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Identification and epidemiological study of an uncultured flavivirus from ticks using viral metagenomics and pseudoinfectious viral particles

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During their blood-feeding process, ticks are known to transmit various viruses to vertebrates, including humans. Recent viral metagenomic analyses using next-generation sequencing (NGS) have revealed that blood-feeding arthropods like ticks harbor a large diversity of viruses. However, many of these viruses have not been isolated or cultured, and their basic characteristics remain unknown. This study aimed to present the identification of a difficult-to-culture virus in ticks using NGS and to understand its epidemic dynamics using molecular biology techniques. During routine tick-borne virus surveillance in Japan, an unknown flaviviral sequence was detected via virome analysis of host-questing ticks. Similar viral sequences have been detected in the sera of sika deer and wild boars in Japan, and this virus was tentatively named the Saruyama virus (SAYAV). Because SAYAV did not propagate in any cultured cells tested, single-round infectious virus particles (SRIP) were generated based on its structural protein gene sequence utilizing a yellow fever virus-based replicon system to understand its nationwide endemic status. Seroepidemiological studies using SRIP as antigens have demonstrated the presence of neutralizing antibodies against SAYAV in sika deer and wild boar captured at several locations in Japan, suggesting that SAYAV is endemic throughout Japan. Phylogenetic analyses have revealed that SAYAV forms a sister clade with the *Orthoflavivirus* genus, which includes important mosquito- and tick-borne pathogenic viruses. This shows that SAYAV evolved into a lineage independent of the known orthoflaviviruses. This study demonstrates a unique approach for understanding the epidemiology of uncultured viruses by combining viral metagenomics and pseudoinfectious viral particles.

flavivirus | tick-borne virus | Saruyama virus | uncultured virus | SRIP

Significance

Recent comprehensive viral genome analyses using next-generation sequencing (NGS) have revealed that ticks, the major arbovirus vectors, harbor a wide variety of viruses. However, most of these viruses are difficult to isolate and culture in vitro, and detailed molecular and epidemiological analyses have not yet been conducted. This study demonstrates that even a difficult-to-culture virus identified in ticks can be applied to elucidate for epidemic dynamics using a unique approach. A previously unknown uncultured flavivirus, Saruyama virus (SAYAV), was detected in Japanese ticks using NGS analysis. Subsequent seroepidemiological studies conducted using single-round infectious particles of SAYAV as antigens demonstrated the presence of neutralizing antibodies against SAYAV in wild animals and viral endemicity in Japan.

Recently, a significant number of infectious diseases affecting humans have been attributed to zoonotic viruses (1, 2). Typically, these viruses are transmitted among and maintained within wild animal populations, with human population epidemics occurring through spillover from natural hosts (1). This spillover process involves various mechanisms, one key route being transmission via blood-feeding arthropods (3).

To proactively identify emerging viruses transmitted by ticks and mosquitoes from wildlife, extensive viral genomic analyses (viral metagenomics) are being conducted globally. This involves using next-generation sequencing (NGS) on these arthropod vectors (4, 5). The majority of the viruses harbored by blood-feeding arthropods are challenging to isolate and culture and remain largely uncharacterized (5, 6). A direct and effective method for assessing the infectious potential and prevalence of these viruses in humans and other vertebrates is to detect the viral genes in host animals directly. However, many vertebrate hosts experience transient viremia (7–12), meaning that viral genes are detectable only for a limited period, which constrains the insights gleaned from nucleic acid-based methods.

Among the plethora of viruses transmitted by ticks and mosquitoes, those belonging to the genus *Orthoflavivirus*, within the family *Flaviviridae*, are notably impactful on human health. For instance, dengue fever, caused by the dengue virus serotypes 1 to 4, stands as the most common mosquito-borne viral infection globally (13). Additionally, tick-borne encephalitis virus (TBEV), transmitted by various tick species, is a prominent tick-borne viral infection. It is endemic across extensive regions of Eurasia, with numerous cases reported annually (14).

