Dengue Virus Infection in *Aedes albopictus* during the 2014 Autochthonous Dengue Outbreak in Tokyo Metropolis, Japan

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Abstract. In 2014 in Japan, 162 autochthonous dengue cases were reported for the first time in nearly 70 years. Here, we report the results of the detection and isolation of dengue virus (DENV) from mosquitoes collected in Tokyo Metropolis in 2014 and 2015. The phylogenetic relationship among DENV isolates from mosquitoes and from patients based on both the entire envelope gene and whole coding sequences was evaluated. Herein, 2,298 female and 956 male *Aedes albopictus* mosquitoes were collected at six suspected locations of DENV infection in Tokyo Metropolis from August to October in 2014 and grouped into 124 and 35 pools, respectively, for viral genome detection and DENV isolation. Dengue virus RNA was detected using reverse transcription polymerase chain reaction and TaqMan assays from 49 female pools; 16 isolates were obtained using C6/36 and Vero cells. High minimum infection rates (11.2–66.7%) persisted until mid-September. All DENV isolates belonged to the genotype I in serotype 1 (DENV-1), and its sequences demonstrated >99% homology to the sequence of the DENV isolated from a patient in the vicinity of Tokyo Metropolis in 2014. Therefore, *Ae. albopictus* was a major DENV vector, and a single DENV-1 strain circulated in Tokyo Metropolis in 2014. Dengue virus was not detected from male mosquitoes in 2014 and wild larvae in April 2015. Thus, the possibility of both vertical transmission and overwintering of DENV was extremely low, even in dengue-epidemic areas. This study reports the first entomological information on a dengue outbreak in a temperate region, where no *Aedes aegypti* mosquitoes are distributed.

INTRODUCTION

On August 27, 2014, the first autochthonous dengue case in Japan was reported by the Ministry of Health, Labor and Welfare.¹ As the patient reported never having traveled overseas, the case was concluded to be an autochthonous dengue case due to the dengue virus (DENV) infection, which occurred in Tokyo Metropolis. Eventually, a total of 162 autochthonous dengue cases were recorded during 2014. During this dengue outbreak, extensive vector control measures, including insecticides targeting on adult mosquitoes, were conducted at almost all locations where DENV transmission was suspected.^{2,3}

Dengue fever is an acute, mosquito-borne febrile illness caused by the DENV, which belongs to the genus *Flavivirus*, family *Flaviviridae*. Dengue outbreaks have occurred worldwide and account for 390 million infections and 250,000 hemorrhagic cases, with 25,000 deaths per year.⁴ A large number of dengue cases have recently been reported, particularly in South and Central America and Southeast Asia. Moreover, dengue outbreaks have occurred continuously in Malaysia, Indonesia, Vietnam, and the Philippines. In Japan, more than 200 imported cases of dengue fever have been reported every year since 2010, and this number has gradually increased annually, for example, 293 cases in 2015 and 338 cases in 2016, which was the highest thus far.⁵

In Japan, the autochthonous dengue outbreaks that occurred between 1942 and 1945 that resulted in approximately 200,000 cases of infection in Nagasaki, Sasebo, Kure, Kobe, and Osaka cities, were caused by returning soldiers who imported the DENV.⁶ However, no subsequent autochthonous epidemics occurred in Japan since the end of 1945 until 2014, except for one suspected case that occurred in 2013. A German tourist who traveled to Japan during middle to late August 2013 became feverish on September 3 after flying directly to Frankfurt Airport from Narita Airport; she was later diagnosed with dengue fever caused by DENV-2.⁷ This case was undeniably diagnosed as dengue most likely acquired within Japan. An autochthonous small-scale spread of a DENV infection might have thus occurred in 2013.

The primary vector mosquito of DENV is *Aedes aegypti* (L.), which widely occurs from tropical to subtropical regions of the world. *Aedes aegypti* once inhabited Okinawa and the Ogasawara Islands of Japan. They also temporarily inhabited Kumamoto prefecture from 1944 to 1947 but disappeared from Japan since 1975.⁸ *Aedes albopictus* (Skuse) is a secondary vector of DENV, which has been distributed in tropical to temperate regions, and currently found worldwide. Particularly in temperate regions, *Ae. albopictus* is regarded as the most important vector of *Aedes*-borne viral diseases.⁹ *Aedes albopictus* is now confirmed to occur throughout Japan except Hokkaido island, and this mosquito is a major *Aedes* species in urban areas of Japan.¹⁰ A previous study suggested that the first dengue case of the 2014 outbreak in Tokyo Metropolis was caused by infection with DENV through a bite from *Ae. albopictus* in Yoyogi Park (YP),^{11,12} a popular forested park frequented by international and local visitors. In

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fact, the density of *Ae. albopictus* in this area has been shown to be high in a previous study.¹³

