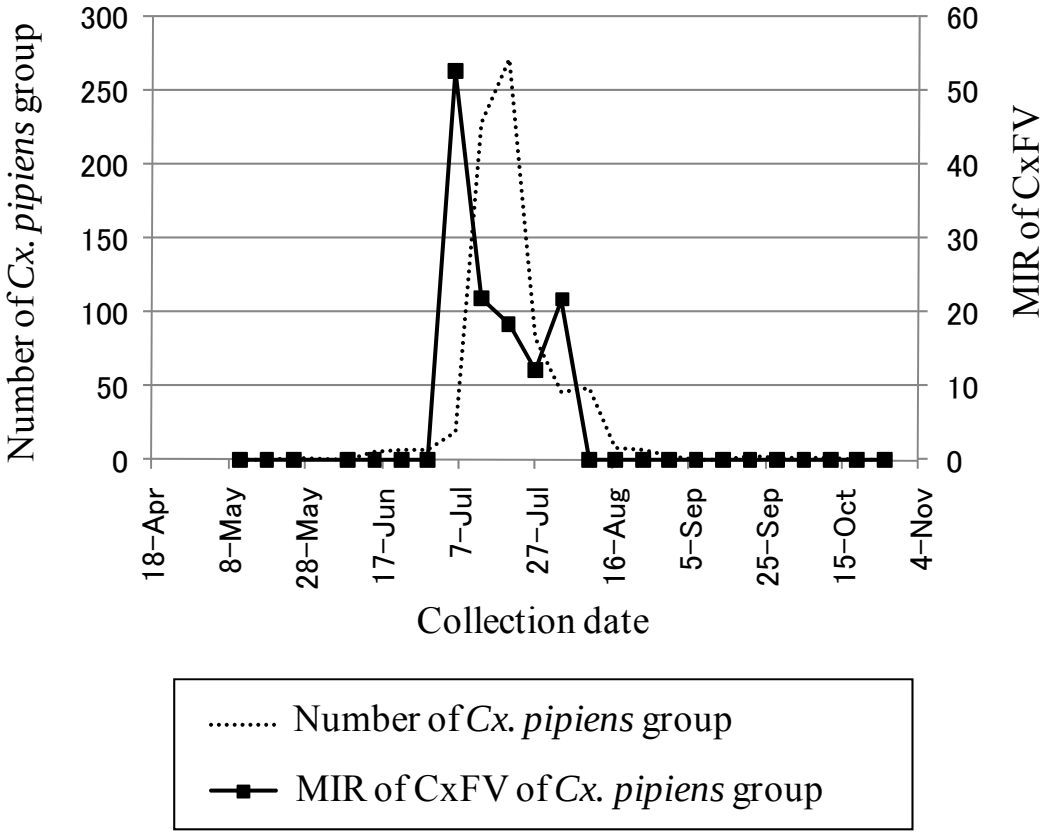

Figure 8. The MIR of CxFV and number of *Cx. pipiens* group in site A (Fig. 7) in 2005. The MIR means CxFV-positive pool number per 1,000 mosquitoes tested. The numbers of mosquitoes were calculated from the weekly collected numbers.

Table 9. The characteristics of CxFV isolated in this study and the reference strains.

Strain names	Accession No.	Mosquito species	Sex	Number of mosquitoes in a pool	Collection sites	Collection date	Full length (nucleotides)	Length of 5'UTR	Length of 3'UTR
Toyama71	AB701766	*Culex tritaeniorhynchus*	female	50	Toyama, Japan	July 13, 2004	10,837	91	654
Toyama734	AB701767	*Cx. tritaeniorhynchus*	female	50	Toyama, Japan	July 6, 2005	10,837	91	654
Toyama1701	AB701768	*Cx. tritaeniorhynchus*	female	25	Toyama, Japan	Sept. 13, 2006	10,837	91	654
Toyama1849	AB701769	*Cx. tritaeniorhynchus*	female	1	Toyama, Japan	May 16, 2007	10,836	91	653
Toyama41	AB701770	*Culex. pipiens* group	female	50	Toyama, Japan	July 7, 2004	10,836	91	653
Toyama75	AB701771	*Cx. pipiens* group	female	12	Toyama, Japan	July 13, 2004	10,837	91	654
Toyama740	AB701772	*Cx. pipiens* group	female	50	Toyama, Japan	July 13, 2005	10,837	91	654
Toyama791	AB701773	*Cx. pipiens* group	female	12	Toyama, Japan	July 13, 2005	10,837	91	654
Toyama861	AB701774	*Cx. pipiens* group	female	3	Toyama, Japan	July 27, 2005	10,836	91	653
Toyama1431	AB701775	*Cx. pipiens* group	female	25	Toyama, Japan	Aug. 2, 2006	10,835	90	653
Toyama2627	AB701776	*Cx. pipiens* group	female	1	Toyama, Japan	April 26, 2008	10,837	91	654
NIID-21-2[a]	AB377213	*Cx. pipiens*	NA	NA	Tokyo, Japan	2003	10,837	91	654
Tokyo[a]	NC_008604	*Cx. pipiens*	NA	NA	Tokyo, Japan	2003	10,837	91	654
Iowa07[b]	FJ663034	*Cx. pipiens*	NA	NA	Iowa, USA	July - Oct., 2007	10,837	91	654
H0901	HQ678513	*Cx. pipiens pallens*	NA	NA	Liaoning, China	2009	10,841	95	654
HOU24518[c]	FJ502995	*Culex quinquefasciatus*	NA	50	Texas, USA	Mar. 5, 2008	10,837	91	654
CxFV-Mex07[d]	EU879060	*Cx. quinquefasciatus*	NA	NA	Yucatan, Mexico	2007	10,837	91	654