Viruses within the *Orthoflavivirus* genus exhibit consistent genome structures, irrespective of their transmission by ticks or mosquitoes. The flaviviral genome is composed of a non-segmented RNA strand, roughly 11,000 nucleotides in length, featuring a large single open reading frame (ORF) (15). This ORF encodes three structural proteins (C, prM, and E)

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alongside eight nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5) (15). Flaviviral polyproteins undergo cleavage into mature proteins, facilitated by proteases originating from either the host cell or the virus itself (15). To date, the International Commission on the Classification of Viruses (ICTV) recognizes 53 viral species, encompassing 75 viruses within the *Orthoflavivirus* genus (16). Although viruses in this genus share a common genome structure, they are phylogenetically categorized into three distinct groups, aligning with their invertebrate vectors: mosquito-borne viruses, tick-borne viruses, and viruses with no known vector (17). Since the early 2000s, "classical insect-specific flaviviruses" (cISFs) (18) have been identified. These viruses, sharing genetic similarities with known *Orthoflavivirus* genus viruses, exclusively infect insects and have been detected in various mosquitoes and other dipteran species since 2000 (19, 20). Moreover, recent advancements in high-throughput sequencing technologies have uncovered several flavivirus-like viruses across a broad spectrum of organisms, further clarifying their evolutionary lineage (21–23). With the identification of this diverse array of flaviviruses, one prior study has categorized flaviviruses within the *Orthoflavivirus* genus as per the traditional ICTV classification as "*Orthoflavivirus* (*sensu stricto*)" (23), setting them apart from other flaviviruses like cISFs. Additionally, all recognized flaviviruses, including *Orthoflavivirus* (*s.s.*) and cISFs, are collectively referred to as "*Orthoflavivirus* (*sensu lato*)" (23).

In a pseudotyped virus, the outer envelope originates from a different virus than the source of the genome or genome replication machinery (24). These viruses can be safely managed in Biosafety Level 2 laboratories and are more manageable experimentally than wild-type viruses (25). Consequently, pseudotyped viruses are favored for assessing cellular tropism, receptor recognition, viral inhibition, and antibody responses, as well as for vaccine development (25). Single-round infectious particles (SRIP) are a type of pseudotyped virus wherein the virion interior contains a flavivirus subgenomic replicon devoid of the prM-E gene, while the virion surface features heterologous flaviviral prM and E proteins (26). Upon entry into a target cell, the SRIP-infected cells do not release progeny virions extracellularly due to the absence of coding regions for prM and E proteins in the subgenomic replicon, thereby restricting the infection to a single round. Our prior research has successfully established SRIP production systems utilizing subgenomic replicons from Japanese encephalitis virus (JEV), dengue virus 1, and yellow fever virus (YFV) (27–29). Moreover, we have developed a serum neutralization test (SNT) utilizing SRIP as an antigen, proving its utility in seroepidemiological studies as a viable alternative to the conventional plaque reduction neutralization test, as evidenced by studies involving human sera (29).

This study aims to present the identification of a hard-to-culture virus in ticks through NGS and to elucidate its epidemic dynamics utilizing SRIP. We hereby report the identification of a previously unknown uncultured virus, the Saruyama virus (SAYAV), in Japanese ticks via NGS and its infection patterns and prevalence in wild mammals as determined by the SNT with SRIP.

Results

Detection of Unique Flavivirus-Like Sequences in Field-Collected Ticks and Analysis of the Virus-Like Sequence. Ticks were collected in May 2018 from the Ishikawa Prefecture, Japan, and 69 ticks belonging to three species (*Haemaphysalis flava*, *Haemaphysalis longicornis*, and *Ixodes ovatus*) were collected. The samples were divided into 12 pools according to species, sex, developmental stage, and collection site (*SI Appendix*, Table S1). A mixture of filtered tick pool homogenates was processed for viral metagenomic analyses. Subsequent NGS using the MiniSeq system yielded 985,732 reads, and de novo assembly with default settings in CLC Genomics Workbench version 21 generated 60 contigs (>500 nucleotides in length). To identify virus-derived sequences from these contigs, a search was performed using BLAST. BLASTN search identified two virus-derived contigs; one contig showed 94% nucleotide sequence identity to Kabuto mountain virus, a tick-borne uukuvirus distributed in Japan (30), and another contig was 99% nucleotide sequence identical to Okutama tick virus [M segment-deficient phenuivirus detected in Japan (31)]. This suggested that these viruses were present in the tick pool used for this analysis. In contrast, in addition to these viruses, BLASTX search identified seven contigs that shared 54 to 75% amino acid sequence identity with the corresponding genomic regions of Muplungu flavivirus (MPFV) and Ngoye virus (NGOV), both of which are flaviviruses found in African ticks (32, 33) (*SI Appendix*, Fig. S1 and Table S2). These results suggested that flavivirus-like viruses were also presented in the pooled tick samples used for the analysis.

