Distribution and Mosquito Hosts of Chaoyang Virus, a Newly Reported Flavivirus From the Republic of Korea, 2008–2011

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ABSTRACT In total, 183,602 female culicine mosquitoes were captured by Mosquito Magnet, black light, and New Jersey light traps, and manual aspiration of resting blood-fed mosquitoes, in the Republic of Korea from 2008 to 2011. Culicine mosquitoes were identified to species, placed in pools of up to 30 mosquitoes each, and screened for flavivirus RNA by using an SYBR green I-based reverse transcription-polymerase chain reaction assay. Thirty-two of the 8,199 pools assayed were positive by quantitative polymerase chain reaction for Chaoyang virus (CHAOV), an insect-specific virus [26 *Aedes vexans nipponii* Theobald, 3 *Culex pipiens* L., 1 *Aedes albopictus* (Skuse), 1 *Aedes bekkui* Mogi, and 1 *Armigeres subalbatus* (Coquillett)]. The maximum likelihood estimations (estimated number of virus-positive mosquitoes/1,000 mosquitoes) for *Ae. bekkui*, *Ae. albopictus*, *Ar. subalbatus*, *Ae. vexans nipponii*, and *Cx. pipiens* positive for CHAOV were 5.37, 3.29, 0.77, 0.27, and 0.26, respectively. CHAOV is an insect-specific virus, and there is currently no evidence to suggest a role in animal or human disease.

KEY WORDS insect-specific flavivirus, Chaoyang virus, *Aedes vexans nipponii*, Republic of Korea

Arboviruses in the family Flaviviridae can be divided into four distinct groups, namely, mosquito-borne, tick-borne, no-known-vector, and arthropod-specific viruses (Kuno et al. 1998, Gaunt et al. 2001, Gould et al. 2003, Cook and Holmes 2006, Hoshino et al. 2007). Mosquito-borne flaviviruses are a large and divergent group and have been further divided into viruses in mosquito-borne *Culex*, causative agents of encephalitis and transmitted by *Culex* spp., and mosquito-borne *Aedes*, causative agents of hemorrhagic complications and transmitted by *Aedes* spp. Insect-specific flaviviruses that appear to only infect mosquitoes have been found worldwide (Hoshino et al. 2007, 2012; Blitvich et al. 2009; Huhtamo et al. 2009; Junglen et al. 2009; Wang et al. 2009; Tyler et al. 2011; Cook et al. 2012). *Culex* flavivirus was first isolated from *Culex pipiens* collected in Japan (Hoshino et al. 2007). The virus replicated in mosquito cell lines, for example, C6/36 and AeA1–2, but did not replicate in mammalian cell lines, for example, baby hamster kidney (BHK-21) or green monkey kidney (Vero cells). Whole-genome sequence and E/NS5 gene analyses clearly showed that *Culex* flaviviruses grouped with arthropod-specific flaviviruses along with cell fusing agent (CFA) and Kamiti River virus and were distantly related to other groups of flaviviruses. Similarly, *Aedes* flavivirus was isolated from *Aedes albopictus* and *Aedes flavipictus* Yamada collected in Japan (Hoshino et al. 2009) and grouped phylogenetically with arthropod-specific arboviruses. NS3 and NS5 sequence analyses showed it was closely related to Kamiti River virus and CFA and formed a separate branch with *Culex* flavivirus. The maintenance and transmission cycle for these insect-specific flaviviruses are not well understood, as some have been shown to cross genera and species. However, vertical transmission and venereal transmission are believed to play an important role in the

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maintenance and transmission of these insect-specific viruses in nature (Cook et al. 2012).

Chaoyang virus (CHAOV) belongs to the family Flaviviridae, genus *Flavivirus* based on its RNA genome organization characteristic of members of the genus *Flavivirus*, which contain one open reading frame encoding a viral protein with putative cleavage sites for protein processing into structural and nonstructural proteins essential for virion assembly (Liu et al. 2011, Lee et al. 2013). CHAOV was first isolated from mosquito hosts collected in Chaoyang, Liaoning province, China (Deming strain, GenBank AFJ883471). Other stains of CHAOV also were isolated from mosquitoes collected in China in 2008 and 2010 (BeiBei strain: GenBank AFJ812035, HLD115 strain: GenBank ANC_017086). Viral complete coding sequences were determined and found to contain a high sequence homology to other mosquito-borne flaviviruses, that is, Sepik virus and dengue virus 2 (Wang et al. 2009, Liu et al. 2011). As part of the U.S. Army mosquito surveillance program in the Republic of Korea (ROK), an unknown flavivirus was isolated from a pool of *Aedes vexans nipponii* Theobald (ROK144) collected in June 2003 at Camp Greaves (closed in 2006) that was located ≈3 km from the southern boundary of the demilitarized zone (DMZ) that separates Democratic People's Republic of Korea (North Korea) from the ROK (South Korea) (Kim et al. 2007a). Subsequently, the isolate (ROK144) was determined to be the same as CHAOV first described from China in 2009. The virus was isolated in cultured *Ae. albopictus* (C6/36) cells, but did not grow in Vero, BHK-21, primary duck, or primary chicken cell lines (Lee et al. 2013). The ROK144 virus was sequenced and found to be 98.9% homologous to CHAOV that was reported from *Ae. vexans* collected in Chaoyang, Liaoning Province, China (Wang et al. 2009, Lee et al. 2013). Phylogenetic analysis with other flaviviruses showed that CHAOV grouped in the same clade as the insect-specific flaviviruses, Lammi virus (LAMV), isolated from *Aedes cinereus* Meigen in Finland (Huhtamo et al. 2009), and Donggang virus (DGV), isolated from *Aedes* spp. in Liaoning province, 2009 (GenBank ANC_016997, AJQ086551). Both CHAOV and LAMV grouped genetically within the mosquito-borne flavivirus cluster, which includes human pathogenic mosquito-borne flaviviruses, for example, dengue virus (DENV) and yellow fever virus (YFV), despite the lack of any association as a causative agent of human and animal diseases.