NA, Not available

[a] Hoshino et al. 2007

[b] Blitvich et al. 2009

[c] Kim et al. 2009

[d] Farfan-Ale et al. 2009

from *Cx. pipiens* group consisted of 10,837 nucleotides, containing 91 nucleotides in the 5'UTR and 654 nucleotides in the 3'UTR, which were the same as the reference strains, except H0901 (Table 9). One isolate from *Cx. tritaeniorhynchus* and three from *Cx. pipiens* group had one nucleotide deletion at position 10,316 (3'UTR), and "Toyama1431" had an additional nucleotide deletion at position 82 (5'UTR).

The nucleotide and amino acid identities of the entire genome sequences were compared among Toyama and reference strains (Table 10). Eleven Toyama and five reference strains (NIID-21-2, Tokyo, Iowa 07, H0901, and HOU24518) showed higher identities in both nucleotides and amino acids (95.2-99.2% and 98.1-99.8%, respectively) than the Mexican reference strain (CxFV-Mex07) (90.7-90.9% and 96.8-97.1%, respectively).

To identify whether there are specific regions in the CxFV genome that might be responsible for the above differences observed among CxFV from different species or countries, their complete genomes were compared by a similarity plot program. No specific region with markedly different homology was observed among these strains, except part of the NS5 region (about no. 9,050-9,100) of the reference "Tokyo" strain (data not shown). Since this region in the reference "Tokyo" strain had low similarity with other reference strains except CxFV-Mex07, it might be unique.

To determine the way in which CxFV is maintained in nature, phylogenetic analysis was performed using 11 Toyama isolates and reference strains. The 11 Toyama isolates were divided into four clusters in the phylogenetic tree (Fig. 9). One cluster consisted of four isolates (Toyama41, 861, 1431, and 1849) from *Cx. pipiens* group or *Cx. tritaeniorhynchus* captured at site A or B. Their nucleotide sequences matched at 99.6 to

Table 10. Nucleotide and amino acid identities of the complete genomes among CxFVs.

No.	Strain names	Accession no.	Isolation source[a]	Collection sites in Figure 1	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	**Toyama71**	AB701766	Ct	C		100.0	100.0	97.7	97.7	100.0	97.9	100.0	97.7	97.7	**97.5**	97.5	97.4	97.8	95.3	97.3	90.7
2	**Toyama734**	AB701767	Ct	C	100.0		100.0	97.7	97.7	100.0	97.9	100.0	97.7	97.7	**97.5**	97.5	97.4	97.7	95.3	97.3	90.7
3	**Toyama1701**	AB701768	Ct	C	100.0	100.0		97.6	97.7	100.0	97.9	100.0	97.7	97.7	**97.5**	97.5	97.3	97.7	95.2	97.3	90.7
4	**Toyama1849**	AB701769	Ct	B	99.3	99.3	99.3		99.6	97.7	98.0	97.6	99.6	99.6	**97.6**	98.8	98.6	99.1	95.3	97.4	90.8
5	**Toyama41**	AB701770	Cp	A	99.3	99.3	99.3	99.9		97.7	98.0	97.7	100.0	99.8	**97.7**	98.9	98.8	99.2	95.3	97.5	90.8
6	**Toyama75**	AB701771	Cp	C	100.0	100.0	100.0	99.3	99.3		97.9	100.0	97.7	97.7	**97.5**	97.5	97.4	97.7	95.2	97.3	90.7
7	**Toyama740**	AB701772	Cp	A	99.5	99.5	99.5	99.3	99.3	99.5		97.9	98.0	98.1	**97.8**	97.9	97.7	98.0	95.5	97.6	90.9
8	**Toyama791**	AB701773	Cp	C	100.0	100.0	100.0	99.3	99.3	100.0	99.5		97.7	97.7	**97.5**	97.5	97.3	97.7	95.2	97.3	90.7
9	**Toyama861**	AB701774	Cp	B	99.3	99.3	99.3	99.9	100.0	99.3	99.3	99.3		99.8	**97.7**	98.9	98.8	99.2	95.3	97.5	90.8
10	**Toyama1431**	AB701775	Cp	A	99.3	99.3	99.3	99.9	99.9	99.3	99.4	99.3	99.9		**97.7**	98.9	98.8	99.2	95.3	97.5	90.8
11	**Toyama2627**	AB701776	Cp	D	**99.0**	**99.0**	**99.0**	**99.1**	**99.2**	**99.0**	**99.1**	**99.0**	**99.2**	**99.2**		97.5	97.4	97.7	95.4	97.2	90.7
12	NIID-21-2	AB377213	Cp		99.3	99.3	99.3	99.6	99.7	99.3	99.4	99.3	99.7	99.7	99.1		99.8	98.9	95.3	97.3	90.5
13	Tokyo	NC_008604	Cp		99.1	99.1	99.1	99.4	99.5	99.1	99.2	99.1	99.5	99.5	98.9	99.8		98.7	95.1	97.2	90.4
14	Iowa07	FJ663034	Cp		99.3	99.3	99.3	99.7	99.8	99.3	99.3	99.3	99.8	99.8	99.1	99.6	99.4		95.4	97.6	90.9
15	H0901	HQ678513	Cp		98.5	98.5	98.5	98.3	98.4	98.5	98.4	98.5	98.4	98.4	98.1	98.3	98.2	98.4		95.0	90.7
16	HOU24518	FJ502995	Cq		99.2	99.2	99.2	99.0	99.1	99.2	99.1	99.2	99.1	99.1	98.7	99.0	98.8	99.0	98.1		90.6
17	CxFV-Mex07	EU879060	Cq		96.9	96.9	96.9	96.9	96.9	96.9	97.1	96.9	96.9	96.9	96.8	96.8	96.7	97.0	96.6	96.8	

