Entomological Surveillance for Flaviviruses at Migratory Bird Stopover Sites in Hokkaido, Japan, and a New Insect Flavivirus Detected in *Aedes galloisi* (Diptera: Culicidae)

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ABSTRACT To investigate the possible spread of West Nile virus (WNV) into Japan, we carried out entomological surveillance for flaviviruses at migratory bird stopover sites in Hokkaido, Japan, during 2003–2006. A total of 3,826 mosquitoes, identified as 15 species in five genera, were collected and 2,465 of these were grouped into 123 pools that were assayed for cytopathic effects on mosquito and mammalian cell cultures and for flavivirus RNA by reverse transcription-polymerase chain reaction using flavivirus universal primer sets for fragments of the NS3 and NS5 genes. Neither WNV nor other mosquito-vertebrate transmitted flaviviruses were detected in mosquitoes collected at any of the sites in Hokkaido, but five Culex flaviviruses and one novel Aedes galloisi flavivirus were identified from *Culex pipiens* L. s. l. and *Aedes galloisi* Yamada, respectively. Genetic and phylogenetic analyses based on the partial NS5 nucleotide sequences classified Aedes galloisi flavivirus with the insect flavivirus, but distant from Cell fusing agent, Kamiti river virus, and Culex flaviviruses, showing <74% sequence identities. Polymerase chain reaction-based bloodmeal analysis of 79 females showed that all of the *Aedes* and *Ochlerotatus* mosquitoes fed on mammals (deer and humans), whereas, *Cx. pipiens* s. l. mosquitoes fed on both of avian (ducks and sparrows, 85.7%) and mammalian hosts (dog, 14.3%). We suggest that to date WNV has not become established in Japan.

KEY WORDS entomological surveillance, Hokkaido, Flavivirus, West Nile virus, host-feeding patter

The genus *Flavivirus* (family *Flaviviridae*) is widespread, with some viruses being etiologic agents of severe human disease. The most important flaviviruses in terms of human health are West Nile virus (WNV), Yellow fever virus, Dengue virus (DENV), and Japanese encephalitis virus (JEV) that often cause severe encephalitis and/or fever in humans. In Japan, only JEV is still endemic, although <10 human cases have been reported annually since the 1990s (National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division 2003), and no other flaviviruses have emerged recently. Therefore, the introduction of WNV, DENV, or other emerging flaviviruses is great concern. In particular, WNV has expanded into new areas neighboring Japan. There was a large outbreak of WNV in southern Russia in 1999 with >500 human cases, and the virus was reported in dead wild birds for the first time in 2004 in the Novosibirsk Region of western Russia and in Vladivostok in far eastern Russia (Ternovoi et al. 2004). It was subsequently suggested that migratory birds could spread WNV to the southern regions of far eastern Russia (Ternovoi et al. 2006), thereby introducing WNV into eastern Asia.

Recently, several insect flaviviruses have been found in natural populations of mosquitoes worldwide. In the Asian region, Culex flavivirus (CXFV) was isolated in Japan and Indonesia (Hoshino et al. 2007), and Quang Binh virus (QBV) was found in Vietnam (Crabtree et al. 2009). Cell fusing agent (CFA) that was originally discovered in an *Aedes aegypti* (L.) cell line >30 yr ago (Stollar and Thomas 1975) was detected in Thailand (Kihara et al. 2007) and isolated in Indonesia (Hoshino et al. 2009). In Japan, we previously reported a high prevalence of Aedes flavivirus (AEFV) in natural populations of mosquitoes (Hoshino et al. 2007, 2009). Although the natural history including their maintenance cycles is unknown, the potential impact of these insect flaviviruses on flavivirus phylogenetic relationships and their relationship to other mosquito-borne flaviviruses is of increasing interest.