To identify samples positive for flavivirus-like sequences from the pooled tick samples, specific primers were designed from the sequences obtained using NGS analysis (*SI Appendix*, Table S3) and RT-PCR-based screening was performed. One pool (pool number 18HKR14, consisting of eight *H. flava* males; *SI Appendix*, Table S1) was identified as positive for flavivirus-like sequences. To isolate the virus, we inoculated tick samples positive for flavivirus-like sequences into cultured cells (BHK-21, derived from baby hamster kidney). BHK-21 cells have been instrumental in our previous research for isolating and cultivating various tick-borne viruses from tick samples (31, 34). Hence, we initially opted for this cell line for virus isolation. Nevertheless, flavivirus-like sequences were not detectable in the extracted RNA from the culture supernatant after two blind passages. Due to the unavailability of *Haemaphysalis*-derived tick cells, we pivoted to isolating the virus using ISE6 cells (sourced from the *Ixodes scapularis* tick) and C6/36 cells (originating from the *Aedes albopictus* mosquito). Both cell types are documented to facilitate the proliferation of various orthoflaviviruses (35). Despite inoculating tick samples into these cells, no viral propagation was discerned. Furthermore, subsequent experiments revealed that the SRIP of SAYAV could enter Vero cells. Hence, the same tick sample, which was positive for flavivirus-like sequences, was inoculated into Vero cells, and the viral RNA levels in both culture supernatants and cells were meticulously monitored over a period. Yet, there was no observed escalation in viral RNA levels in either of the samples (*SI Appendix*, Fig. S2). This indicated that flavivirus-like viruses were not competent enough to infect and propagate in these cells.

Since culturing was unsuccessful, further sequencing of the viral genome was performed using total RNA extracted from the virus-positive tick homogenate. The sequence gaps between each contig were filled by RT-PCR using primer sets (*SI Appendix*, Fig. S1 and Table S3), followed by direct sequencing using the Sanger method. Additionally, efforts were made to complete the 5′ and 3′ ends of the cDNA using rapid amplification of cDNA ends (RACE) based on methodologies from prior studies (36, 37). Consequently, a contiguous sequence of 10,896 nucleotides was assembled, which included a 3′ terminal sequence with a large ORF encoding 3,408 amino acids (Fig. 1*A*). Thus, the seven flavivirus-like contigs detected using NGS (*SI Appendix*, Fig. S1 and Table S2) were derived from a single sequence.

Recent studies have reported the endogenous flavivirus-like elements (EVEs) into the genomic DNA of various organisms (22, 38).