The percent nucleotide sequence differences of the complete genomes are presented at the upper right.
The percent amino acid sequence differences of the complete genomes are shown in the lower left.
The identities of Toyama2627 and 10 other isolates are denoted in bold.

[a] Ct, *Cx. tritaeniorhynchus*; Cp, *Cx. pipiens* group; Cq, *Cx. quinquefasciatus*

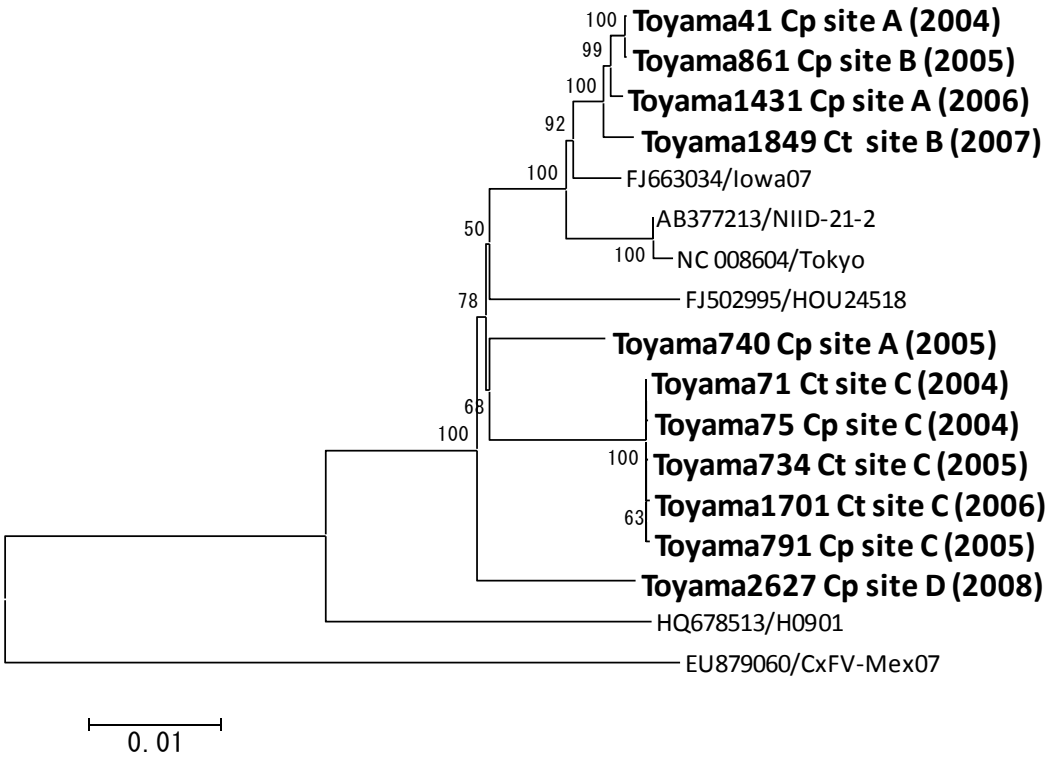

Figure 9. Phylogenetic tree of the complete genome of CxFV. The tree was constructed by the neighbor-joining method. CxFV isolates in Toyama Prefecture are denoted in bold and shown as strain name, isolation source (Ct, *Cx. tritaeniorhynchus*; Cp, *Cx. pipiens* group), site name (Fig. 7), and year of isolation. The reference strains are shown by "accession no./strain name". The scale shows the genetic distance in nucleotide substitutions per site. Numbers at branches indicate bootstrap values (%) greater than 50%. Bootstrap replications were performed 1,000 times.

99.8% (Table 10). Two of them, Toyama 1849 and 861 isolated from *Cx. tritaeniorhynchus* and *Cx. pipiens* group, respectively, were captured at site B. Another cluster consisted of five isolates (Toyama71, 75, 734, 791, and 1701) from *Cx. pipiens* group or *Cx. tritaeniorhynchus* exclusively captured at site C. Their nucleotide sequences almost completely matched (Table 10). The other two clusters consisted of two unique isolates of either Toyama740 or Toyama2627 from *Cx. pipiens* group captured at A or D (airport) site, respectively (Fig. 9).