To ensure that an outbreak of dengue fever that will inevitably occur in Japan in the near future is better managed, it is important to understand the conditions underlying the 2014 dengue outbreak in vector mosquitoes in Japan. We therefore conducted entomological surveys of adult mosquitoes to evaluate the mosquito density¹³ and DENV infection in *Ae. albopictus* in several parks in the vicinity of Tokyo Metropolis during the dengue outbreak period in Japan in 2014. Because vertical transmission of DENV by *Ae. albopictus* has been observed both experimentally^{14–16} and naturally,^{17–19} the possibility of overwintering of DENV in mosquitoes was considered. Therefore, we also conducted surveys of the larvae in the spring of 2015 to evaluate the overwintering of DENV in an epidemic area. Herein, we report the results of the detection and isolation of DENV from mosquitoes collected in Tokyo Metropolis in 2014 and 2015. We also report here the phylogenetic relationship between DENV isolates from mosquitoes and isolates from patients based on both the entire envelope (E) gene and whole coding sequences.

MATERIALS AND METHODS

Study sites. Local transmission of DENV was suspected mainly at YP and other sites in Tokyo Metropolis in 2014 (Figure 1). Adult mosquitoes were collected at six locations from August 28 to October 23, 2014: YP (35°40′19.11″N, 139°41′51.67″E), Shinjuku Central Park (SCP: 35°41′23.3″N, 139°41′21.8″E), Meiji Jingu (MJ: 35°40′34″N, 139°41′57″E), National Olympics Memorial Youth Center (NYC: 35°40′28.7″N, 139°41′36.4″E), Sotobori Park (35°41′20.19″N, 139°43′52.05″E), and Shinjuku Gyoen National Garden (SGNG: 35°41′29.9″N, 139°42′16.62″E). Mosquito surveys were carried out at 10–20 points in each location. In addition,

to assess the possibility of overwintering of DENV, mosquito larvae were collected at SCP and SGNG on April 20, 2015.

Mosquito collection. We collected adult mosquitoes by the 8-minute human bait-sweep net method¹³ at all collection sites, except for SGNG. At SGNG, mosquito collection was carried out by the CO₂ dry ice-baited trap method, which used a Centers for Disease Control miniature light trap enhanced with a dry ice.¹⁰ All field-collected mosquitoes were transported to the National Institute of Infectious Diseases (NIID), Tokyo, Japan, while keeping them alive. The species were identified according to established identification keys.²⁰ Subsequently, *Ae. albopictus* were pooled in a 2-mL microtube (Eppendorf, Hamburg, Germany) according to collection site and gender with a maximum of 34 mosquitoes per pool and stored at –80°C until detection and isolation of DENV.

Larvae were collected at SCP and SGNG using a ladle on April 20, 2015. Larvae were transported to the NIID and maintained with SWIMMIY BABY (Nippon Pet Food, Tokyo, Japan) at 25°C until adult emergence. Emerged adults were identified according to the established identification keys²⁰ described previously, and *Ae. albopictus* were used for the detection and isolation of DENV.

Dengue virus RNA detection from mosquitoes. The pooled *Ae. albopictus* specimens were homogenized in Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 2% heat-incubated fetal bovine serum (Sigma-Aldrich), 2% nonessential amino acids (aa) (Sigma-Aldrich), 200 U penicillin (Life Technologies, Carlsbad, CA)/mL, 200 µg streptomycin (Life Technologies)/mL, and 5 µg fungizone (Life Technologies)/mL. The homogenates were centrifuged and the supernatants were passed through sterile 0.45 µm filters (Merck Millipore, Darmstadt, Germany). Aliquots of each filtrate were used to extract total RNA using Isogen II (Nippon Gene, Tokyo, Japan) for the reverse transcription polymerase chain reaction (RT-PCR)-based virus detection, whereas the residuals were used as inocula for virus

FIGURE 1. Map of the vicinity of Tokyo Metropolis showing locations where dengue virus (DENV) transmission was suspected during dengue outbreak in 2014. At the six locations (closed circles) among these, mosquito collections were carried out in this study. Open circles describe locations where DENV transmission was suspected but mosquito survey was not performed. Shinjuku Gyoen National Garden (SGNG)* was not a suspected location of DENV transmission, but mosquito collection was carried out. Abbreviation of the collection sites is described in Materials and Methods. This figure appears in color at www.ajtmh.org.

isolation (described below). Reverse transcription polymerase chain reaction for DENV detection was performed using the PrimeScript One-Step RT-PCR Kit Ver. 2 (Takara Bio Inc., Shiga, Japan) with a specific primer set for DENV-1 (D1s; 5'-GGA CTG CGT ATG GAG TTT TG-3' and D1c; 5'-ATG GGT TGT GGC CTA ATC AT-3').²¹ The thermal program consisted of reverse transcription (RT) at 50°C for 30 minutes, termination of RT at 94°C for 2 minutes, and 35 cycles for polymerase chain reaction (PCR) at 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds. The amplified products were then analyzed by agarose gel electrophoresis.