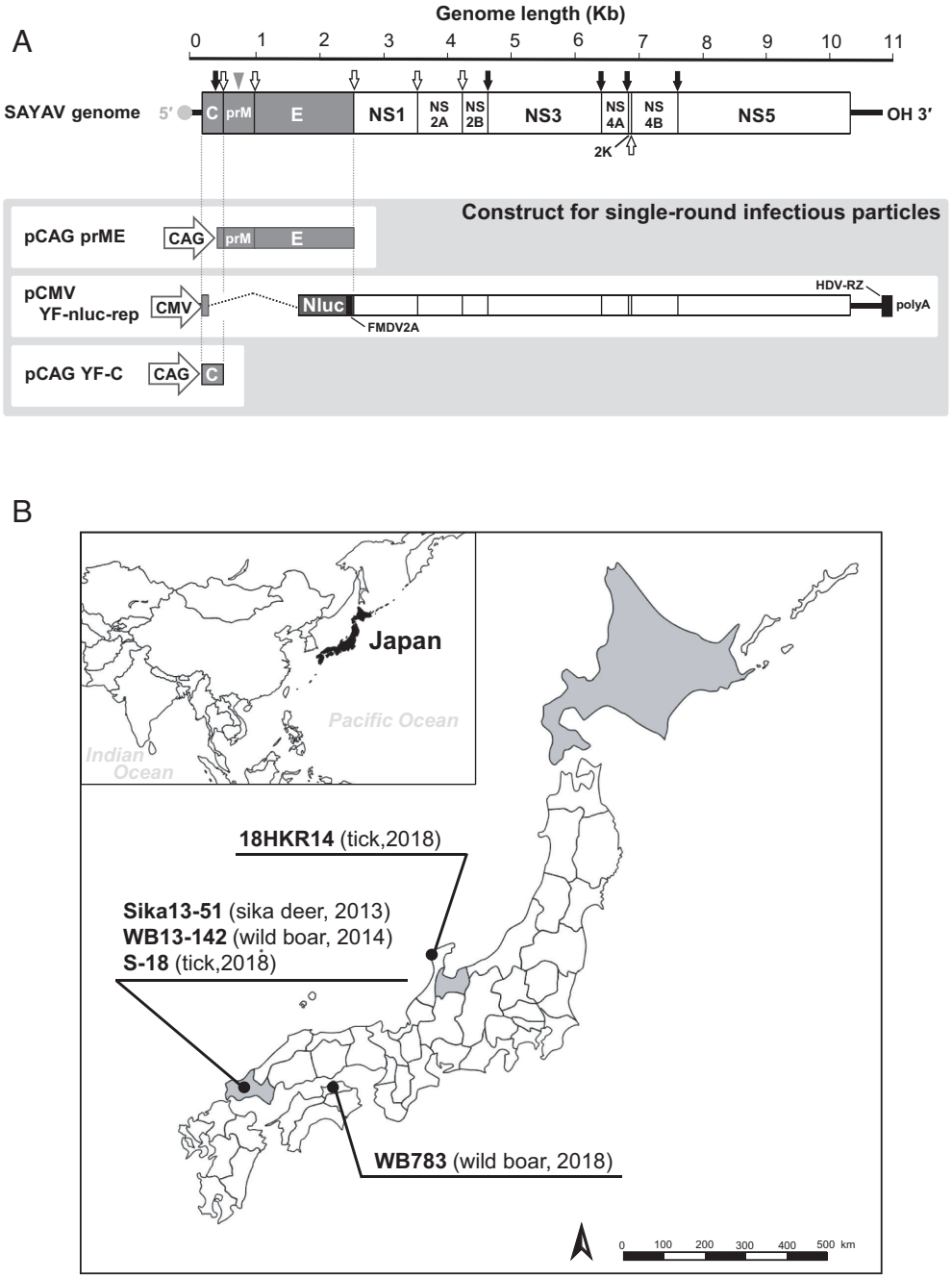

Fig. 1. Genome organization and distribution of the SAYAV. (*A*) Schematic illustration of the SAYAV genome organization. Black and white arrows indicate cleavage sites within the viral polyprotein by NS3 protease and signal peptidase, respectively. The gray triangle indicates the cleavage site by furin protease. The gray squares below show the structural protein expression plasmid (pCAG prM-E) encoding the prM-E gene of SAYAV, the replicon (pCMV YF-nluc-rep), and the YFV capsid (pCAG YF-C) used for SAYAV SRIP production in schematic illustrations. The cytomegalovirus promoter (CMV), nanoluciferase (Nluc) gene, foot-and-mouth disease virus 2A protein sequence (FMDV2A), hepatitis delta virus ribozyme (HDV-RZ), polyadenylation signal (poly A), and CAG promoter (CAG) are shown. The SRIP of SAYAV was generated by transfection of these plasmids into 293T cells. (*B*) Areas in which SAYAV RNA and neutralizing antibodies were detected. Areas where SAYAV RNA was detected are indicated by black circles, and the name of each viral strain, source of viral detection, and year of detection are indicated. Areas shown in gray are locations where wild animals (sika deer and wild boar) were captured and neutralizing antibodies against SAYAV were detected.

The virus-like sequence detected in this study was considered a potential EVEs because viral infection and propagation were not observed during virus isolation using a cell culture system. To investigate whether the virus-like sequence could be derived from EVEs in the tick genome, DNA extracted from the homogenate of a virus-like-sequence-positive tick sample was tested for the presence of the DNA form of the sequence using PCR. Analysis was performed using several viral-gene-specific primer sets (*SI Appendix*, Table S3); no amplified fragment amplicons were detected (*SI Appendix*, Fig. S3). Thus, flavivirus-like sequences were present in the RNA form and were not derived from the host tick genome.