Because Hokkaido, in northern Japan, is only ≈700 km from Vladivostok in eastern Russia, the possibility of WNV being carried to Japan by migrating birds from Eurasia is a concern. In the current study, we carried out entomological surveillance for flaviviruses at mi-

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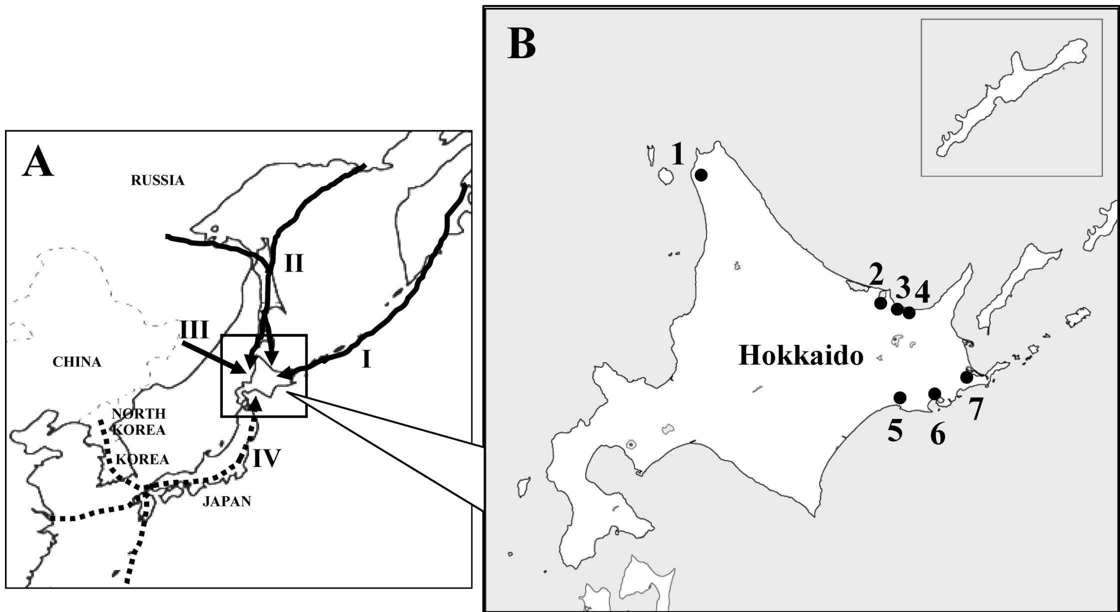

Fig. 1. Flyways of migratory *Anatidae* to Japan and mosquito collection sites in Hokkaido. (A) The black lines are recognized flyways to Japan for migratory birds. Route I is the Sakhaline-Kuril route, route II is the Kamchatka route, and route III is the Crossing of the Sea of Japan route. The dotted line indicates the Korean Peninsula route (route IV), which is from eastern and northeastern Asia to Hokkaido through mainland Japan. (B) Locations of the seven mosquito collection sites in this study are shown here and described in Materials and Methods.

gratory bird stopover sites in Hokkaido, Japan, during 2003–2006, to detect WNV transmission from Eurasian birds. To obtain data on the blood-feeding patterns, a polymerase chain reaction (PCR)-based bloodmeal analysis was carried out on all blood-fed females collected.

Materials and Methods

Study Areas. Several south bound species of migratory Anseriformes may be competent hosts of WNV and stop over in Hokkaido from November to April, whereas others are summer residents in Hokkaido from April to October (Miyabayashi and Mundkur 1999). Although there are annual changes in the migratory flyways, birds generally migrate to Japan via four flyways (routes I–IV in Fig. 1A) (Saito et al. 2009, Ministry of the Environment 2010). Three of these are from Eastern Siberia to Japan through Hokkaido: the Sakhalin–Kurile route (route I), the Kamchatka route (route II), and the Crossing of the Sea of Japan route (route III). In addition, migratory birds have also used the Korean Peninsula route (route IV), which is from eastern and northeastern Asia to Hokkaido through mainland Japan. Therefore, we chose seven sites in three areas of Hokkaido where migratory birds stop over for entomological surveillance for flaviviruses.