The adult *Ae. albopictus* collected at SGNG from September to October were pooled, with a maximum of 50 individuals per pool, and homogenized. Viral RNA was extracted by using an RNeasy Mini Kit (QIAGEN, Valencia, CA). TaqMan RT-PCR was carried out according to the method established in a previous study.²² Briefly, extracted RNA was mixed with a specific primer and probe set for DENV-1 (primers: D1MGBEn469s, 5'-GAA CAT GGR ACA AYT GCA ACY AT-3'; D1MGBEn536r, 5'-CCG TAG TCD GTC AGC TGT ATT TCA-3'; probe: D1MGBEn493p, 5'-ACA CCT CAA GCT CC-3') using a TaqMan RT-PCR Ready-Mix kit (Applied Biosystems, Foster City, CA). The thermal profile of the TaqMan RT-PCR assay consisted of RT at 48°C for 30 seconds and 45 cycles for the PCR at 95°C for 15 seconds and 57°C for 60 seconds. Polymerase chain reaction products were directly detected by monitoring the increase in fluorescence of a dye-labeled oligonucleotide probe with a STEP ONE sequence detector (Applied Biosystems).

Dengue virus isolation using cultured cells. Virus isolation was carried out using C6/36 cells (Health Science Research Resources Bank, Osaka, Japan) and Vero cells (American Type Culture Collection, Manassas, VA). The method of isolation is described in a previous study.²³ The filtrates of the mosquito homogenates were inoculated into monolayers of C6/36 cells and incubated at 28°C at 5% CO₂ for 7 days. Subsequently, the cell culture supernatant from the C6/36 cells was passaged onto Vero cells and further incubated at 37°C at 5% CO₂ for 7 days. To confirm virus recovery and propagation, the culture supernatants obtained after the incubation were subjected to an immunochromatographic strip test for the detection of DENV nonstructural protein 1 (NS1) antigens (Dengue NS1 Ag Strip; Bio-Rad, Hercules, CA) and an RT-PCR assay for the detection of viral RNA as described previously.

Analysis of the viral genome sequence. Dengue virus-1-positive RNAs extracted from the filtrates were used to determine the nucleotide (nt) sequence of the entire E gene.

Reverse transcription polymerase chain reaction was performed using the same conditions as described previously, using four primer sets specific for the DENV genome (Table 1). The amplicons were purified by the AMPure XP kit (Beckman Coulter, Brea, CA) and sequenced using the ABI PRISM Big-Dye Terminator Cycle Sequencing Kit Ver. 1.1 (Applied Biosystems) and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

For the analysis of whole coding sequences, viral cDNAs were synthesized using the ThermoScript RT-PCR System for First-Strand cDNA Synthesis (Thermo Fisher Scientific, Waltham, MA) from RNA extracted from the supernatant of the Vero cells. The cDNAs were amplified by a PCR using PrimeSTAR Max DNA polymerase (Takara Bio Inc.). Primer sets for cDNA amplification were as follows: DENV1_F1, 5'-CGG AAG CTT GCT TAA CGT AG-3'; DENV1_R2, 5'-GCC CTA CAG CAA ACA TTG GT-3'; DENV1_F3.2, 5'-GCT GAT GAC TGG AAC ACT GG-3'; DENV1_R4.2, 5'-CCG TTG TGT TTT CGA TTG TG-3'; DENV1_F5.2, 5'-TTC AGC CTG GAC CCT TTA TG-3'; and DENV1_R6.2, 5'-CAG CCT CCC AGG TTT TTA CA-3'. The adaptor-ligated cDNA libraries were prepared using an Ion Plus Fragment Library Kit (Thermo Fisher Scientific) and analyzed using the Ion PGM system (Thermo Fisher Scientific). The sequencing data were assembled using CLC Genomic Workbench Software (QIAGEN).

Phylogenetic analysis of DENV. The molecular phylogenetic analysis was performed based on the nt sequences of the viral E gene and whole coding sequences. Multiple alignment of the sequences from selected DENV strains/isolates was carried out using the Clustal W program.²⁴ The phylogenetic analysis was carried out on the basis of a maximum likelihood method²⁵ using MEGA ver. 7.²⁶ The statistical significance of the resulting tree was evaluated using a bootstrap test with 1,000 replications. GenBank accession numbers for sequences used in the phylogenetic analysis are shown in Table 2.