To analyze the virus-like sequence, we deduced the genes and amino acid sequence encoded in the sequence. The polyprotein encoded by the sequence contained several protein domains that were conserved among flaviviruses and had the same genome organization as those of typical flaviviruses (*SI Appendix*, Table S4). We then predicted all cleavage sites of the SAYAV polyprotein and compared the amino acid sequences around these sites with those of other flaviviruses. The cleavage sites of viral serine proteases (VirC/AnchC, NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/2K, and NS4B/NS5) were located immediately after the two-amino acid motifs, RK, RR, and QR, which are well-conserved among

other flaviviruses (Fig. 1*A* and *SI Appendix*, Table S5). Overall, the amino acid residues around the cleavage site were more similar to those of MPFV and NGOV than to those of other tick-borne, mosquito-borne, and vector-unknown flaviviruses (*SI Appendix*, Table S5). Interestingly, the predicted 2K/NS4B cleavage site sequence was highly conserved among MPFV, NGOV, and SAYAV (*SI Appendix*, Table S5). Thus, the virus-like sequence obtained in this study had essentially the same genomic organization as viruses of the genus *Orthoflavivirus* (Fig. 1*A* and *SI Appendix*, Table S6).

Moreover, this virus-like sequence had the highest amino acid sequence identity of 48.0 to 78.2% with the proteins of MPFV and NGOV (*SI Appendix*, Table S7). However, other flaviviruses showed lower amino acid sequence identity with the virus-like sequence; for example, tick-borne flaviviruses showed 20.9 to 46.7% and 20.6 to 61.1% sequence identity for structural (C-E) and nonstructural (NS1 to NS5) proteins, respectively (*SI Appendix*, Table S7).

Investigation of SAYAV-Infected Hosts via Viral RNA Detection. To investigate whether this virus-like sequence is present in other ticks and other animals, we performed retrospective viral RNA detection using the total RNA extracted from ticks and wild mammal sera previously collected from different regions of Japan. Due to limited knowledge regarding the genetic diversity among the virus-like sequences, the RT-PCR-based assay was conducted using universal primers targeting the highly conserved region of the NS5 gene among flaviviruses.

A total of 174 pools (consisting of 3,690 individuals) including 10 species (*Amblyomma testudinarium*, *Dermacentor taiwanensis*, *I. ovatus*, *Ixodes turdus*, *H. flava*,*Haemaphysalis formosensis*, *Haemaphysalis hystricis*, *Haemaphysalis kitaokai*, *H. longicornis*, and *Haemaphysalis megaspinosa*) were screened for extracted RNA using RT-PCR. The virus-like sequence was detected in one tick pool (containing 20 *H. flava* nymphs), designated as strain S-18 (Fig. 1*B* and Table 1). Since the virus-like sequence prototype 18HKR14 was also detected in ticks of the same species, it may be specifically harbored by *H. flava* ticks.

To further investigate the presence of the sequence in wildlife, we performed the same virus-like sequence screening in sika deer and wild boars, the preferred host species of *H. flava* ticks, using total RNA extracted from both animals captured from different locations in Japan (*SI Appendix*, Fig. S4). The sequences were also detected in the sera from sika deer in the Yamaguchi Prefecture, and in two wild boars from the Yamaguchi and Kagawa prefectures (Fig. 1*B* and Table 1). The nucleotide sequences of the four different virus-like sequences detected in this study were compared with those of 18HKR14, indicating that the nucleotide and amino acid sequences were 98.0 to 100% and 100% identical, respectively (*SI Appendix*, Table S8). This high degree of similarity suggests that the virus-like sequence represents an infectious virus, as evidenced by its independent detection in ticks from geographically distinct locations and in multiple mammal species, including wild boars and sika deer. Consequently, this virus was provisionally named SAYAV, after the Point Saruyama collection site where the prototype strain, 18HKR14, was detected.