Genetic identities among CxFV strains isolated in Toyama. To characterize Toyama740 and Toyama2627 more precisely, 210 bp of NS5 genes were compared with all of the other Toyama isolates, because 210 bp of NS5 sequences of all Toyama CxFV isolates have been obtained and this region was not characteristic in the complete genome sequence of Toyama isolates (data not shown). The result that 210 bp of the NS5 sequence of "Toyama740" matched 100% with those of six out of 94 isolates indicates that "Toyama740" is not unique. "Toyama2627" formed a unique cluster according to both complete genome (Fig. 9) and 210 bp of the NS5 gene (data not shown), and its identities to 10 other Toyama isolates were relatively low (97.5-97.8% nucleotides). Since the strain "Toyama2627" was isolated from *Cx. pipiens* group captured in an aircraft from Taiwan, it is highly possible that the mosquito originated from Taiwan. On the other hand, 210 bp of the NS5 gene of 20 out of 22 strains isolated at site C from 2004 to 2009 matched 100% with each other. The other two strains isolated in 2008 and 2009 had 99.5% nucleotide identity with 20 isolates. The above observations suggest that CxFV is maintained in a habitat-dependent manner in nature in Toyama Prefecture irrespective of the mosquito species.

Genetic identities among CxFV strains isolated from different regions of the world. To date 61 nucleotide sequences of CxFV strains whose host mosquitoes have been identified have been deposited in GenBank, nucleotide identities among these sequences including 11 Toyama isolates were compared to reveal the relationship between the CxFV sequences and mosquito species (Table 11). When 30 sequences of CxFV strains isolated from *Cx. quinquefasciatus* were compared, five isolates (Cq-5 isolated in Indonesia and the U.S.A.) were revealed to be closely related to the isolates from *Cx. pipiens* group, *Cx. restuans*, and *Cx. tritaeniorhynchus*, while the other 25 isolates (from Mexico, Indonesia, Guatemala, Trinidad, and Uganda) were closely related to those from *Cx. interrogator*. The identities between Cq-5 and Cq-25 were 88.7-90.9%. Therefore, CxFV strains from *Cx. quinquefasciatus* seem to be divided into two types. On the other hand, sequences from *Cx. pipiens* group, *Cx. restuans*, and *Cx. tritaeniorhynchus* possessed more than 94.1% identities with each other. Thus, CxFV seems to possess closely related nucleotide sequences across species boundaries.

Next, to reveal the relationship between the sequences and the country of isolation, 69 nucleotide sequences of CxFV available in GenBank and 11 Toyama isolates were compared (Table 12). The sequences of CxFV strains isolated in Japan and the U.S.A. were closely related (96.8-100% identical), and those isolated in Mexico, Guatemala, Trinidad, Brazil, and Uganda possessed 97.7-100% nucleotide identities. One of the nucleotide sequences of CxFV strains isolated in Indonesia (Indonesia-1 in Table 12) was closely related to those isolated in Japan and the U.S.A. (97.1-99.8% identical), whereas those of three and one CxFV strains isolated in Indonesia (Indonesia-3 in Table 12) and China, respectively, possessed relatively low identities with those in other countries. The above results indicated that CxFV strains were divided into several

groups: Japan/U.S.A. including Indonesia-1, Latin America/Africa (including Mexico, Guatemala, Trinidad, Brazil, and Uganda), China, and Indonesia-3 types. Indonesia-1 might have been imported into Indonesia from abroad.

Table 11. Ranges of nucleotide sequence identities of CxFV from mosquitoes within species and between species.

Isolation source[a]	No. of sequences	Cq-5	Cq-25	Cp	Cr	Ct	Ci
Cq-5[b]	5	**97.3-99.9**	88.7-90.9	**94.4-99.9**	**97.3-100**	**97.0-99.6**	89.9-90.3
Cq-25[c]	25		**94.2-100**	88.8-91.4	89.5-90.9	88.8-91.4	**98.0-99.8**
Cp	31			**94.1-100**	**94.3-99.9**	**94.8-99.7**	89.3-90.4
Cr	3				**99.6-99.7**	**97.0-97.3**	89.8-90.3
Ct	6					**97.2-100**	89.8-90.1
Ci	2						**99.5**
Total	72[d]						

Identities from minimum to maximum percent of the nucleotide sequences are shown.
The identities higher than 94% are denoted in bold.

[a] Cq, *Cx. quinquefasciatus*; Cp, *Cx. pipiens* group; Cr, *Cx. restuans*; Ct, *Cx. tritaeniorhynchus*; Ci, *Cx. interrogator*

[b] Cq-5, 5 sequences of CxFV isolated from *Cx. quinquefasciatus* in Indonesia and U.S.A. (accession no. AB262766, FJ502995, FJ502999, FJ503000, FJ503001)

[c] Cq-25, 25 sequences of CxFV isolated from *Cx. quinquefasciatus* in Mexico, Indonesia, Guatemala, Trinidad, and Uganda

[d] Out of 72, 11 are Toyama isolates and 61 are available from GenBank.