RESULTS

A total of 2,298 *Ae. albopictus* females and 956 males were collected at six locations in Tokyo Metropolis from August 28 to October 23, 2014. The *Ae. albopictus* collected were divided into 124 female pools and 35 male pools and examined for DENV infection. Forty-nine female pools yielded positive RT-PCR results for DENV-1, and viable viruses were successfully recovered from 16 of 29 RT-PCR-positive pools (Table 3). Dengue virus-positive *Ae. albopictus* were found in five locations in Tokyo Metropolis: 14 pools from YP, seven

TABLE 1
Primers used for reverse transcription polymerase chain reactions and sequence analysis

Primer name	Position*	Polarity	Sequence (5'-3')
D1-E-1FW	846–870	Forward	TAGGAACATCCATCACCAGAAAGG
D1-E-1RV	1,272–1,296	Reverse	TTTCCTTCCAGTTTGTACACACT
D1-E-2FW	1,194–1,218	Forward	GAACGTTTGTGGACAGAGGCTGGGG
D1-E-2RV	1,634–1,658	Reverse	CCTGCTTCTTCGCATGAGCTGTCTT
D1-E-3FW	1,528–1,552	Forward	ATGGCTAGTCCACAAACATGGTTT
D1-E-3RV	2,066–2,090	Reverse	TGAACCAGCTTAGTTTCAAAGCTTT
D1-E-4FW	1,986–2,101	Forward	TCACTGACAAAGAAAACCAAGTCAA
D1-E-4RV	2,480–2,504	Reverse	CCTGGAATTGTATTGCTCTGTCCA

NIID = National Institute of Infectious Diseases.

* Region where each primer is hybridized on the genome sequence of a dengue virus strain D1/Hu/Saitama/NIID/100/2014 (GenBank accession no. LC011945).

TABLE 2
The strains of DENV used for the phylogenetic analysis

Strain	Year	Location	Source	Genotype	GenBank accession no.	
MJ03/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335820*, LC335875
MJ06/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335876
MJ07/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335877
MJ09/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335878
MJ11/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335821*
NYC02/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335822*, LC335880
SCP17/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335823*, LC335881
SCP24/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335824*
YP02/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335825*, LC335871
YP04/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335872
YP10/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335873
YP11/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335874
YP18/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335879
YP19/Mosq/Tokyo/2014	2014	Japan	Tokyo	<i>Ae. albopictus</i>	I	LC335826*
D1/Hu/Chiba/NIID153/2014	2014	Japan	Chiba	Serum of patient	I	LC011948
D1/Hu/Hyogo/NIID188/2014	2014	Japan	Hyogo	Serum of patient	I	LC016760
D1/Hu/Saitama/NIID100/2014	2014	Japan	Saitama	Serum of patient	I	LC011945
D1/Hu/Shizuoka/NIID181/2014	2014	Japan	Shizuoka	Serum of patient	I	LC011949
D1/Hu/Tokyo/NIID111/2014	2014	Japan	Tokyo	Serum of patient	I	LC011947
D1/Hu/Tokyo/NIID149/2014	2014	Japan	Tokyo	Serum of patient	I	LC011946
SG(EH)D1/05723Y14	2014	Singapore	—	Patient	I	KJ806962
SG(EH)D1/04009Y13	2013	Singapore	—	Patient	I	KJ806953
CHN/GuangDong/ZhongShan/13/2014	2014	China	Guangdong	Human	I	KP055774
DENV1/CN/GZ35/2014	2014	China	Guangdong	Human	I	KP723476
China/GD-D13202(Guangzhou)	2013	China	Guangdong	Serum of patient	I	KJ545459
D1/IDN/Bali_068/2011	2011	Indonesia	Bali	Human	I	KM216688
Bali 2010a	2010	Indonesia	Bali	Patient	I	JN415489
D1/SG/05K2402DK1/2005	2005	Singapore	—	Blood of patient	I	EU081230
S393/04	2004	Singapore	—	—	I	EU069606
T3179/04	2004	Singapore	—	—	I	EU069619
S330/05	2005	Singapore	—	—	I	EU069595
D1/Singapore/0508aTw	2005	Singapore	—	Serum of patient	I	EU448399
T3317/04	2004	Singapore	—	—	I	EU069622
D1/Malaysia/36000/05	2005	Malaysia	—	Patient	I	FR666924
765101	1987	Taiwan	—	Human	I	AF425628
DENV-1/MX/BID-V3748/2008	2008	Mexico	Yucatan	Cell supernatant	V	GU131983
D1/IN/RGCB294/2007	2007	India	Kerala	Serum of patient	V	JN903578
SG(EH)DED06807	2007	Singapore	—	Serum of patient	V	GQ357690
SG(EH)DED65008	2008	Singapore	—	Serum of patient	V	GQ357692
16007	1964	Thailand	—	Serum of patient	II	AF180817
2543-63	1963	Thailand	—	Human	II	AF425629
TH-SMAN	1958	Thailand	—	Patient	II	D10513
P72-1244	1972	Malaysia	—	Monkey	III	AF425622
02-07-1HuNIID	2002	Indonesia	—	Serum of patient	IV	AB111073
01-37-1HuNIID	2001	Samoa	—	Serum of patient	IV	AB111068
PF08/070308-138	2008	French Polynesia	—	Serum of patient	IV	JQ915073
D1/Tonga/0803aTw	2008	Tonga	—	Serum of patient	IV	JF967797
Fiji 2012a	2012	Fiji	—	Human	IV	JX298567
Niue 2012b	2012	Niue	—	Human	IV	JX298573