Production of SAYAV SRIP and Cross-Reactivity between SAYAV and Other Known Flaviviruses. In vertebrates, flavivirus-induced viremia is generally transient, making it difficult to determine the actual status of flavivirus prevalence using serum RNA analysis (8, 10–12). In contrast, antibodies produced by viral infections are more potent indicators of epidemic dynamics than is viral RNA, since antibodies exist in blood for prolonged durations. Although SNTs using isolated viral strains effectively detect antibodies specific to the virus of interest, SAYAV isolates have not been obtained. Therefore, we used the SRIP method established in our previous study (29). The SRIP of SAYAV was generated using a sequence of its structural protein genes (prM-E) with the YFV-based replicon system (Fig. 1*A*) and used as a viral antigen in its seroepidemiological study.

To serologically examine SAYAV infections in animals, we constructed SRIP based on SAYAV, JEV, Langat virus (LGTV), TBEV, and Powassan virus (POWV). JEV and Yamaguchi virus (YGV), a close relative of LGTV, are prevalent mainly in western Japan (39–41), and TBEV is distributed in Hokkaido, Japan (42), while POWV infects deer in North America. Polyclonal antibodies against prM-Es for these flaviviruses, apart from TBEV, and an additional antibody for MPFV were produced by immunizing rabbits with their expression plasmids. Subsequently, the SNT was performed using each SRIP and polyclonal antibody against prM-E. The SRIP of SAYAV reacted only with the anti-SAYAV antibody, and not with other polyclonal antibodies, including anti-MPFV antibodies (Table 2). Furthermore, the anti-SAYAV antibody did not react with SRIP other than those of SAYAV. Both, the anti-JEV antibody and the SRIP of JEV, showed strong specific reactions, whereas LGTV, TBEV, and POWV, which belong to the same tick-borne flavivirus clade,

Table 1. Summary of the SAYAV strains detected in this study

	Source					
	Species					
Strain name	Common name	Scientific name		Collection site	Collection date	Accession no.
Sika13-51	Sika deer	*Cervus nippon*	Serum of male (38 kg*)	Yamaguchi Pref., Japan	11 August, 2013	LC658948
WB13-142	Japanese wild boar	*Sus scrofa leucomystax*	Serum of female (26 kg*)	Yamaguchi Pref., Japan	11 January, 2014	LC658949
S-18	Tick	*H. flava*	Pooled 20 nymphs	Shunan City, Yamaguchi Pref., Japan	28 March, 2014	LC658947
18HKR14	Tick	*H. flava*	Pooled 8 males	Wajima City, Ishikawa Pref., Japan	6 May, 2018	LC657549
WB783	Japanese wild boar	*Sus scrofa leucomystax*	Serum of male (30 kg*)	Kagawa Pref., Japan	22 October, 2018	LC658950

*Body weight at time of capture.

Table 2. Cross-reaction among each flaviviral SRIPs

Polyclonal antibodies	Inhibition titers against SRIPs (IC_{75})				
	SAYAV	JEV	LGTV	TBEV	POWV
SAYAV-prME	1:670	<1:10	<1:10	<1:10	<1:10
MPFV-prME	<1:10	<1:10	<1:10	<1:10	<1:10
JEV-prME	<1:10	1:25,882	<1:10	1:17	<1:10
LGTV-prME	<1:10	<1:10	1:5,495	1:7,594	1:476
POWV-prME	<1:10	<1:10	1:3,732	1:2,981	1:14,825

The titer is shown as geometric mean of two independent experiments.
Homologous titers are underlined.

showed cross-reactivity (Table 2). An immunofluorescence assay (IFA) was also performed to compare cross-reactivity among flaviviruses; however, IFA showed higher cross-reactivity than that using the SNT (*SI Appendix*, Table S9).