Mosquitoes were collected at the Sarobetsu Peat Land, Toyotomi Town (N45°06′ E141°41′) (site 1 in Fig. 1B) in northern Hokkaido from 10 to 11 July 2006. Three additional sites were located in the Abashiri area of northeastern Hokkaido: Lake Notoro (N44°03′ E144°10′), Lake Tofutsu (N44°57′ E144°19′), and Lake Mokoto (N44°55′ E144°24′) (sites 2–4 in Fig. 1B, respectively). Mosquitoes were collected from 1 to 3 August 2003 at Lake Notoro, and from 6 to 9 August 2004 at the other two sites. There were three mosquito collection sites in the Kushiro–Nemuro area of southeastern Hokkaido: the Kushiro Wetlands, the largest wetlands in Japan (N43°04′ E144°19′); Itoigawa, Akeshi Town (N43°05′ E144°53′); and Lake Furen, Nemuro City (N43°16′ E145°23′) (sites 5–7 in Fig. 1B, respectively). Collections conducted in 2003 and 2004 (sites 2–7) were previously described by Higa et al. (2006), and included here for completeness.

Mosquito Collection and Identification. Mosquitoes were collected by dry ice-baited CDC light traps, sweep nets, and human baited traps (Higa et al. 2006). In the field, all mosquitoes frozen and then were transported on dry ice to the National Institute of Infectious Diseases (NIID) in Tokyo, where they were promptly identified by morphological examination using the identification keys of Tanaka et al. (1979). Because it was difficult to distinguish *Ochlerotatus hokkaidensis* Tanaka, Mizusawa & Saugstad from *Oc. punctor* Kirby (Tanaka et al. 1979), these mosquitoes are designated *Oc. hokkaidensis* (*Oc. punctor*) in the text. For the blood-fed *Cx. pipiens* s. l., a PCR-based assay that detects polymorphism in the acetylcholinesterase (ACE) two gene (ACE2-assay) (Kasai et al. 2008) was used to distinguish between *Cx. p. pallens* and *Cx. p.* form *molestus.* Mosquitoes were sorted at −80°C into

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pools containing 20–50 adults in 2 ml centrifuge tubes according to species, study site, and date of collection.

Virus Isolation. The mosquito C6/36 cell line, derived from *Aedes albopictus* Skuse (Health Science Research Resources Bank [HSRRB], Osaka, Japan), and two mammalian cell lines, Vero-9013 derived from African green monkey kidney (HSRRB) and BHK-21 derived from baby hamster kidney (a gift from Dr. H. Bando, Hokkaido University, Sapporo, Japan), were used for virus isolation as described previously (Hoshino et al. 2007, 2009). Briefly, the pools of mosquitoes were homogenized in the presence of 500 μl ice-cold MEM with 2% heat-inactivated FBS, 2% NEAA, 200 U penicillin/ml, 200 μg streptomycin/ml, and 10 μl Fungizone (Gibco BRL)/ml using a Mixer Mill (model MM300, Retsch GmbH, Haan, Germany). The homogenates were clarified by centrifugation, and the supernatants passed through sterile 0.45 μm filters (Ultrafree MC, Millipore, Bedford, MA). The filtrates were diluted 10-fold with the same medium and inoculated onto monolayers of C6/36, Vero, or BHK-21 cells in 24-well culture plates. The plates were incubated for 2 h at 28°C (mosquito cell line) or at 37°C in 5% CO_2 (mammalian cell lines) to allow virus adsorption. After addition of 500 μl fresh medium, the cell cultures were incubated under the same conditions for ≈7 d. Cytopathic effects (CPE) were observed daily by phase-contrast microscopy until the culture supernatants were harvested. Culture supernatants were collected after at least three blind passages and used as virus stocks. These virus stocks were stored at −80°C.

Viral RNA Detection by Reverse Transcription-PCR and Sequence Analysis. Viral RNA was extracted from cell culture supernatants using a High Pure Viral RNA Kit (Roche Diagnostics, Mannheim, Germany) or a QIAamp Viral RNA Mini Kit (QIAGEN Inc., Valencia, CA). Reverse transcription-PCRs (RT-PCR) were conducted using a TaKaRa One Step RNA PCR Kit (Takara Bio, Shiga, Japan). Flavivirus universal primer sets for fragments of the NS3 gene (Fla-U5004/Fla-L5457) (Briese et al. 1999) and NS5 gene (FU1/cFD2 and FU2/cFD3) (Kuno et al. 1998) were used. RT-PCR was carried out according to the manufacturer's instructions. The amplified products were purified by agarose gel electrophoresis, followed by extraction of the amplified fragments using a QIAEXII Gel Extraction Kit (QIAGEN). Purified DNA fragments were directly cycle-sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Kit v.1.1 (Applied Biosystems, Foster City, CA) and the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Sequence analyses were performed using the program GENETYX-WIN v.8 (Genetyx Corp., Tokyo, Japan).