The purpose of the mosquito-borne disease surveillance program conducted by the U.S. military in the ROK was to determine the prevalence and distribution of mosquito-borne pathogens, for example, Japanese encephalitis (JEV), that affect human health in the ROK. Characterization of non-JEV was conducted to determine the presence of endemic, newly reported, or possibly introduced mosquito arboviruses in the ROK. This study reports on the prevalence and geographical distribution of the CHAOV detected in culicine mosquito hosts collected during the mosquito season (May through October) throughout the ROK from 2008 to 2011.

Materials and Methods

Mosquito Collections. In total, 86 collection sites distributed over eight provinces and five Metropolitan areas throughout the ROK were surveyed from 2008 to 2011. These included 22 U.S. Army/Air Force installations/training sites where U.S. military reside and/or train, Daeseongdong (village) and the Neutral Nations Supervisory Commission (NNSC) camp near the military demarcation line inside the DMZ, and Tongilchon, a village 3 km from the DMZ, to identify mosquito population densities and as a measure for instituting mosquito control measures (Fig. 1). An additional 55 cowsheds and seven piggeries were surveyed to determine the distribution of *Anopheles* spp. and detection of JEV in the ROK.

Adult mosquitoes were collected continuously at 3–7-d intervals from May to October during 2008–2011 by using Mosquito Magnets (Pro-model, Woodstream Corp., PA), weekly by using New Jersey light traps (Model 1112, John W. Hoch, Gainesville, FL) at U.S. Army installations and military training sites, and periodically by black light traps (model "Black Hole" by BioTrap, Seoul, Korea, http://www.bio-trap.com) and mouth aspiration for outdoor resting mosquito collections during the early evening hours (2100–2400 hours) from villages, cowsheds, and piggeries in the ROK. Mosquitoes were removed from the traps, placed in a Styrofoam cooler containing wet ice, and transported to the Entomology Section, Fifth Medical Detachment, Seoul, ROK. Mosquitoes collected in resting collections were mouth-aspirated, transferred to screened pint cartons, and similarly placed in a Styrofoam cooler and transported to the Fifth Medical Detachment. Culicine mosquitoes collected from traps and resting collections were identified to species according to Tanaka et al. (1979) and Lee (1998); sorted by species, collection site, collection date, and trap type; and placed in 2-ml cryovials (1–30 mosquitoes/vial) and stored at −70°C until transported on dry ice to the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand, where they were assayed for flaviviruses. Flavivirus-positive pools were then assayed for JEV and CHAOV.

CHAOV Detection. *RNA Extraction.* Mosquito pools were homogenized in 750 μl of Roswell Park Memorial Institute medium by using a TissueLyser II (Qiagen, Hilden, Germany). The homogenate was centrifuged at 12,000 rpm for 10 min at 4°C. A subsample of 250 μl of the supernatant was used for flavivirus RNA isolation. The remaining homogenate was maintained at −70°C for future use. Total RNA was extracted from mosquito homogenates by using TRIzol reagent (Invitrogen, Foster City, CA) according to the manufacturer's instruction. In addition, 50 μg/ml of glycogen (Sigma–Aldrich) was added during RNA precipitation to enhance nucleic acid precipitation. Finally, the RNA pellet was resuspended in 50 μl