Table 12. Ranges of nucleotide sequence identities of CxFV from mosquitoes within a country and between countries.

Country	No. sequences	Japan	Indonesia-1	Indonesia-3	China	USA	Mexico	Guatemala	Trinidad	Brazil	Uganda
Japan	16	**96.9-100**	**97.2-99.8**	88.8-91.1	**94.8-96.3**	**96.8-99.5**	89.2-91.4	89.4-90.4	89.3-90.4	90.5-91.7	90.4-91.1
Indonesia-1[a]	1		-	-	**95.0**	**97.1-97.7**	90.0-90.3	90.2	90.1-90.5	-	90.9
Indonesia-3[b]	3			-	89.0-90.2	88.7-90.6	**94.2-95.1**	**95.5**	-	-	**95.1-95.2**
China	1				-	**94.1-95.6**	89.1-90.7	89.5-89.5	89.1-89.5	89.4-90.0	90.6
USA	21					**97.5-100**	89.8-91.5	89.4-90.4	89.5-90.4	90.4-91.0	90.5-91.1
Mexico	19						**99.2-100**	**99.0-99.5**	**98.3-98.8**	**97.7-98.2**	**97.8-98.3**
Guatemala	2							-	**98.4-98.5**	-	**98.0-98.5**
Trinidad	2								**99.5**	-	**98.0-98.2**
Brazil	3									**98.8-99.3**	**96.8-97.3**
Uganda	1										-
Total	80[c]										

Identities from minimum to maximum percent of the nucleotide sequences are shown.
The identities higher than 96% are underlined.
The identities higher than 94% are denoted in bold.
Pairs that could not be aligned are shown by "-".

[a] Indonesia-1, 1 sequence of CxFV isolated in Indonesia (accession no. AB262766).

[b] Indonesia-3, 3 sequences of CxFV isolated in Indonesia (accession no. AB488431, AB488432, AB488433).

[c] Out of 80, 11 are Toyama isolates and 69 are available from GenBank.

Discussion

CxFV has been sporadically isolated from mosquito species collected from several countries; however, it is not clear how CxFV is maintained in nature. Herein, CxFV was isolated from *Cx. tritaeniorhynchus* and *Cx. pipiens* group mosquitoes collected at several sites in Toyama Prefecture, Japan, over several years and the complete genome sequences of these isolates were compared with those in GenBank. The results of this study combined with those of others suggested that CxFVs are genetically stable over years in a habitat-dependent manner and that transmission of CxFVs occurs among different species of mosquitoes (inter-mosquito-species transmission). In particular, nucleotide sequences of CxFV at one site in Toyama Prefecture were strongly conserved from 2004 to 2009. CxFV may be stable because the virus may not be harmful to the host mosquito. Other reasons are that NS5 may not be influenced by the immunity of mosquitoes or that the nucleotide sequences analyzed in this study were short.

The above assertions of habitat-dependent maintenance and inter-mosquito-species transmission of CxFV were supported by the fact that the entire genome sequences of CxFV strains almost completely matched among *Cx. tritaeniorhynchus* and *Cx. pipiens* group mosquitoes at collection site C when the nucleotide sequences of CxFV strains from different sites in Toyama Prefecture were compared (Fig. 9). The comparison of nucleotide sequences of CxFV strains from different species of mosquito in different areas in the world (Table 11) showed the similar identities among the viruses from *Cx. tritaeniorhynchus*, *Cx. pipiens* group, *Cx. quinquefaciatus*, and *Cx. restuans* mosquitoes, which also supports the above assertions. However, to ask whether contamination of

mosquitoes during collection was occurred, PCR was performed to detect *Cx. pipiens* group DNA from 12 *Cx. tritaeniorhynchus* pools with CxFV positive (Table 8) using the method as described (Kasai et al. 2008). The DNA of *Cx. pipiens molestus* were detected in eight out of 12 pools. If CxFV was contaminated from *Cx. pipiens* group, CxFV should have been isolated from not only pools of *Cx. tritaeniorhynchus* but also those of *Cx. pipiens* group collected in the same trap. Only two pools (strains Toyama71, Toyama734, Toyama1701, Toyama1849 in Table 9 did not contain these two pools) out of eight pools met this condition. Therefore, CxFV strains isolated from at least 10 pools of *Cx. tritaeniorhynchus* do not seem to be derived from *Cx. pipiens* group.

CxFV strains were found to be divided into Japan/U.S.A., Latin America/Africa, China, and Indonesia types by comparison of the nucleotide sequences in GenBank and those of Toyama isolates. Previous studies also support our assertion that the nucleotide sequences of CxFV strains were divided by area independent of mosquito species (Kim et al. 2009, Blitvich et al. 2009).

In contrast, the nucleotide sequences of CxFV isolates collected at sites A and C were different and the isolate from a mosquito in an aircraft at site D from a foreign country had relatively low nucleotide and amino acid identities with other Toyama isolates. This implies that nucleotide sequences of CxFV may vary with an area and CxFV may move across country borders by air.