Mosquito homogenates from the Sarobetsu Peat Land collected in 2006 were tested using the ABI Prism 7900 Sequence Detection System (Applied Biosystems) with the RNA UltraSense One-Step Quantitative RT-PCR System (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol for TaqMan RT-PCR. Viral RNA was extracted from mosquito homogenates using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The primer-probe sets used were WN-ENV for amplification of the E gene and WN3′NC for amplification of the 3′ noncoding region (Lanciotti 2000), and the WNV-multi primer-probe set (Shirato et al. 2005). For the sequencing of the viral RNA positive pool, RT-PCR was carried out using the flavivirus universal primer sets for fragments of the NS3 and NS5 genes described above.

Phylogenetic Analysis. To analyze the genetic relatedness of a novel virus isolate made from *Ae. galloisi* Yamada, we constructed a phylogenetic tree based on nucleotide sequence. For this analysis, two conserved regions including RNA dependent RNA polymerase motifs A, B, C, and D (nucleotides 8803–9013 and 9203–9652 in AEFVs) were selected and concatenated. The corresponding sequence data were used for nine strains of six insect flaviviruses: CFA, Kamiti river virus (KRV) strain SR-75, CXFV strain Tokyo, CXFV strain Mex07, CXFV strain HOU24518, CXFV strain Iowa07, QBV strain VN180, Nakiwogo virus (NAKV) strain Uganda08, and AEFV strain Narita-21, which were available in the GenBank database (M91671, AY149904, AB262759, EU879060, FJ502995, FJ663034, FJ644291, GQ165808, AB488408, respectively). Dengue virus type 2 (DEN2, GenBank NC001474) was included as an outgroup sequence. The sequences were aligned by CLUSTALX ver. 2.0.8 (Larkin et al. 2007) and analyzed by neighbor-joining (NJ) algorithms using MEGA ver. 4.1 (Tamura et al. 2007). Statistical significance of the resulting NJ tree was evaluated using a bootstrap test with 1,000 replications.

Bloodmeal Analysis. Field collected blood-fed females were used for bloodmeal analysis with primers for the mtDNA cytochrome b gene (Avian-3, -4, -8; Mammalian-1, -2, -7; and AvMa-5, -6) and 18S rRNA (VerU-1, -2) to identify the vertebrate host on which the mosquitoes had fed (Sawabe et al. 2010). Briefly, genomic DNA was extracted from each mosquito using a REDExtract-N-Amp Tissue PCR Kit (Sigma). The PCR was carried out as described previously (Sawabe et al. 2010). Sequences of PCR amplified DNA fragments then were identified using BLAST searches against the GenBank nucleic acid database (NCBI Web site, http://www.ncbi.nlm.nih.gov/BLAST/). Only those with >98% sequence identity were reported. It was difficult to differentiate among some closely related species from sequence information on their cytochrome b and 16S rDNA; for example, spot-billed duck (*Anas poecilorhyncha*), mallard (*A. platyrhynchos*), domestic duck (*A. platyrhynchos* ver. *domesticus*), and hybrid duck, and for pig and wild boar. Therefore, the former was designated as ducks (*Anas* spp.) and the latter as pigs, because wild boar have not been found in Hokkaido.

Results

Mosquito Collection in Hokkaido. In total, 3,826 mosquitoes, identified as 15 species in five genera, were collected in three areas of Hokkaido during the

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Table 1. Mosquitoes collected at migratory bird stopover sites in Hokkaido, Japan, during mosquito seasons of 2003–2006