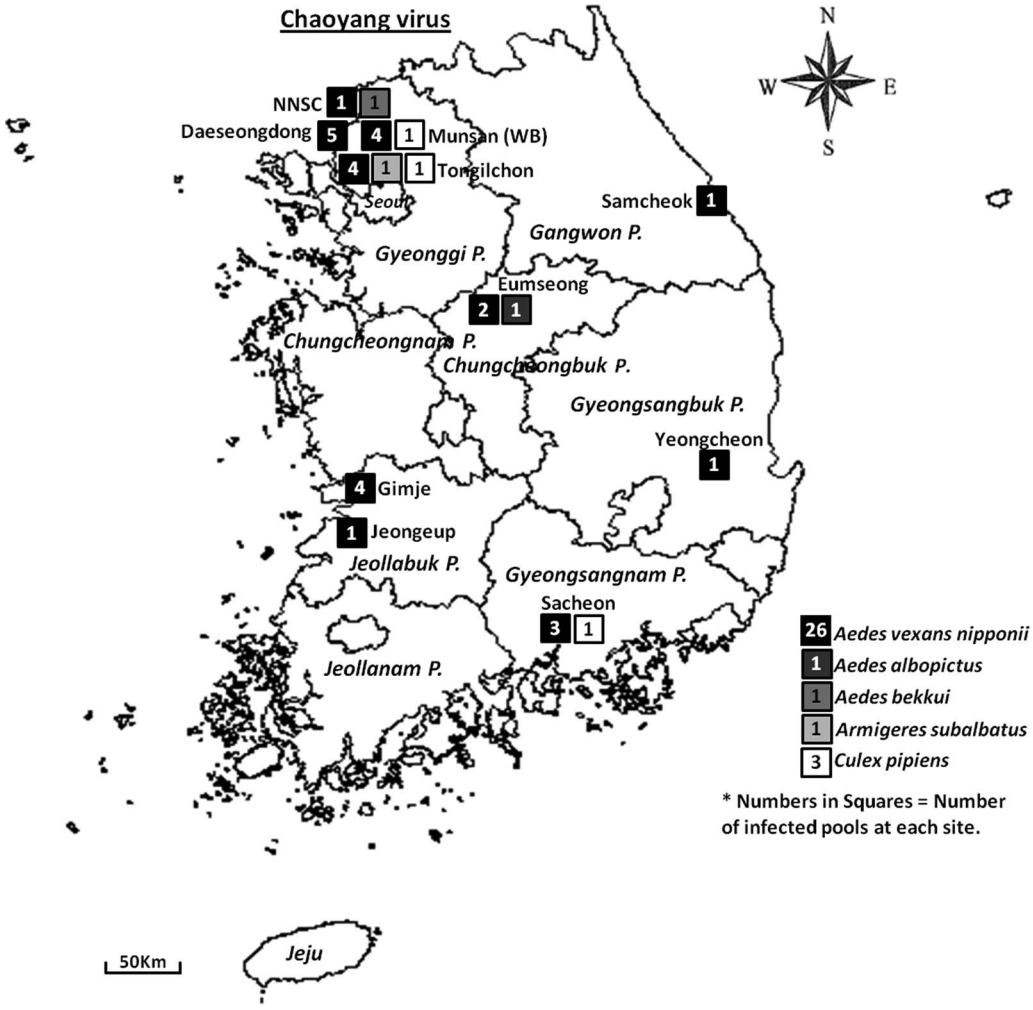

Fig. 1. Ten of 86 collection sites surveyed from 2008 to 2011 that were positive (number of positive pools) for CHAOV, by mosquito species, *Ae. vexans nipponii*, *Ae. albopictus*, *Ae. bekkui*, and *Ar. subalbatus*, and *Cx. pipiens*, in the Republic of Korea. [NNSC (37° 57′ 16.39″ N, 126° 40′ 50.03″ E), Daeseongdong (37° 56′ 26.92″ N, 126° 40′ 37.42″ E), Warrior Base (37° 55′ 17.01″ N, 126° 44′ 30.22″ E), Tongilchon (37° 54′ 32.18″ N, 126° 44′ 01.88″ E), Samcheok (37° 23′ 56.54″ N, 129° 12′ 24.80″ E), Eumseong (36° 55′ 00.81″ N, 127° 33′ 10.08″ E), Gimje (35° 46′ 59.58″ N, 126° 52′ 27.34″ E), Jeongeup (35° 31′ 44.20″ N, 126° 50′ 50.60″ E), Yeongcheon (36° 10′ 30.94″ N, 128° 52′ 18.95″ E), Sacheon (35° 05′ 49.52″ N, 128° 08′ 27.35″ E)]. NNSC, Neutral Nations Supervisory Commission camp.

of RNase-free water containing 10 U of RNasinPlus RNase Inhibitor (Promega).

cDNA Synthesis. First-strand cDNA synthesis by using random hexamers for two-step reverse transcription-polymerase chain reaction assay was carried out according to the SuperScript III (Invitrogen) manufacturer's protocol. The maximum amount of viral RNA (8 μl) was used in place of water for 20 μl cDNA synthesis reaction.

Flavivirus Detection by Quantitative Real-Time Polymerase Chain Reaction Assay. Flavivirus RNA detection was performed by quantitative polymerase chain reaction (qPCR) by using a chromo4 Real-Time PCR detector (Bio-Rad). The flavivirus consensus primers, mFU1 and cFD2, were designed based on the most conserved domain of the flavivirus NS5 gene, as previously described by Kuno (1998). Dengue virus serotype 4-inoculated *Aedes aegypti* (L.) and noninfected mosquitoes were included in every experimental run as positive and negative controls, respectively, as well as nontemplate controls. The qPCR reaction was carried out by using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) in accordance with the manufacturer's protocol. Briefly, the 25 μl qPCR reaction mixture contained 5 μl of cDNA synthesis reaction, 12.5 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.5 μl of reference dye (ROX), and 0.2 μM concentrations of mFU1 and cFD2 primers (Chao et al. 2007). qPCR conditions consisted of uracil DNA glycosylase (UDG) incubation at 50°C for 2 min, followed by one cycle at 95°C for 2 min and 40 cycles at 95°C for 15s and 60°C for 1 min with fluorescence acquisition at the

end of each annealing/extension cycle. Amplification was immediately followed by a melt curve analysis program consisting of 1 min at 95°C, 1 min at 65°C, and a stepwise temperature increase of 0.2°C/2s until 95°C, with fluorescence acquisition at each temperature transition. Melting curve analysis was used to identify flavivirus RNA-positive samples. The amplification products from the qPCR reactions were subjected to DNA sequencing for confirmation and pathogen identification.

DNA Sequencing. The entire CHAOV envelope (E) gene was amplified by using iProof High-Fidelity DNA Polymerase kit (Bio-Rad) by using in-house primers EnvCY792 (5′-GGCTATCGGATGGCTATTGGG-3′) and EnvCY2489 (5′-GGAGAGCCAGGGTGAAAGAC-3′). The amplification products of the E gene and the qPCR reaction were sequenced by the AITbiotech Pte. Ltd. (AITbiotech, Singapore). The E gene (1,600 bp) was sequenced by using four overlapping in-house primers EnvCY792, EnvCY2489, CY1494 F (5′-CAAGCTAGCTCAATAGAGGCGG-3′), and CY2046R (5′-CTATGAATGGGTGTGCTGTCAC-3′). Sequences were edited and assembled by using the Applied Biosystems DNASequencer program v4.1.4 (Applied Biosystems) and compared with sequences in the BLASTN database (National Center for Biotechnology Information).