Vertical transmission of CxFV is suggested because it replicates only in mosquito cells, has been isolated from both male and female mosquitoes, and detected in eggs and larvae of mosquitoes (Hoshino et al. 2007, Farfan-Ale et al. 2009, Bolling et al. 2011, Saiyasombat et al. 2011). However, to explain inter-mosquito-species transmission, CxFV may infect mosquitoes not only vertically but also horizontally via an oral route,

such as drinking infected liquid, or eating microbes contaminated with virus. Because CxFV reportedly did not multiply in adult mosquitoes orally exposed to the virus (Kent et al. 2010), oral route transmission may only occur during the larval stage. *Cx. tritaeniorhynchus* and *Cx. pipiens* group share the same larval habitat (Tanaka et al. 1979). The larvae of *Cx. tritaeniorhynchus* are found most frequently in rice fields and various types of impounded water. Those of *Cx. pipiens* group occur in a very wide variety of artificial containers or other types of stagnant water. The larvae of *Cx. tritaeniorhynchus* and *Cx. pipiens* group were actually found in the same water (data not shown), so horizontal infection between *Cx. tritaeniorhynchus* and *Cx. pipiens* group might occur. Although vertical transmission and horizontal infection in the larva stage may be the main mechanism of the maintenance of CxFV in nature, interspecies mating can not be ruled out for the transmission of CxFV among different mosquito species.

In this study, CxFV infection rate of *Cx. tritaeniorhynchus* was found to be much lower than that of *Cx. pipiens* group. There was no site where CxFV was isolated from only *Cx. tritaeniorhynchus*. Low infection rate of *Cx. tritaeniorhynchus* might be due to few infection opportunities, low efficiency of infection, or the presence of only a few colonies of infected mosquitoes. The infection rate of CxFV in *Cx. pipiens* group and *Cx. tritaeniorhynchus* peaked in July when the number of *Cx. pipiens* group peaked, while the number of *Cx. tritaeniorhynchus* peaked in August to September in Toyama Prefecture in Chapter I. *Cx. tritaeniorhynchus* might have opportunities to be infected with CxFV from *Cx. pipiens* group in July.

JEV and CxFV were simultaneously isolated from three pools in the same collection of mosquitoes. Flaviviruses reportedly show some influence on each other (Kent et al. 2010, Pepin et al. 2008, Cologna et al. 2005). Our preliminary results show that CxFV

suppressed the growth of JEV maximally to about one-hundredth in a virus-dose-dependent manner (data not shown) when the mosquito cultured cells (C6/36) were simultaneously infected with CxFV and JEV. Further investigation is required to confirm the above result, using cell lines of genus *Culex* mosquitoes, but also mosquitoes in vivo.

In this study, the complete genome sequences of CxFV strains isolated from two species of mosquito in the same area were analyzed. Furthermore, ecological features of CxFV in terms of the host species of mosquitoes and the distributed area were characterized. Evolution of CxFV seemed to depend not on mosquito species but on mosquito habitat.

Summary

CxFV is an insect-specific flavivirus that was first reported in 2007 in Japan. CxFV strains were isolated from *Cx. tritaeniorhynchus* and *Cx. pipiens* group mosquitoes and genetically characterized in Toyama Prefecture, Japan, from 2004 to 2009, to reveal host specificity, mode of transmission, and seasonal and geographical distribution. The MIR of CxFV within *Cx. tritaeniorhynchus* populations was 0.3 and much lower than that within *Cx. pipiens* group (17.9). The complete genome sequences of 11 CxFV isolates (four from *Cx. tritaeniorhynchus* and seven from *Cx. pipiens* group) consisted of 10,835-10,837 nucleotides. When these 11 isolates and five reference strains (NIID-21-2 and Tokyo strains from Japan, Iowa07 and HOU24518 strains from U.S.A., H0901 strain from China) were compared, there were 95.2-99.2% nucleotide and 98.1-99.8% amino acid identities. Phylogenetic analysis showed that the 11 isolates were divided into four clusters. One cluster consisted of five isolates from *Cx. pipiens* group and *Cx. tritaeniorhynchus* from one site and their nucleotide sequences almost completely matched. Another cluster consisted of an isolate with a unique sequence from a *Cx. pipiens* group mosquito captured in an aircraft from Taiwan, suggesting that it was introduced from abroad. CxFV strains were divided into several groups according to countries when nucleotide sequences of CxFV available in GenBank and 11 Toyama isolates were compared. These results suggest that CxFV is maintained in nature among *Culex* mosquitoes in a mosquito habitat-specific but not a species-specific manner.

Conclusion

JEV and CxFV belong to the genus *Flavivirus* within the family *Flaviviridae*. JEV exists in an enzootic cycle between mosquitoes and vertebrate hosts such as pigs and birds. JEV is distributed in East and South Asia, and causes severe encephalitis in humans. CxFV is an insect-specific flavivirus and genetically related with the other insect flaviviruses.

The author isolated 87 JEV strains from mosquitoes and pigs in Toyama Prefecture during 2005-2009. The result suggests JEV still circulates between mosquitoes and pigs in Toyama Prefecture, and circulation of JEV is correlated with the prevalence of mosquitoes. According to the nucleotide sequence of the E and C/prM genes, all isolates belong to genotype I. These isolates were divided into the major type and the two minor types. The major type of JEV might have remained in Toyama Prefecture and gradually changed over five years, while two minor types of JEV might have migrated from other countries and then become extinct. All isolates collected in 2008 and 2009 had a novel deletion in the 3'UTRs. Furthermore, the author reports that the peak level of JEV circulation occurs later in the year than in the past. This might be related to the recent decrease in the prevalence of JE in Japan, in addition to the effects of human vaccinations. JEV strains circulating in areas of the temperate zone may consist of locally maintained viruses and the viruses migrated from other areas.