Mosquito species	No. collected[a]	Sarobetsu Peat Land in 2006 (site 1)		Abashiri area in 2003–2004 (sites 2–4)[b]		Kushiro–Nemuro areas in 2004 (sites 5–7)[b]	
		No.	%	No.	%	No.	%
Anopheles sp.	1					1	0.2
Ae. esoensis	624	527	39.2	2	0.1	95	18.5
Ae. galloisi	11	8	0.6	1	<0.1	2	0.4
Ae. vexans	66	1	<0.1	60	3.0	5	1.0
Ae. yamadai	178	38	2.8	131	6.6	9	1.8
Aedes (*Stegomyia*) spp.	11			10	0.5	1	0.2
Cx. orientalis	145	1	<0.1	144	7.3		
Cx. pipiens s. l.	1,552	1	<0.1	1,428	72.5	123	24.0
Culicoides sp.	4					4	0.8
Cu. nipponica	13			1	<0.1	12	2.3
Oc. dorsalis	378			193	9.8	185	36.5
Oc. excrucians	552	551	41.1			1	0.2
Oc. hokkaidensis/punctor	200	200	14.9				
Oc. japonicus	1	1	<0.1				
Ochlerotatus spp.	75					75	14.6
Unidentified species	15	15	1.1				
Total (%)	3,826	1,343[c]	100	1,970[d]	100	513[e]	100

[a] Collection sites are in Fig 1.
[b] The part of the collections conducted in 2003 and 2004 were previously presented by Higa et al. (2006).
[c] Includes 63 blood-fed females (32 *Oc. excrucians*, 27 *Oc. hokkaidensis* (or *Oc. punctor*), and 4 *Ae. esoensis*).
[d] Includes eight blood-fed females (2 *Oc. dorsalis* and 6 *Cx. pipiens* s. l.).
[e] Includes eight blood-fed females (6 *Oc. dorsalis*, 1 *Ae. Esoensis*, and 1 *Cx. pipiens* s. l.).

mosquito seasons of 2003–2006 (Table 1). In Sarobetsu Peat Land in northern Hokkaido (site 1 in Fig. 1B), a total of 1,343 mosquitoes were collected near bushes in 2006 and identified as members of four *Aedes*, two *Culex* and three *Ochlerotatus* species (Table 1). Of these nine species, *Oc. excrucians* Walker (41.1% of the total) and *Ae. esoensis* (39.2%) were the predominant species in this area, followed by *Oc. hokkaidensis* (*Oc. punctor*) (14.9%).

In total, 1,970 and 513 mosquitoes were collected in the Abashiri area of northeastern Hokkaido (sites 2–4) and in the Kushiro–Nemuro areas of southeastern Hokkaido (sites 5–7), respectively (Fig. 1B). Collections conducted at sites 2–7 were previously presented by Higa et al. (2006). Briefly, of nine species in the Abashiri area, the most abundant species was *Cx. pipiens* s. l. (72.5% of the total). At the Kushiro–Nemuro areas, of 12 species, *Oc. dorsalis* Meigen was the predominant species (36.5%), followed by *Cx. pipiens* s. l. (24%) and *Ae. esoensis* (18.5%).

Virus Isolation and Identification. In total, 2,465 mosquitoes, excluding the 79 blood-fed females, were grouped into 123 pools and tested for flaviviruses (Table 2). Five pools of *Cx. pipiens* s. l. and one of *Ae. vexans* collected from Lake Notoro (site 2) in 2003 produced CPE on C6/36 cells and were identified as flavivirus by RT-PCR with primer sets for both the NS3 and NS5 genes. The flavivirus sequences from the five pools of *Cx. pipiens* s. l. were similar to that of CXFV-Tokyo strain (AB262759) (Hoshino et al. 2007), and previously designated as CXFV-Hokkaido strain (AB262762) (Hoshino et al. 2007). Although CPE was observed from one pool of *Ae. vexans*, the sequence did not match any virus in the GenBank database. Of 605 mosquitoes in 31 pools collected from five sites in the eastern Hokkaido (sites 3–7) in 2004, none were positive for flavivirus, based on both production of CPE on Vero, BHK-21 and C6/36 cells, and testing for flavivirus RNA (data not shown). Of the 1,279 mosquitoes in 74 pools collected in Sarobetsu Peat Land (site 1) in 2006, one pool of *Ae. galloisi* was positive for flavivirus RNA using primer sets for the NS5 gene (Table 2). The nucleotide sequence of this flavivirus

Table 2. Number of mosquitoes and pools from Hokkaido processed for the virus isolation and detection