Phylogenetic Analysis. Multiple sequence alignment was performed by using the CLUSTALW program (Thompson et al. 1997, Larkin et al. 2007). The phylogenetic tree of the NS5 gene (210 nt) sequences was constructed by using the maximum likelihood method in MEGA5 program (Tamura et al. 2011). The robustness of branching patterns was tested by using 1,000 bootstrap pseudo replicates. Percentage identity of the E and NS5 regions was calculated by using BioEdit's Sequence Identity Matrix in the BioEdit v7.1.11 program (Hall 1999).

Data Analysis. The maximum likelihood estimation with 95% CIs for estimating the number of viral RNA-positive mosquitoes/1,000 mosquitoes from pooled samples (1–30/pool) was calculated by using PooledInfRate statistical software package (Biggerstaff 2006).

Results

In total, 183,601 female culicine mosquitoes, belonging to 22 species and seven genera, were collected in the ROK from 2008 to 2011 (Table 1). *Ae. vexans nipponii* was the most frequently collected culicine species (52.0%), followed by *Culex tritaeniorhynchus* Giles (29.5%), *Culex bitaeniorhynchus* Giles (8.9%), *Cx. pipiens* sensu lato (s.l.) L. (6.3%), and *Ochlerotatus dorsalis* (Meigen) (1.7%). The remaining 17 species comprised only 1.0% of the total number of mosquitoes collected. Female culicine mosquitoes were placed in 8,199 pools, sorted by species, collection site, date of collection, and trap type. Of the 8,199 pools that were screened, 98 pools were positive for JEV (reported separately) and 32 pools were positive for CHAOV RNA, including 26/3,664 pools (0.7%) of *Ae. vexans nipponii*, 1/130 pools (0.8%) of *Ae. albopictus*, 1/30 pools (3.3%) of *Aedes bekkui* Mogi, 3/866 pools (0.3%) of *Cx. pipiens*, and 1/182 pools (0.5%) of *Armigeres subalbatus* (Coquillett) (Kim et al. 2011, Takhampunya et al. 2011) (Table 1).

CHAOV was detected in mosquitoes from 10/86 (11.6%) collection sites distributed over eight provinces: Gyeonggi Province (Tongilchon [six positives], Warrior Base [five], Daeseongdong [five], and the NNSC camp [two]), Gangwon Province (Samcheok [one]), Chungcheongbuk Province (Eumseong [three]), Jeollabuk Province (Gimje [four] and Jeongeup [one]), Gyeongsangbuk Province (Yeongcheon [one]), and Gyeongsangnam Province (Sacheon [four]) (Fig. 1; Table 2). Different collection methods were used throughout the ROK, with black light and NJ light traps used predominately south of Seoul, whereas Mosquito Magnets were used predominately in/near the DMZ (Table 3). Therefore, analysis of collection methods versus detection of CHAOV was not done.

Sequence analysis of the full-length E gene (1,170 nt) showed minimal variation among CHAOV detected from the ROK and China during 2003–2011, with nucleotide sequence identities in the range of 97.2–99.7%. The multiple alignment of putative amino acid sequence similarities of the E gene (390 aa) among strains from the ROK and China showed a high degree of similarity and were in the range of 99.4–100% (Table 4) with only five amino acid (aa) residue differences (only three aa residue differences were noted for CHAOV isolated from the ROK). In comparisons of the E protein sequences of CHAOV and those of other flaviviruses, the highest homology percentages were observed between CHAOV and LAMV (73.5–74.1%), followed by CHAOV and DGV (60.7–60.9%) (Table 4), whereas the lowest homology percentages were found between CHAOV and arthropod-specific viruses that included CFA and *Aedes* and *Culex* flaviviruses (15.6–16.8%) (data not shown).

Nucleotide (210 nt) and amino acid (70 aa) sequence analysis of the partial NS5 region showed highly conserved sequences among strains detected from China (two isolates) and the ROK (27 isolates), including the ROK144 strain with homologies in the ranges of 94.2–100% and 97.1–100% (Table 4), respectively. The most conserved NS5 gene of CHAOV shares >70% protein sequence identity with other mosquito-borne flaviviruses, and the highest protein sequence identities were observed between CHAOV and LAMV (88.5–90.0%), followed by CHAOV and DGV (78.5–80.0%) (Table 4). However, the sequence homology was relatively low when amino acid sequences of CHAOV were compared with those of other arthropod-specific viruses, including CFA and *Aedes* and *Culex* flaviviruses (54.2–58.5%) (data not shown).

The alignment of partial NS5 sequence (210 nt) of 26 Chaoyang isolates detected in the ROK from 2008 through 2011 (this study), CHAOV detected from China (Deming and HLD115 strains), and the ROK (ROK144 strain) along with 25 other insect and medically relevant flaviviruses was performed by using the

Table 1. Number of mosquitoes collected (percent of total) by year, number of pools assayed for flaviviruses, and number of pools positive by qPCR for CHAOV, Republic of Korea, 2008–2011