The author isolated and genetically characterized CxFV strains from *Cx. tritaeniorhynchus* and *Cx. pipiens* group mosquitoes. The MIR of CxFV within *Cx. tritaeniorhynchus* populations was much lower than that within *Cx. pipiens* group. The complete genome sequences of 11 CxFV isolates and five reference strains had 95.2-

99.2% nucleotide and 98.1-99.8% amino acid identities. Phylogenetic analysis showed that the 11 isolates were divided into four clusters. One cluster consisted of five isolates from *Cx. pipiens* group and *Cx. tritaeniorhynchus* from one site and their nucleotide sequences almost completely matched. An isolate had a unique sequence, suggesting that it was introduced from abroad. CxFV strains were divided into several groups according to countries when nucleotide sequences of CxFV available in GenBank and 11 Toyama isolates were compared. These results suggest that CxFV is maintained in nature among *Culex* mosquitoes in a mosquito habitat-specific but not a species-specific manner.

Acknowledgements

The author would like to sincerely thank the chief supervisor, Prof. Hiroaki Kariwa, Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, for his continuous guidance, support and warm encouragement throughout the experimental work.

The author also appreciates the kind guidance and advice of supervisors, Prof. Kazuhiko Ohashi, Laboratory Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Associate Prof. Yoshihiro Sakoda, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, and Lecturer Rie Hasebe, Laboratory of Veterinary Hygiene, Department of Applied Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University.

The author expresses special thanks to Prof. Emeritus Ikuo Takashima, Hokkaido University, for his encouragement and helpful discussion.

The author is deeply grateful to Dr. Takenori Takizawa, Department of Virology, Toyama Institute of Health, for his constructive comments and warm encouragement. The author would like to thank Dr. Takeo Yamauchi, Toyama Institute of Health, and Dr. Mamoru Watanabe, Department of Medical Entomology, National Institute of Infectious Diseases (a former staff member of Toyama Institute of Health), for surveillance of mosquitoes and insightful comments and suggestions. The author also thanks to Dr. Takeshi Kurata, International University of Health and Welfare (a former director of Toyama Institute of Health) and all the members of Department of Virology,

Toyama Institute of Health. The author thanks Ms. Miyuki Maekawa for her technical assistance.

The author is also grateful to volunteers and staff of Toyama-Airport Detached Office of Niigata Quarantine Station, Takaoka Health Center, and Toyama Prefectural Meat Inspection Center for surveillance of mosquitoes and pigs. The author also thanks Department of Virology 1 and Department of Medical Entomology, National Institute of Infectious Diseases, for advice on virus isolation and PCR.

This work was supported by a Health Labor Sciences Research Grant for Research on Emerging and Re-emerging Infectious Disease (H17-shinkou-ippan-018, H20-shinkou-ippan-015) from the Japanese Ministry of Health, Labor and Welfare.

Finally the author's deepest appreciation goes to the author's family for their encouragement, love, and devoted support to the author's education.

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和文要旨

富山県における日本脳炎ウイルスと Culex flavivirus の生態学的及び分子疫学的研究

日本脳炎ウイルス（JEV）はフラビウイルス科フラビウイルス属に属し、I～V の 5 つの遺伝子型に分けられる。通常、蚊（主にコガタアカイエカ *Culex tritaeniorhynchus* Giles）と増幅動物であるブタの間で JEV の感染サイクルが形成されている。ヒトが JEV 保有蚊に吸血されて感染すると、高熱を発して重篤な脳炎を発症する場合がある。JEV の感染による脳炎を日本脳炎と呼び、東アジアから東南アジア、南アジアまでアジア地域を中心に広範な地域で流行し、世界で年間数万人の患者発生がある。日本脳炎の致死率は 30%にも及ぶ上、回復後も後遺症が残る場合が多いため、公衆衛生上の大きな問題となっている。日本では、1960 年代には年間数千人の患者が報告されていたが、近年の患者数は 10 人未満に抑えられている。患者数の減少の要因として、ワクチン接種の勧奨、ブタの飼育農場が住宅地から離れたところに設置されるようになったことなどが推測されるものの、その原因は十分には明らかにされていない。また、1990 年代に、日本における主要な JEV の流行型が遺伝子型の III 型から I 型へ変化したことが知られているが、同一地点における JEV の詳細な年次変化については、世界的にもほとんど研究されていない。

富山県は水稲栽培が盛んで JEV の増幅動物であるブタも飼育されていることから、JEV の感染サイクルが維持されやすい環境に富んでいると考えられる。また、感染症流行予測調査により、JEV に対する抗体を持ったブタがほぼ毎年確認されていることからも、現在でも JEV が流行していることが示唆される。そこで、富山県において継続的な JEV の生態学的調査を行い、長期的な JEV の季節消長や流行状況の変化について解析を試みるとともに、JEV 分離株の遺伝子解析により、JEV 流行株の年次変化について解明を試みた。