Mosquito species	No. mosquitoes tested	No. pools tested	No. flavivirus-positive pools	
			CPE on cells	Virus identified
Ae. esoensis	611	32	0	0
Ae. galloisi	8	1	0	1[c]
Ae. vexans	60	7	1[a]	0
Ae. yamadai	178	9	0	0
Aedes (*Stegomyia*) spp.	10	1	0	0
Cx. orientalis	145	4	0	0
Cx. pipiens s. l.	372	10	5[b]	5[b]
Cu. nipponica	10	2	0	0
Oc. dorsalis	363	18	0	0
Oc. excrucians	519	27	0	0
Oc. hokkaidensis (or *Oc. punctor*)	173	10	0	0
Oc. japonicus	1	1	0	0
Unidentified species	15	1	0	0
Total	2,465	123	6	6

[a] An *Ae. vexans* pool from Lake Notoro in 2003 produced CPE on C6/36 cells but was unidentified by RT-PCR for both NS3 and NS5 genes.
[b] Five pools of *Cx. pipiens* s. l. from Lake Notoro produced CPEs on C6/36 cells and CXFV genes were confirmed by RT-PCR for both NS3 and NS5 genes.
[c] AGFV was detected in one pool of *Ae. galloisi* mosquito homogenate collected in Sarobetsu Peat Land in 2006 by TaqMan RT-PCR and identified by RT-PCR for NS5 gene.

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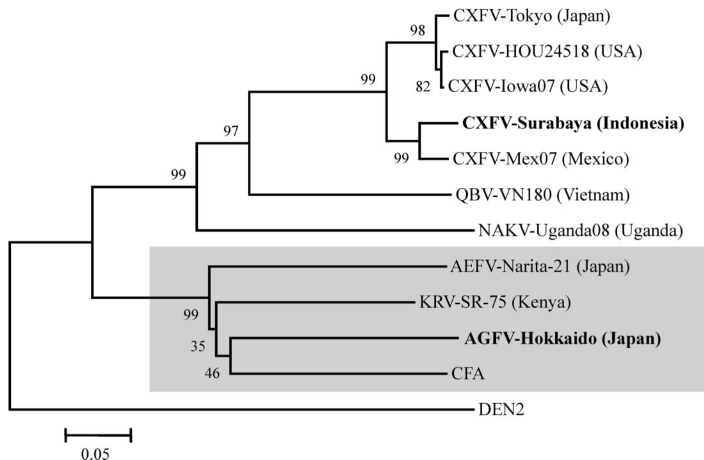

Fig. 2. NJ dendrogram showing phylogenetic relationships of insect flaviviruses based on the 661-nucleotide sequences of the NS5 gene. Bootstrap values correspond to 1,000 replications. Bar denotes the nucleotide similarity distance. Shaded background indicates insect flaviviruses from *Aedes* mosquitoes. GenBank accession numbers for sequences used in the phylogenetic analysis are as follows: CXFV strain Tokyo from Japan, AB262759; HOU24518 from the United States, FJ502995; Iowa07 from the United States, FJ663034; Surabaya from Indonesia, AB639348 and AB639349; Mex07 from Mexico, EU879060; QBV strain VN180 from Vietnam, FJ644291; NAKV strain Uganda08 from Uganda, GQ165809; AEFV strain Narita-21 from Japan, AB488408; KRV strain SR-75 from Kenya, AY149904; AGFV strain Hokkaido from Japan, AB639346 and AB639347; CFA, M91671; and DEN2, NC001477. The branches for flavivirus sequences published for the first time in the current study (i.e., CXFV strain Surabaya and AGFV strain Hokkaido) are in bold.

RNA from *Ae. galloisi* had 73.0, 72.8, 70.9, and 62.6% identity to the corresponding sequences of KRV, CFA, AEFV, and CXFV, respectively. Therefore, considering the criteria of Kuno et al. (1998), the flavivirus from *Ae. galloisi* was considered to be a new insect flavivirus and tentatively designated Aedes galloisi flavivirus (AGFV). Neither WNV nor other mosquito-borne flaviviruses were detected at any of the sites in Hokkaido during our surveillance in 2003–2006.