DENV = dengue virus; MJ = Meiji Jingu; NIID = National Institute of Infectious Diseases; NYC = National Olympics Memorial Youth Center; YP = Yoyogi Park; SCP = Shinjuku Central Park. The contents denoted in bold indicate DENV isolates from the present study and the accession numbers with or without asterisk show viral envelope sequences only or complete coding sequences, respectively.

from SCP, six from MJ, two from NYC, and 20 from SGNG. During the period of our survey, DENV RNA-positive *Ae. albopictus* were first detected on August 29 in YP and last on September 26 at SGNG, although DENV RNA was not detected from all male *Ae. albopictus* (Supplemental Table 1).

The minimum infection rate (MIR) of DENV among the tested *Ae. albopictus* was defined as follows: number of DENV-positive pools/number of mosquitoes tested $\times$ 1,000. The MIR ranged from 11.2 to 66.7 and varied depending on the collection location and date (Table 3). The highest MIR (66.7) was obtained at YP on August 29, although only 15 specimens in one pool were examined. At SGNG, DENV-positive *Ae. albopictus* were found during September (from September 12 to 26). On the other hand, all male pools yielded negative

RT-PCR results for any DENV-1 (Supplemental Table 1). On April 20, 2015, 135 and one larvae were collected at SCP and SGNG, respectively; herein, 66 females and 69 males from SCP and one female from SGNG were grouped into six, five, and one pools, respectively. No DENV RNA was detected on RT-PCR (Table 3 and Supplemental Table 1).

We determined the nt sequences of entire E regions of 29 DENVs from mosquito pools obtained from YP, SCP, MJ, and NYC. The E gene sequences (1,500 nt) from 26 mosquito pools were 100% identical to those from DENV strain D1/Hu/Saitama/NIID100/2014, which is a DENV-1 isolate obtained from the patient diagnosed with the first case of autochthonous dengue in 2014 in Japan (GenBank accession no. LC002828). On the other hand, single nt substitutions in the

TABLE 3
Result of detection and isolation of DENV from female *Ae. albopictus* mosquitoes collected in Tokyo Metropolis in 2014

Mosquito collection sites	Collection date	No. of mosquitoes tested	No. of pools tested	No. of DENV-positive pools	
				Virus gene detected (MIR*)	Virus isolated
Yoyogi Park	August 28, 2014	36	2	0	0
	August 29, 2014	15	1	1 (66.7)	NT
	September 4, 2014	412	21	13 (31.6)	9
Shinjuku Central Park	September 5, 2014	422	21	6 (14.2)	1
	September 8, 2014	32	2	0	0
	September 19, 2014	89	4	1 (11.2)	1
	April 20, 2015†	66	6	0	0
Meiji Jingu	September 5, 2014	241	12	6 (24.9)	4
	September 8, 2014	11	1	0	0
National Olympics Memorial Youth Center	August 29, 2014	46	2	1 (21.7)	NT
	September 4, 2014	78	4	1 (12.8)	1
Sotobori Park	September 9, 2014	71	4	0	0
Shinjuku Gyoen National Garden	September 12, 2014	296	15	10 (33.8)	NT
	September 19, 2014	424	15	9 (21.2)	NT
	September 26, 2014	37	7	1 (27.0)	NT
	October 3, 2014	7	2	0	NT
	October 8, 2014	14	4	0	0
	April 20, 2015†	1	1	0	0
Total		2,298	124	49	16

DENV = dengue virus; MIR = minimum infection rate; NT = not tested.

* MIR is defined as (number of DENV-positive pools/number of mosquitoes tested) × 1,000.

† Larvae collected were maintained in the laboratory and emerged adults were tested.

E gene were observed in virus sequences detected in three mosquito pools, when compared with the viral sequences obtained from patients in the outbreak. A single nt substitution was observed at position 446 (A–G) in the E gene of the virus detected from a mosquito pool from SCP on September 19 (strain SCP24/Mosq/Tokyo/2014). This substitution was a non-synonymous substitution (from H to R at the position 149 in the aa sequence of the E gene). Moreover, a single nt polymorphism (C or T) was observed at the position 467 in a mosquito pool from YP on September 4 (strain YP19/Mosq/Tokyo/2014) and at the position 518 in a mosquito pool from MJ on September 5 (strain MJ11/Mosq/Tokyo/2014).