Detection of Antibodies against SAYAV from Wild Animal Sera. Since SAYAV RNA was detected in ticks from the Ishikawa and Yamaguchi Prefectures, the SNT was performed using the sera from wild animals in the Toyama Prefecture, which is contiguous to the Ishikawa Prefecture, and the Yamaguchi Prefecture. To detect antibodies against SAYAV, SNTs against SAYAV SRIP were performed using 10-fold diluted serum samples. Neutralizing antibody titers were ascertained by the serum dilution (inhibitory concentration) that neutralized 75% of SRIP infections (IC75), a measure chosen due to its reported minimal variation, bias, and variance in titer measurement (43). Antibodies against SAYAV were detected in 14 of 15 wild boar samples in Toyama and 15 of 45 sika deer samples in Yamaguchi (Tables 3 and 4 and *SI Appendix*, Fig. S5). To confirm whether these positive serum samples were specific for SAYAV, SNTs were performed using SRIP for SAYAV, JEV, LGTV, TBEV, and POWV (Tables 3 and 4). Seven wild boars and three sika deer had SAYAV-specific antibodies because the SNT titers against SAYAV SRIP in these individuals were significantly higher (more than fourfold) than the titers against other SRIP (Table 3). Notably, serum samples T-1396, T-1430, and T-1431 from wild boars and 20 to 34 from sika deer reacted only with the SRIP of SAYAV (Tables 3 and 4). In contrast, many serum samples reacted with several other SRIP. In particular, the sera from many sika deer in Yamaguchi reacted strongly to the SRIP of JEV, which is widespread in this prefecture (Table 4). To further explore the potential direct link between these wild animals, suspected to be infected with SAYAV, and the vector ticks, *H. flava* ticks were collected from locations in Toyama Prefecture where wild boars positive for SAYAV antibodies were found. Despite testing 111 pooled samples comprising 701 *H. flava* ticks, no positive detections of SAYAV RNA were made (*SI Appendix*, Table S10).

SAYAV-specific antibodies were detected in the sera of sika deer from the Yamaguchi Prefecture and in the sera of wild boars from the Toyama Prefecture; however, many serum samples also reacted with other flavivirus SRIP (Tables 3 and 4), indicating the prevalence of multiple flaviviruses in these areas. JEV is widespread in southern and western Japan (44), and flaviviruses, including JEV, are less active in northern Japan. Hence, we performed the SNT using sera from sika deer in northern Japan, Aomori Prefecture, and Hokkaido (*SI Appendix*, Fig. S5). First, 10-fold-diluted sera were subjected to the SNT using SAYAV SRIP. As a result, sera from 6 of 18 sika deer in Aomori and 9 of 22 sika deer in Hokkaido exhibited antibodies against SAYAV (Table 4). In the Aomori Prefecture, four serum samples showed significantly high SNT titers against SAYAV, and three of them reacted only with the SRIP of SAYAV. In Hokkaido, three serum samples showed more than fourfold higher antibody titers against SAYAV than those against other flaviviruses, and two of those serum samples reacted only with the SRIP of SAYAV (Table 4).

Table 3. Detection of anti-SAYAV antibodies in wild boars in Toyama Prefecture

Wild boar ID	SNT titers against SRIPs (IC_{75})				
	SAYAV	JEV	LGTV	TBEV	POWV
T-1390	1:30	<1:10	1:19	1:33	<1:10
T-1391	1:86	1:51	1:72	1:130	1:81
T-1392	1:**687**	1:10	<1:10	<1:10	<1:10
T-1394	1:27	<1:10	<1:10	1:12	<1:10
T-1395	1:167	1:17	1:19	1:45	<1:10
T-1396	1:**43**	<1:10	<1:10	<1:10	<1:10
T-1397	1:**113**	1:26	<1:10	1:13	<1:10
T-1398	1:302	1:54	1:74	1:203	1:20
T-1428	1:92	1:68	1:77	1:63	1:95
T-1430	1:**604**	<1:10	<1:10	<1:10	<1:10
T-1431	1:**270**	<1:10	<1:10	<1:10	<1:10
T-1463	1:47	<1:10	1:17	<1:10	<1:10
T-1471	1:**481**	1:33	1:27	1:11	1:24
T-1472	1:**119**	1:21	1:20	<1:10	<1:10

Bold letters indicate more than fourfold difference in comparison with SNT titers against the other SRIPs.

Sequence-Based Estimates of SAYAV Host Range and the Phylogenetic Relationships of Other Flaviviruses. Viral RNA detection in ticks and wild mammals suggests that SAYAV is hosted by these organisms. However, because viral isolates have not yet been obtained, it is not possible to directly examine the infectivity and growth ability of SAYAV in these organisms through laboratory infection experiments. Therefore, we attempted to estimate the host range of the virus by analyzing the SAYAV genome sequence obtained in this study.