近年、蚊が保有するフラビウイルス属のウイルスとして、Culex flavivirus（CxFV）が発見された。本ウイルスは昆虫の細胞でしか増殖せず、人や動物の病気とは関連性がないことが知られている。これまでに CxFV はアカイエカ *Culex pipiens* L.やネッタイイエカ *Culex quinquefasciatus* Say などの *Culex* 属蚊から分離されているものの、生態学的にも不明な点が多い。本研究では、富山県における CxFV の分布、感染様式やウイルスの遺伝子性状などについて解明を試みた。

第一章では、JEV の自然界での維持と進化について明らかにするため、蚊とブタから JEV を分離し、解析を行った。蚊は 51,265 個体（2,740 プール）、ブタは 1,451 個体の血清を用いた。雌のコガタアカイエカから 77 株、ブタから 10 株、合計 87 株の JEV が分離された。他にアカイエカ群 *Culex pipiens* group（アカイエカ *Culex pipiens pallens*、チカイエカ *Culex pipiens molestus* を含む）、ヒ

トスジシマカ *Aedes albopictus* Skuse 等からもウイルス分離を行ったが、コガタアカイエカ以外の種類の蚊からは JEV は分離されなかった。

JEV の季節消長をウイルス感染率（蚊 1,000 匹当たりの最小感染率；minimum infection rate ＝MIR）の増減からみたところ、2005～2009 年の富山県においては、雌のコガタアカイエカの発生数のピークは 8～9 月頃であったのに対し、JEV の流行は、9～10 月頃で、両者のピークにはずれがあった。また、1966～1972 年における富山県の JEV の流行は 7 月下旬から 9 月上旬であったため、近年では流行時期が遅くなっていると考えられる。

分離された JEV のエンベロープ（E）領域全長 1,500 塩基と カプシド／プレメンブレン（C/prM）領域 240 塩基について遺伝子解析したところ、87 株の分離株は全て遺伝子型 I に属した。これらの株は、2005～2009 年にわたり継続して分離された主要なタイプと、2007 年にだけ分離された稀なタイプに分けられた。主要なタイプは、年々少しずつ変化しており、2008 年と 2009 年には 3’側非翻訳領域（3’UTR）において 7 塩基の新たな欠損がみられた。

第二章では、CxFV の宿主の特異性や伝播方法、季節性、地域分布を調べるため、2004～2009 年に捕集されたコガタアカイエカとアカイエカ群から CxFV を分離した。その結果、94 株の CxFV 分離株が得られた。うち 3 株は JEV と同時に分離された。コガタアカイエカの MIR は 0.3 だったのに対し、アカイエカ群の MIR は 17.9 であり、両者には有意な差がみられた。4～11 月の蚊から分離されたが、7 月が最も多かった。

11 の CxFV 分離株（コガタアカイエカ由来 4 株、アカイエカ群由来 7 株）についてウイルス遺伝子の全塩基配列を決定したところ、全長は 10,835-10,837 塩基であった。これらをこれまで報告された日本や他の国由来の CxFV 5 株の全長配列と比較したところ、塩基で 95.2～99.2% 、アミノ酸で 98.1～99.8% と非常に高い一致率を示した。しかし、既報のメキシコ由来株 CxFV-Mex07 とは 90.7～90.9%、アミノ酸で 96.8～97.1%とやや低い一致率を示した。

今回の CxFV 分離株 11 株は系統樹解析により 4 つのクラスターに分けられた。同じ地点のコガタアカイエカとアカイエカ群から分離された 5 株で構成されたクラスターでは、配列がほとんど一致（塩基、アミノ酸とも 100%一致）していた。台湾からの飛行機の中で捕獲されたアカイエカ群から分離された株は、他の分離株とは異なる独自のクラスターを形成しており、この結果は今回得られた 94 株を NS5 領域の 210 塩基について比較した場合でも同様であった。従って、この株は海外から蚊とともに持ちこまれた可能性が示唆された。

今回分離した 11 株と、これまでに報告された CxFV の部分的な塩基配列を含む 61～69 配列とを比較したところ、CxFV は蚊の種類よりも分離された国・地域に応じていくつかのグループに分けられることが明らかになった。また、前述のとおり、富山県内の同じ地点の異なる種類の蚊から非常に近縁な CxFV が得られていることからも、CxFV は *Culex* 属蚊の中で蚊の種類特異的というよりも地域特異的に維持されていると考えられた。

本研究によって、近年の JEV の動向や年次変化の詳細が明らかとなった。本研究で得られた知見は、今後の流行予測や他の地域での JEV の動向を推察す

る際のモデルとして役立つものと考えられる。また、不明な点の多い CxFV についても、7 月に最も多く分離されること、地域特異的に維持されていることなどが判明した。JEV と同時に分離された蚊のプールがあったことから、コガタアカイエカでは JEV と CxFV が同時に感染しうると考えられる。自然界における JEV と CxFV の分布や蚊体内における両ウイルスの相互関係などについて、本研究成果を基礎として、今後、さらに詳細が明らかにされることが期待される。

本論文の掲載元は以下の URL を参照してください。

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