Species	2008			2009			2010			2011			Total (2008–2011)		
	Total no. tested	Tested pools	CHAOV (MLE[a])	Total no. tested	Tested pools	CHAOV (MLE)	Total no. tested	Tested pools	CHAOV (MLE)	Total no. tested	Tested pools	CHAOV (MLE)	Total no. tested (%)	Tested pools	CHAOV (MLE)
Aedes albopictus	27	17		33	17	1 (28.0)	237	92		5	4		302 (0.2)	130	1 (3.3)
Aedes bekkui	0	0		0	0		3	3		172	27	1 (5.5)	175 (0.1)	30	1 (5.4)
Aedes esoensis	0	0		0	0		0	0		124	40		124 (<0.1)	40	
Aedes lineatopennis	0	0		0	0		89	13		42	21		131 (<0.1)	34	
Aedes vexans nipponii	7,791	425	1 (0.1)	36,880	1250	9 (0.2)	29,700	1,228	6 (0.2)	21,016	761	10 (0.5)	95,387 (52.0)	3,664	26 (0.3)
Aedes nipponicus	0			0	0		0	0		1	1		1 (<0.1)	1	
Culex bitaeniorhynchus	51	25		1	1		1,564	123		14,732	533		16,348 (8.9)	682	
Culex inatomii	4	1		0	0		42	11		5	4		51 (<0.1)	16	
Culex mimeticus	0	0		0	0		1	1		1	1		2 (<0.1)	2	
Culex orientalis	12	11		2	2		107	61		2	2		123 (<0.1)	76	
Culex pipiens	1,822	210		3,595	144	1 (0.3)	5,084	386		1,126	126	2 (1.8)	11,627 (6.3)	866	3 (0.3)
Culex rubensis	1	1		0	0		0	0		1	1		2 (<0.1)	2	
Culex tritaeniorhynchus	4,903	205		16,679	572		30,673	1,133		1,808	138		54,063 (29.5)	2,048	
Culex vagans	46	23		0	0		100	25		1	1		147 (<0.1)	49	
Armigeres subalbatus	208	32		812	56		229	66		35	28	1 (28.4)	1,284 (0.7)	182	1 (0.8)
Coquillettidia ocharacea	3	3		0	0		46	24		0	0		49 (<0.1)	27	
Mansonia uniformis	23	15		0	0		331	68		14	9		368 (0.2)	92	
Ochlerotatus dorsalis	10	5		3,151	109		11	4		2	2		3,174 (1.7)	120	
Ochlerotatus Japonicus	0	0		0	0		1	1		0	0		1 (<0.1)	1	
Ochlerotatus koreicus	55	28		18	10		128	69		37	26		238 (0.1)	133	
Ochlerotatus togoi	2	2		0	0		0	0		0	0		2 (<0.1)	2	
Tripteroides bambusa	0	0		0	0		2	2		0	0		2 (<0.1)	2	
Total	14,958	1,003	1 (0.1)	61,171	2161	11 (0.2)	68,348	3,310	6 (0.1)	39,124	1,725	14 (0.4)	183,601 (100)	8,199	32 (0.2)

[a] MLE, Maximum Likelihood Estimation, estimated numbers of mosquitoes positive for CHAOV/1,000 mosquitoes.

Table 2. Collection sites by city/village, province, dates of collections, and mosquito species for 32 CHAOV-positive mosquito pools, 2008–2011

Serial no.	Collection site	Province	Trap type	Date	Species	Accession no. (NS5, E genes)
A8.0864	Samcheok	Gangwon	MM	3 Aug. 2008	*Aedes vexans nipponii*	KC756391, n/a
A9.0015	Gimje	Jeollabuk	BL	11 June 2009	*Aedes vexans nipponii*	KC756392, n/a
A9.0457	Gimje	Jeollabuk	BL	29 June 2009	*Aedes vexans nipponii*	KC756394, n/a
A9.0487	Gimje	Jeollabuk	BL	29 June 2009	*Aedes vexans nipponii*	n/a
A9.1116	Gimje	Jeollabuk	BL	21 June 2009	*Aedes vexans nipponii*	n/a
A9.0715	Eumseong	Chungcheongbuk	BL	27 June 2009	*Aedes vexans nipponii*	n/a
A9.0805	Eumseong	Chungcheongbuk	BL	27 June 2009	*Aedes vexans nipponii*	KC756395, n/a
A9.2136	Eumseong	Chungcheongbuk	BL	24 Sept. 2009	*Aedes albopictus*	KC756398, n/a
A9.1145	Sacheon	Gyeongsangnam	BL	21 June 2009	*Aedes vexans nipponii*	KC756396, n/a
A9.1555	Sacheon	Gyeongsangnam	BL	27 Aug. 2009	*Aedes vexans nipponii*	n/a
A9.1559	Sacheon	Gyeongsangnam	BL	27 Aug. 2009	*Aedes vexans nipponii*	KC756397, n/a
A9.0375	Sacheon	Gyeongsangnam	BL	25 June 2009	*Culex pipiens*	KC756393, n/a
A10.0123	Yeongcheon	Gyeongsangbuk	BL	19 July 2010	*Aedes vexans nipponii*	KC756399, n/a
A10.0775	Jeongeup	Jeollabuk	BL	27 Aug. 2010	*Aedes vexans nipponii*	n/a, KC756390
10.0570	Warrior Base[a]	Gyeonggi	MM	13 July 2010	*Aedes vexans nipponii*	KC756400, n/a
10.1149	Warrior Base[a]	Gyeonggi	MM	10 Aug. 2010	*Aedes vexans nipponii*	KC756402, KC756389
10.1075	Daeseongdong	Gyeonggi	MM	7 Aug. 2010	*Aedes vexans nipponii*	KC756401, KC756388
10.1342	Daeseongdong	Gyeonggi	MM	14 Aug. 2010	*Aedes vexans nipponii*	n/a
A11.0346	NNSC[b]	Gyeonggi	MM	27 June 2011	*Aedes vexans nipponii*	KC756407, n/a
A11.1459	NNSC[b]	Gyeonggi	MM	29 Aug. 2011	*Aedes bekkui*	KC756415, n/a
A11.0286	Daeseongdong	Gyeonggi	MM	23 June 2011	*Aedes vexans nipponii*	KC756403, n/a
A11.0333	Daeseongdong	Gyeonggi	MM	27 June 2011	*Aedes vexans nipponii*	KC756405, n/a
A11.1010	Daeseongdong	Gyeonggi	MM	25 July 2011	*Aedes vexans nipponii*	KC756413, n/a
A11.0302	Warrior Base[a]	Gyeonggi	MM	27 June 2011	*Aedes vexans nipponii*	KC756404, n/a
A11.1547[c]	Warrior Base[a]	Gyeonggi	MM	14 Sept. 2011	*Aedes vexans nipponii*	KC756416, KC756387
A11.0472	Warrior Base[a]	Gyeonggi	MM	2 July 2011	*Culex pipiens*	KC756408, n/a
A11.0341	Tongilchon	Gyeonggi	MM	27 June 2011	*Aedes vexans nipponii*	KC756406, n/a
A11.0574	Tongilchon	Gyeonggi	MM	4 July 2011	*Aedes vexans nipponii*	KC756410, n/a
A11.0664	Tongilchon	Gyeonggi	MM	7 July 2011	*Aedes vexans nipponii*	KC756411, n/a
A11.1065	Tongilchon	Gyeonggi	MM	25 July 2011	*Aedes vexans nipponii*	KC756414, n/a
A11.0568	Tongilchon	Gyeonggi	MM	4 July 2011	*Armigeres subalbatus*	KC756409, n/a
A11.0842	Tongilchon	Gyeonggi	MM	14 July 2011	*Culex pipiens*	KC756412, n/a