Molecular phylogenetic analyses of 1,485 nt sequences of the E genes were carried out to examine the phylogenetic relationships between the previously reported 35 DENV strains from the GenBank database and the mosquito-derived DENV sequences in this study (Table 2). All DENV sequences from *Ae. albopictus* in this study formed a clade with DENV-1 isolates from patients in this outbreak in Japan in 2014, within genotype I strains of DENV-1 (Figure 2). Overall, the epidemic DENV-1 strains isolated in this outbreak in Japan in 2014 were closely related to recent strains from Indonesia (strain D1/IDN/Bali_068/2011, GenBank accession no. KM216688), Singapore [SG(EH)D1/04009Y13, KJ806953 and SG(EH)D1/05723Y14, KJ806962], and China (CHN/GuangDong/ZhongShan/13/2014, KP055774 and CN/GZ35/2014, KP723476), indicating more than 99% in nt sequence identities (Figure 2).

The 11 DENVs isolated from *Ae. albopictus* were subjected to next-generation sequencing. The phylogenetic analysis based on the whole coding sequences (10,179 nt) of DENV-1 isolates was conducted using 11 sequences derived from mosquitoes and six from patients in Japan in 2014 (Figure 3). All DENV sequences belonged to only one clade indicating 100% in aa sequence identities, except for the Shizuoka strain (D1/Hu/Shizuoka/NIID181/2014), indicating 99% in aa sequence but 98% in nt sequence identities. Our analysis thus

revealed that two independent autochthonous epidemics occurred in Japan in 2014 caused by DENV-1 strains and only one genotype (DENV-1) was circulated and spread during the dengue outbreak period in Tokyo Metropolis in 2014. Furthermore, the whole coding sequences of 10,179 nt of the mosquito isolates were compared with the sequence of the Tokyo strain (D1/Hu/Tokyo/NIID111/2014) (Table 4). Although all the DENVs isolated from *Ae. albopictus* belonged to only one clade, there were slight differences in the nt and aa sequences. The nt changes were found in four strains (MJ-4, YP-2, YP-10, and YP-18), and nt changes accompanied by aa changes were detected in MJ-4 and YP-10.

DISCUSSION

In this study, we detected and isolated multiple DENVs from *Ae. albopictus* collected in the six suspected locations of DENV infection during the outbreak in Tokyo, Japan, in 2014. The DENVs from *Ae. albopictus* belonged to the genotype I in DENV-1, and its sequences demonstrated > 99% homology with the sequences of the DENVs isolated from patients in the outbreak (strains D1/Hu/Saitama/NIID100/2014, D1/Hu/Tokyo/NIID111/2014, D1/Hu/Tokyo/NIID149/2014, D1/Hu/Chiba/NIID153/2014, and D1/Hu/Hyogo/NIID188/2014). We therefore confirmed that *Ae. albopictus* was a major vector of DENV in that outbreak. Moreover, the DENVs observed in this outbreak were closely related to the DENV isolates from China, Singapore, and Indonesia identified in 2013 and 2014. We therefore concluded that a single strain of DENV-1 circulated and spread in Tokyo Metropolis in 2014. Although only the Shizuoka strain (D1/Hu/Shizuoka/NIID181/2014) belonged to a different clade from that of the Japanese DENV strains in the dendrogram, the strain was similar to those isolated from Taiwan and Thailand in 2009.^{27,28} Thus, the 2014 dengue outbreak in Japan was caused by two different DENV strains introduced to Japan via two separate foreign areas.

FIGURE 2. Phylogenetic analysis of dengue virus (DENV)-1 sequences derived from *Ae. albopictus* collected in Tokyo Metropolis, Japan, in 2014, among previously described strain/isolates of DENV-1 based on the E gene sequences. Phylogenetic analysis was performed by maximum likelihood method based on the E gene of selected DENV-1 strains including genotype I–V. The percentages of 1,000 bootstrap replication were indicated at the nodes. The DENV-1 strains derived from *Ae. albopictus* in this study are indicated with closed circles. Moreover, six from the patients of autochthonous dengue outbreak in Japan in 2014 are indicated with open circles. GenBank accession numbers for sequences used in the phylogenetic analysis are shown in Table 2.

Japan shares one of the busiest Asian flight routes with China and other Southeast Asian countries. A previous study suggested that air passenger transportation might have played a role in DENV introduction in Japan.²⁹ Another study showed that air transportation contributes to the expansion and influence of the dynamics of DENV circulation.³⁰ Another concern is the reestablishment of *Ae. aegypti* in Japan. Recently, *Ae. aegypti* has often been discovered at the Narita International Airport³¹ and in several other international airports since 2012. Cases in which adult *Ae. aegypti* were captured inside the airplane of an international flight have been reported, and the number of such reports has been increasing annually.