Phylogenetic Analysis of AGFV. The two partial nucleotide sequences of the NS5 gene of flavivirus have been submitted to DDBJ, EMBL, and GenBank databases. The former is AGFV strain Hokkaido (AB639346 and AB639347), which was newly isolated from *Ae. galloisi* in this study, and the latter is CXFV strain Surabaya (AB639348 and AB639349), which was previously reported by Hoshino et al. (2007). A phylogenetic tree was constructed based on 661 nucleotide sequences of the NS5 gene of eight flaviviruses (Fig. 2). The tree showed that AGFV clustered with insect flaviviruses and distant from the DENV2 outgroup. CXFVs formed one robust clade despite differences in species of origin and isolation site, whereas AGFV was in the *Aedes* mosquito-associated clade in the insect flavivirus cluster. The topological order among AGFV, CFA, and KRV could not be determined because of the low bootstrap values.

Host Animal Identification. Overall, bloodmeal hosts were identified from 79 blood-fed female mosquitoes (Table 3). All of the *Aedes* and *Ochlerotatus* fed on mammals, regardless of their collection site. Hokkaido Sika deer was the predominant host for *Ae. esoensis*, *Oc. excrucians*, and *Oc. hokkaidensis* (*Oc. punctor*), followed by humans. Humans were the predominant host for *Oc. dorsalis*, followed by pigs. In addition, one *Oc. dorsalis* mosquito had fed on the American Bullfrog *Rana catesbeiana.* In contrast, birds were the predominant host of the seven *Cx. p. pallens* mosquitoes tested. Of six *Cx. p. pallens* mosquitoes collected near Lake Notoro, five fed on ducks and one on a sparrow, and the one *Cx. p. pallens* from the Kushiro area had fed on a dog (14.3%). In addition, two *Oc. excrucians* had multiple host bloodmeals, one on a human and a cow, and the other on a dog and a cow.

Discussion

WNV has spread to several Eurasian countries since early August 2010. The Russian Federation reported 448 registered WNV infections and six fatal cases from July to September 2010. Although a majority of these cases were in western Russia, Volgograd, Rostov, and Voronezh oblasts as of 3 September 2010 (European Centre for Disease Prevention and Control [ECDC] 2010), WNV may spread to far eastern Russia and near Japan. The current study found no evidence of WNV infection in mosquitoes at any collection site in Hokkaido, Japan. The predominant *Aedes* and *Ochlerotatus* species at most of the collection sites mostly fed on mammalian hosts, mainly Hokkaido Sika deer followed by humans, whereas the few *Cx. pipiens* s. l. fed on avian hosts. During our nationwide survey of the host-feeding patterns of mosquitoes throughout Japan, a similar tendency was found in *Aedes*, *Ochlerotatus*, and *Culex* mosquitoes (Sawabe et al. 2010). If WNV is transported by migratory birds to these areas, *Aedes* and *Ochlerotatus* are probably not significant

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Table 3. Bloodmeal analysis of the species on which the 79 blood-fed female mosquitoes in this study had fed

Species	*Ae. esoensis*	*Oc. excrucians*[a]	*Oc. hokkaidensis* (*Oc. punctor*)	*Oc. dorsalis*[b]	*Cx. p. pallens*[b]
	No. tested (%)	No. tested (%)	No. tested (%)	No. tested (%)	No. tested (%)
Hokkaido sika deer (*Cervus nippon yesoensis*)	3 (60.0)	18 (52.9)	21 (77.8)	0	0
Human (*Homo sapiens*)	2 (40.0)	11 (32.4)	3 (11.1)	5 (62.5)	0
Dog (*Canis familiaris*)	0	3 (8.8)	1 (3.7)	0	1 (14.3)
Cow (*Bos taurus*)	0	2 (5.9)	2 (7.4)	0	0
Pig (*Sus scrofa*)[c]	0	0	0	2 (25.0)	0
Ducks (*Anas* spp.)[d]	0	0	0	0	5 (71.4)
Russet sparrow (*Passer rutilans*)	0	0	0	0	1 (14.3)
Others	0	0	0	1 (12.5)[e]	0
Total (%)	5 (100)	34 (100)	27 (100)	8 (100)	7 (100)