Locations where positive pools were detected are graphically presented in Figure 1.
[a] Warrior Base, U.S. military training site.
[b] NNSC (Neutral Nations Supervisory Commission) camp, 50 m south of the military demarcation line in the demilitarized zone.
[c] Isolate was cultured in C6/36 cells and the supernatant then passed several times in the C6/36 cell cultures.
MM, Mosquito Magnet trap; BL, black light trap.

CLUSTALW program (Thompson et al. 1997), and the alignment data were tested by the bootstrap method. Maximum likelihood tree was constructed by using Kimura-2 parameter model implemented in MEGA five program (Tamura et al. 2011). Phylogenetic analysis of the partial NS5 sequence showed that all isolates from the ROK and China grouped together within the *Aedes*-transmitted flavivirus subgroups and formed a separate clade situated between DENV and YFV groups (Fig. 2). Our results were supported by Lee et al. (2013), where similar tree topologies were observed based on the analyses of full-genome sequences of CHAOV and other flaviviruses.

Discussion

CHAOV, including the isolate ROK144, more closely grouped with two insect-specific flaviviruses, LAMV and DGV, as well as several mosquito-borne flaviviruses that are medically important, for example, DENV, YFV, JEV, and West Nile virus, than with the other arthropod-specific *Culex* and *Aedes* flaviviruses that received recent attention in the literature (Huhtamo et al. 2009, Wang et al. 2009, Gao et al. 2010, Liu et al. 2011, Cook et al. 2012, Lee et al. 2013). To date, no vertebrate cell line culture that supports the replication of CHAOV in vitro has been found (Lee et

Table 3. Total number of mosquitoes collected by each trap type and number of pools positive for CHAOV, 2008–2011

Trap type	2008			2009			2010			2011			Total (2008–2011)		
	Total capture	Pools assay	Pos pools	Total capture	Pools assay	Pos pools	Total capture	Pools assay	Pos pools	Total capture	Pools assay	Pos pools	Total capture	Pools assay	Pos pools
MA	327	16	0	—	—	—	—	—	—	—	—	—	327	16	0
BL	—	—	—	61,171	2,161	11	23,292	901	2	—	—	—	84,463	3,062	13
MM	7,011	387	1	—	—	—	32,605	1,395	4	39,124	1,725	14	78,740	1,782	19
NJ	4,228	452	0	—	—	—	6,828	750	0	—	—	—	11,056	2,927	0
NJC	3,392	148	0	—		—	5,623	264	0	—	—	—	9,015	412	0
Total	14,958	1,003	1	61,171	2,161	11	68,348	3,310	6	39,124	1,725	14	183,601	8,199	32

MA, manual aspiration; BL, black light trap; MM, Mosquito Magnet trap; NJ, New Jersey light trap; NJC, New Jersey light trap with CO_2.

Table 4. Percent identity comparison of CHAOV protein sequences and other flaviviruses

Protein	CHAOV ROK144		CHAOV deming		LAMV		DGV		DENV3		YFV		JEV	
	aa[a]	%[b]	aa	%	aa	%	aa	%	aa	%	aa	%	aa	%
E	390	99.7–100	390	99.4–99.7	390	73.5–74.1	392	60.7–60.9	386	42.6–42.8	386	37.2–37.4	393	48.9–49.2
NS5	70	97.1–100	70	97.1–98.5	70	88.5–90.0	70	78.5–80.0	70	77.1–78.5	70	78.5–81.4	70	78.5–80.0

[a] Amino acid sequence length of viral protein.
[b] Percent amino acid identity.

al. 2013). The property of infecting only mosquito cells in culture was noted in several viruses in the mosquito-borne group such as LAMV, DGV, and Nounane virus (Huhtamo et al. 2009, Junglen et al. 2009). Likewise, the biological properties and phylogenetic position of Entebbe bat virus, which has no known arthropod vector, group separately from insect-specific and mosquito-borne flaviviruses (Kuno et al. 1998). However, the possible role of CHAOV in human or veterinary diseases remains to be further investigated.