Our results also showed a high MIR of the DENV, ranging from 11.2 to 66.7 among female *Ae. albopictus* collected from several parks in Tokyo Metropolis during the dengue outbreak. Generally, *Ae. aegypti* and *Ae. albopictus* are

distributed in endemic countries of DENV; however, *Ae. aegypti* plays a more important role than *Ae. albopictus* as a DENV vector in the region.³² Our previous study indicated that several Japanese *Ae. albopictus* colonies were as susceptible to DENV-1 and DENV-2 infection as the *Ae. aegypti* and *Ae. albopictus* obtained from dengue-endemic countries.³³ These results indicate that *Ae. albopictus* can be a vector for DENV in regions where no *Ae. aegypti* are distributed.

Although the number of reports available is limited, a few dengue epidemics probably caused by *Ae. albopictus* have been reported: 122 patients in 2002 and four in 2011 on the island of Maui, Hawaii, United States³⁴; two patients in Nice in 2010³⁵ and one in Aix-en-Provence in 2013³⁶ in France; 68 and 54 dengue cases in Taipei city and New Taipei city, respectively, in Taiwan in 2014.³⁷ In Taiwan, *Ae. aegypti* does not inhabit northern cities and only *Ae. albopictus* is distributed there; however, *Ae. aegypti* exists in the southern areas.

FIGURE 3. Phylogenetic analysis of dengue virus (DENV)-1 sequences derived from *Ae. albopictus* and patients isolated during dengue outbreak period in Japan in 2014, among previously described strain/isolates of DENV-1 based on whole genome sequences. Phylogenetic analysis was performed by maximum likelihood method based on whole genome of selected genotype 1 of DENV-1 strains. The percentages of 1,000 bootstrap replication were indicated at the nodes. The 11 DENV-1 strains derived from *Ae. albopictus* and the six from patients in Japan in 2014 are indicated with closed circles and open circles, respectively. GenBank accession numbers for sequences used in the phylogenetic analysis are in Table 2.

Therefore, the DENV could be easily introduced from the south to the north. In the Hawaii islands, *Ae. albopictus* was identified across all islands surveyed, but *Ae. aegypti* was found only on the Hawaii island during the outbreak.³⁸ *Aedes aegypti* was distributed in the southern part of France before 1960, but it cannot be detected currently.^{39,40} However, *Ae. albopictus* invaded the Mediterranean area, and it was confirmed that *Ae. albopictus* was established in the southern part of France when dengue cases were reported.^{35,36} Thus, *Ae. albopictus* probably served as a vector of DENV in the autochthonous dengue cases in Hawaii in 2002 and 2011, and in France in 2010 and 2013. However, in these cases, there is little entomological information available. In comparison with previous dengue cases that involved *Ae. albopictus*, the present study is the first report to confirm the vector potential of *Ae. albopictus* for DENV in natural settings.

In Tokyo Metropolis in 2014, DENV-positive *Ae. albopictus* were detected from five locations (YP, SCP, MJ, NYC, and SGNG) within a 1.5-km radius. In these locations, YP, MJ, and NYC are adjacent to each other, forming Yoyogi area. In a recent survey, 128 of 160 patients with an autochthonous DENV infection reported being bitten by mosquitoes at the YP

and the surrounding areas.⁴¹ Therefore, the YP was assumed to be a central location for DENV transmission in this outbreak. Our previous study suggested that infections from an *Ae. albopictus* bite could disperse through Yoyogi area over several days.¹³ On the other hand, there are busy railways and traffic loads between SCP, SGNG, and Yoyogi area, although SCP and SGNG are about 1 km and 600 m away from Yoyogi area, respectively. Therefore, it is unlikely that *Ae. albopictus* migrates directly from Yoyogi area to SCP and SGNG. Thus, the DENV may have spread to the separate locations from Yoyogi area via patients because asymptomatic dengue patients could also be infectious.⁴² In fact, a great number of domestic and overseas travelers visit Yoyogi area, and most people usually jog around or commute through this area. A study showed that 5% of 207 employees and groups who may have visited or resided in this area tested positive for dengue antibodies on epidemiological investigations consisting of active surveillance.⁴³ In addition, this study also showed that the number of positive cases for dengue antibodies increased significantly as the cumulative staying time was longer. The possibility that the presence of long-term residents commonly found in YP and SCP had affected DENV expansion in Yoyogi area is undeniable.

Between September 4 and October 30, the YP was partially closed, and five rounds of vector control using an adulticide were conducted. An area of SCP adjacent to the Tokyo Metropolitan Government office had restricted access. Although no one reported being bitten by mosquitoes at SGNG, the park was completely closed just after the closure of YP until the beginning of October, and adulticides were applied several times. In a laboratory in NIID, the average longevity of female *Ae. albopictus* collected at YP on August 27 was 32 days and the longest was 54 days (K. Ogawa et al., unpublished data). If they harbored DENV, it is possible that DENV-positive mosquitoes were present in the park until the middle of October. To our knowledge, this was the first time a public park was closed to conduct vector control measures for the prevention of the spread of mosquitoes harboring DENV to other areas by confinement.