[a] Includes two bloodmeal hosts for each of two mosquitoes for which two bloodmeals were identified (human and cow, dog and cow).
[b] Some of these results were part of a previous study (Sawabe et al. 2010).
[c] Pig and boar could not be differentiated from the available sequence data.
[d] Ducks include the spot-billed duck (*Anas poecilorhyncha*), mallard (Anas *platyrhynchos*), and domestic duck (*Anas platyrhynchos* ver. *Domesticus*).
[e] One specimen had fed on the American Bullfrog (*Rana catesbeiana*).

bridge vectors for WNV transmission without prior amplification by *Cx. pipiens* s. l.

When WNV spreads to far eastern Asia, the virus will probably eventually reach Japan via migratory birds. For monitoring the possible entry of viruses into Japan via migratory birds, Hokkaido is an excellent area, because there are several major stopover areas for migratory birds and this area still has a rich natural environment. We suggest that the probability of WNV entry into Japan using the northern flyway (route I–III) is very low, because the mosquito season in Hokkaido, from July to October (Takashima et al. 1989), does not coincide with the season when migratory birds stop over, from November to April. Furthermore, transient viremias of WNV would not persist to the beginning of the mosquito season in July. In contrast, the southern flyway (route IV) may be more important than the northern flyway for WNV introduction as well as JEV. There have been JEV infections almost every year in Korea, eastern China, and Taiwan. It has been suggested that JEV might be transported from these countries to Japan by migratory birds and/or mosquitoes (Nga et al. 2004, Nabeshima et al. 2008, Morita, 2009, Saito et al. 2009). Subsequently, a 2005–2006 serologic survey of flaviviruses in wild birds in the Tokachi area of eastern Hokkaido by Saito et al. (2009) suggested that wild ducks there had been exposed to flaviviruses, including WNV and JEV, somewhere along their flyway, but not in Hokkaido. In South Korea as of 2008, no evidence of WNV activity was reported, but serologic evidence strongly suggested that some of the birds had been exposed to WNV or JEV (Yeh et al. 2011a, b). In Japan, there were no reported cases of WNV in mosquitoes and dead birds through 2007 (Tabei et al. 2007, Shirafuji et al. 2011), and in humans as of 1 June 2011 (Infectious Disease Surveillance Center [IDSC] 2011). Therefore, we concluded that WNV has not been introduced into Hokkaido to date.

During our flavivirus surveillance, two insect flaviviruses were identified from Hokkaido. One from *Cx. pipiens* s. l. collected at Lake Notoro was previously reported as CXFV-Hokkaido strain (Hoshino et al. 2007), which is one of the CXFV strains isolated from *Culex* spp. mosquitoes worldwide: for example, in Japan (Hoshino et al. 2007); Central America (Morales-Bettoulle et al. 2008, Kim et al. 2009); North America (Blitvich et al. 2009, Farfan-Ale et al. 2009, Kim et al. 2009); and Uganda, Africa (Cook et al. 2009). The other from *Ae. galloisi* collected at Sarobetsu Peat Land was considered a new insect flavivirus, designated AGFV in this study, and was shown to be in the *Aedes* mosquito-associated phylogenetic branch, although the virus isolation has not succeeded to date. Further sequence analysis is needed to establish the detailed taxonomic status of this virus. These results indicated that each insect flavivirus is maintained in a host genus-specific manner in Hokkaido, as in the case with CXFV, AEFV, and/or CFA in Japan and Indonesia (Hoshino et al. 2009). Additionally, in Japan all *Aedes* mosquito-associated insect flaviviruses have only been isolated from the subgenus *Stegomyia*, for example, AEFVs from *Ae. albopictus* and *Ae. flavopictus* Yamada (Hoshino et al. 2009), and AGFV from *Ae. galloisi*. They are reminiscent of the intimate relation between *Aedes* mosquito-borne flaviviruses, such DENV, and the specific vectors in the subgenus *Stegomyia*. Therefore, ongoing surveillance for other *Aedes* mosquitoes in Hokkaido, including *Ae. flavopictus* from which AEFV was isolated, will be important for understanding the flavivirus-mosquito association and will also provide data on the evolution of insect flaviviruses.

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