CHAOV is most commonly collected from *Ae. vexans nipponii*, but in Korea, CHAOV was detected in five species belonging to three genera, *Ae. vexans nipponii*, *Ae. albopictus*, *Ae. bekkui*, *Cx. pipiens*, and *Ar. subalbatus* (Wang et al. 2009, Lee et al. 2013). CHAOV was detected in collections of *Ae. albopictus*, *Ae. bekkui*, and *Cx. pipiens* during collection periods when there were no positive *Ae. vexans nipponii*, reducing the potential for cross-contamination. However, one *Ar. subalbatus* was positive for CHAOV on the same collection date when *Ae. vexans nipponii* was positive, which may have been contamination of infected legs during sorting. Additional arbovirus surveillance is needed in the ROK to determine the mosquito host range of CHAOV in nature.

CHAOV was most commonly detected in *Ae. vexans nipponii*, a common floodwater mosquito found throughout the ROK during the early spring and summer months (Kim et al. 2007b, 2009, 2010). *Ae. vexans nipponii* is zoophilic (commonly collected at pig, chicken, and beef farms), readily bites man, and is frequently collected at military training sites, installations, and villages (Strickman et al. 2000). *Cx. pipiens* s.l. is a complex of species, subspecies, and forms that includes *Cx. pipiens pallens* Coquillett and *Cx. pipiens* form *molestus* Forskal that are distributed throughout the ROK and cannot be identified by current morphological techniques. *Cx. pipiens pallens* and *Cx. pipiens* form *molestus* are considered primarily nuisance mosquitoes in the ROK, but are reported as vectors of arboviruses in other parts of the world (Lee and Lee 1992; Sohn and Park 1998; Turell et al. 2003, 2006). *Ae. bekkui* was first reported in ROK in 1987, with larvae commonly collected from ground pools in forested habitats and adults collected at human landing collections during daytime and evening hours (Lee et al. 1987). Adults are infrequently collected in light traps, which often leads to the misconception that they are uncommon (Bekku 1954, Mizuta 2011). *Ae. albopictus*, although not routinely collected in light traps, is more commonly collected in forested habitats during the daytime in human landing collections (K-CDC [Korean Centers for Disease Control and Prevention] 2008). Immatures of *Ae. albopictus* are generally collected from artificial containers and tree holes and are not usually collected in combination with *Ae. vexans nipponii.* Similarly, *Ar. subalbatus* is a day- and dusk-biting mosquito commonly found in forested environments and that readily bites humans, whereas immatures are frequently collected from artificial and natural containers. Although field infections of JEV have not been detected in *Ar. subalbatus*, the species was incriminated in the laboratory as a competent vector of JEV (Chen et al. 2000). While *Ae. vexans nipponii* was the most commonly mosquito species positive for CHAOV, our results demonstrate a wide range of mosquito host specificities for CHAOV replication. Some mosquito-borne flaviviruses are known to be transmitted vertically from infected female mosquitoes to their offspring, as well as by venereal transmission from infected males to females during mating (Thompson and Beaty 1976, Barreau et al. 1997, Higgs and Beaty 2005). It is unclear how CHAOV is maintained and transmitted within and between mosquito populations. Additional studies are needed to determine the maintenance and transmission cycle of CHAOV in nature.

Surveillance of mosquitoes is dependent on efficient and effective traps. New Jersey and black light traps are effective in capturing large numbers of *Cx. tritaeniorhynchus* and *Ae. vexans nipponii* for the detection of arboviruses, including JEV and CHAOV (Burkett et al. 2002). However, they do not efficiently collect day-biting mosquitoes such as *Ae. albopictus*, *Ochlerotatus japonicus* (Theobald), *Ochlerotatus koreicus* (Edwards), and of CHAOV, and therefore, only small numbers of these species were collected. Whereas one pool of *Ae. albopictus* tested positive for CHAOV, insufficient numbers of adult *Oc. japonicus* and *Oc. koreicus* were collected for analysis. The use of improved Mosquito Magnets and BG-Sentinel traps (Biogents AG, Regensburg, Germany) baited with CO_2, attractants specific for *Aedes* spp., and placement of traps in appropriate habitats (i.e., forests), may provide better estimates for Chaoyang infection rates and the range of day-biting mosquito hosts. Mosquito-borne disease surveillance programs provide U.S. Forces with a more accurate analysis of arbovirus threats in the ROK. Both current and retrospective studies provide for improved disease risk analysis, which demonstrates the need for personnel and funding resources for improved health surveillance, accu-