The vertical transmission of DENV by *Ae. albopictus* has been observed both experimentally and naturally. After oral inoculation in a laboratory, vertical transmission of DENV-1 occurred at a rate of approximately 11% in both of the Brazil and Florida strains of *Ae. albopictus*.^{15,16} However, in natural settings, the incidence of vertical transmission of DENVs via *Ae. albopictus* ranges from 1% to 4%.⁴⁴ Therefore, vertical transmission of DENVs in *Ae. albopictus* generally occurs in nature, but it is unlikely that vertical transmission was important to affect the ecological dynamics of DENVs.⁴⁴ In our study, DENV was not detected in 956 males collected during

TABLE 4
Differences in whole coding sequences of DENVs isolated from mosquitoes and D1/Hu/Tokyo/NIID111/2014

DENV strain	Nucleotide substitution at indicated position (viral protein)				
	376 (preM-12)	2328 (NS1-1)	2523 (NS1-66)	4047 (NS2B-4)	7858 (NS5-127)
D1/Hu/Tokyo/NIID111/2014*	A	T	C	C	C
MJ-7	G (M126V)	T	C	T	C
YP-2	A	C	C	C	C
YP-10	A	T	C	C	T (H2620Y)
YP-18	A	T	T	C	C

DENV = dengue virus; MJ = Meiji Jingu; NIID = National Institute of Infectious Diseases; NS = nonstructural protein; YP = Yoyogi Park. The nucleotide denoted in bold indicate mutations compared with the D1/Hu/Tokyo/NIID111/2014 strain.

* Data for the D1/Hu/Tokyo/NIID111/2014 strain has been submitted to the GenBank database (accession no. LC011946).

the dengue outbreak period in 2014. In addition, the virus was not detected in the larvae (67 females and 69 males after emergence) collected in April in the following year. Taken together, these results indicate that the possibility of vertical transmission or overwintering is extremely low, although the number of specimens examined was small. This is also supported by the fact that the DENV isolated from a German tourist suspected infection in Japan in 2013 was DENV-2,⁷ but no DENV-2 was isolated in the 2014 autochthonous dengue cases.

Recent studies showed that one source of the genetic diversity in the *Flavivirus* genus is the natural alternation between invertebrate and vertebrate hosts, which imposes different selective pressures on the viral population,^{45–47} whereas during the Chikungunya virus (CHIKV; genus *Alphavirus*, family *Togaviridae*) outbreak in Réunion island in 2005–2006, the virus evolved to be highly susceptible to *Ae. albopictus* with only one aa substitution (A226V mutation in viral E1 gene) occurring within 5 months in a natural setting.^{48,49} Furthermore, the CHIKV that caused the autochthonous outbreak in Italy in 2007 had this mutation, and the virus was transmitted via the *Ae. albopictus* distributed there.⁵⁰ An outbreak of CHIKV also occurred in Cameroon in 2006 and Gabon in 2007, where *Ae. albopictus* has displaced *Ae. aegypti*.^{51,52} Interestingly, despite the virus isolates from Cameroon and Gabon being classified into a different clade from that of the Réunion and Italian isolates (the Central Africa clade), the A226V mutation was observed in both isolates.⁵³ This suggested that an independent adaptive mutation occurred under similar situations of transmission by *Ae. albopictus*. These facts indicate that the mosquito-borne virus easily adapts to new vector mosquito species depending on the situation, and it results in the expansion of an outbreak. In our results, several nt and aa substitutions that were not detected in the DENVs isolated from patients were observed in the DENVs isolated from *Ae. albopictus*. These substitutions might be important for the adaptation to *Ae. albopictus*. Therefore, further study may give us new insight into DENV microevolution and the factors influence their fitness or the susceptibility of vector mosquitoes.

In conclusion, we reported that the MIR among *Ae. albopictus* populations with DENV was high during the dengue outbreak in Japan in 2014, and a single strain of DENV-1 circulated and spread in Tokyo Metropolis during the outbreak period. Our study also clarified that *Ae. albopictus* served as a vector of DENV in the outbreak. This is the first report to confirm the vector potential of *Ae. albopictus* for DENV in natural settings. Tokyo, an international metropolis, in 2014 was likely to have satisfied all requirements, the pathogen, the vector, and the host, for autochthonous dengue infection and its spread even though it is located in a temperate region. Fortunately, no cases of autochthonous dengue infection have occurred in Japan since 2014. However, as such, the 2014 dengue outbreak in Japan is not a rare case, and the frequency of such epidemics can increase in the future.

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