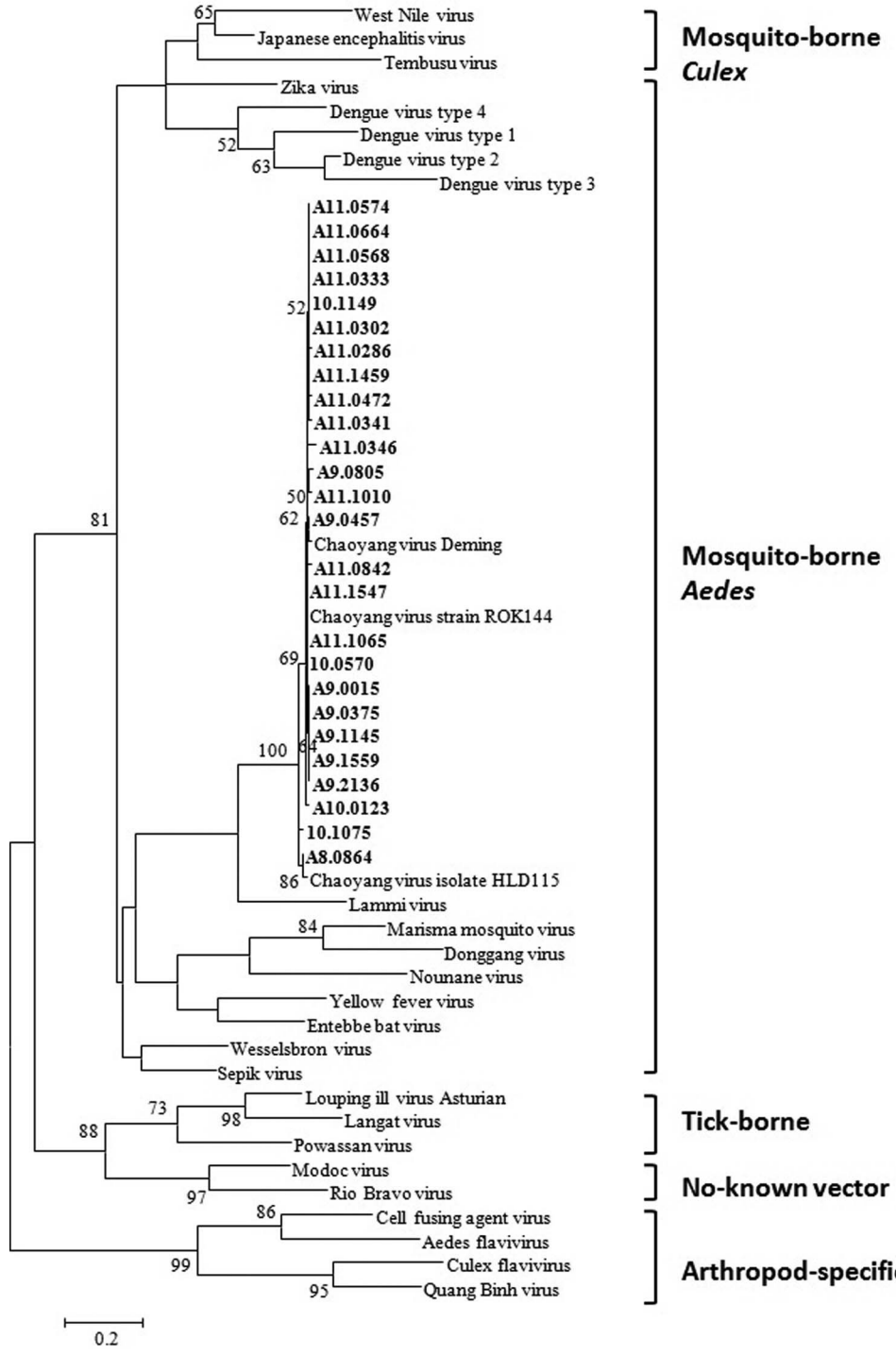

Fig. 2. Phylogenetic tree showing evolutionary relationships among CHAOV detected from mosquitoes collected from the ROK (2008–2011) and CHAOV isolated from China (HLD115 and Deming strains) along with other insect and medically relevant flaviviruses (54 nucleotide sequences; Table 5). The tree was constructed from the 204-nt NS5 sequence by using the maximum likelihood method. Evolutionary distances were computed by using the Kimura 2-parameter model in the MEGA5 program. The bootstrap values were calculated with 1,000 replicates. Only higher bootstrap values (>50) are shown at the nodes. Scale bar represents substitutions per site.

Table 5. Description of Chaoyang virus and reference sequences retrieved from GenBank database

Flaviviruses	Strain	Accession no.
Aedes flavivirus	Narita-21	AB488408
Chaoyang virus	Deming	FJ883471
Chaoyang virus	HLD115	JQ308185
Chaoyang virus	BeiBei	FJ812035
Chaoyang virus	ROK144	JQ068102
Cell fusing agent virus	H9–84	EU074055
Culex flavivirus	Tokyo	AB262759
Dengue virus type 1	Clone 45AZ5	U88536
Dengue virus type 2	16681	U87411
Dengue virus type 3	D3/H/IMTSSASRI/2000/1266	AY099336
Dengue virus type 4	recombinant clone	AF326825
Donggang virus	DG0909	JQ086551
Entebbe bat virus	UgIL30	AF013373
Japanese encephalitis virus	FU	AF217620
Langat virus	TP 64	EU073999
Lammi virus	N/A	FJ606789
Louping ill virus	Asturian	JQ646028
Marisma mosquito virus	HU4528/07	JN603190
Modoc virus	3321	EU074050
Nounane virus	B31	FJ711167
Powassan virus	647062	AF310945
Quang Binh virus	VN180	FJ644291
Rio Bravo virus	M64	AF013396
Sepik virus	MK7148	AF013404
St. Louis encephalitis virus	1A059	EU906871
Tembusu virus	MM1775	JX477685
Wesselsbron virus	SAH-177 99871–2	DQ859058
West Nile virus	TX_AR10–5718	JX015522
Yellow fever virus	17D vaccine	X03700
Zika virus	MR766	HQ234498

rate risk assessments, and institution of timely and effective vector and disease mitigation strategies. Although the phylogenetic relationship of CHAOV aligns with other mosquito-borne flaviviruses, some of which are medically important, its relative importance as a zoonotic pathogen and potential influence on other viruses or mosquito populations is unknown. The mosquito hosts of CHAOV are widely distributed and commonly found throughout the ROK. As a result of recent JEV outbreaks in the ROK (2010, 26 cases, 7 deaths; 2012, 20 cases, 5 deaths) (K-CDC 2012), and potential for the introduction of other arboviruses, longitudinal surveillance of arboviruses is needed. In addition, an integrated biosurveillance program will provide information on the distribution and prevalence of insect-specific and mosquito-borne arboviruses and avoid their misidentification throughout the ROK.

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