Because viruses utilize host translation systems, viral genomes are expected to have the same nucleotide composition as the host genome and thus exhibit similar dinucleotide biases (45). Therefore, the host range of a virus can be estimated by analyzing the bias of the nucleotide sequences that constitute the viral genome. This approach has been used to estimate the host ranges of various flaviviruses (22, 33, 46). To predict the host range of SAYAV, we analyzed the dinucleotide composition of the SAYAV coding sequence and its various flaviviruses (*SI Appendix*, Table S11). Linear discriminant analysis (LDA), a technique for predicting qualitative outcomes from quantitative data, was employed on the dinucleotide odds

Table 4. Serological surveillance of SAYAV infection among sika deer

Prefectures	Sika deer ID	SAYAV	SNT titers against SRIPs (IC_{75})			
			JEV	LGTV	TBEV	POWV
Aomori	A-1399	1:**20**	<1:10	<1:10	<1:10	<1:10
	A-1400	1:11	1:11	<1:10	1:13	<1:10
	A-1423	1:**56**	<1:10	<1:10	1:12	<1:10
	A-1425	1:21	<1:10	<1:10	<1:10	1:11
	A-1466	1:**52**	<1:10	<1:10	<1:10	<1:10
	A-1469	1:**41**	<1:10	<1:10	<1:10	<1:10
Yamaguchi	20-5	1:40	1:46	1:17	1:51	1:15
	20-6	1:**134**	1:18	<1:10	1:13	<1:10
	20-9	1:30	1:16	1:39	1:23	<1:10
	20-21	1:59	1:29	1:26	1:29	1:21
	20-27	1:28	1:122	1:17	1:35	1:43
	20-28	1:80	1:54	1:19	1:25	1:21
	20-30	1:27	1:27	<1:10	1:18	<1:10
	20-34	1:**302**	<1:10	<1:10	<1:10	<1:10
	20-41	1:47	1:12	1:11	1:74	<1:10
	20-47	1:29	1:39	<1:10	1:41	1:14
	20-48	1:37	1:71	1:16	1:15	1:41
	20-52	1:**128**	1:17	<1:10	1:16	1:23
	20-53	1:64	1:87	<1:10	1:29	1:16
	20-55	1:57	<1:10	<1:10	1:11	1:21
	20-67	1:29	1:38	1:15	1:50	1:37
Hokkaido	H-56	1:146	1:42	1:87	1:100	1:39
	H-62	1:29	1:55	1:45	1:56	1:35
	H-65	1:30	<1:10	<1:10	1:11	<1:10
	H-229	1:**93**	<1:10	<1:10	<1:10	<1:10
	H-244	1:**148**	<1:10	<1:10	1:12	<1:10
	H-276	1:18	1:24	<1:10	1:53	1:17
	H-277	1:**64**	<1:10	<1:10	<1:10	<1:10
	H-295	1:233	1:311	<1:10	1:183	1:148
	H-325	1:211	1:223	1:65	1:216	1:208

Bold letters indicate more than fourfold difference in comparison with SNT titers against the other SRIPs.

ratio data. By comparing the dinucleotide composition odds ratio of flaviviruses with known host ranges, the potential host range category for the SAYAV was assessed. The LDA result showed accurate grouping of each host category for flaviviruses, and SAYAV was grouped with tick-borne flaviviruses (Fig. 2). This suggests that SAYAV has a host range similar to that of tick-borne flaviviruses.

Next, a phylogenetic dendrogram was constructed to evaluate the phylogenetic relationship between SAYAV and diverse flaviviruses (*SI Appendix*, Table S12), including viruses of the genus *Orthoflavivirus* (*s.s.*) and cISF, flaviviruses from marine organisms, MPFV, and the Tamana bat virus. Using the aligned polyprotein sequences (approximately 2,000 amino acids), phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI) methods. Dendrograms constructed using both methods showed similar tree topologies (*SI Appendix*, Figs. S6 and S7). Interestingly, SAYAV formed a robust clade with MPFV (Fig. 3) and was located just outside the *Orthoflavivirus* (*s.s.*) clade in the phylogenetic dendrogram. The monophyly of the clade of SAYAV and MPFV and the *Orthoflavivirus* (*s.s.*) clade was fully supported (bootstrap value = 100%, posterior probability = 1.0) (Fig. 3). The crustacean flavivirus clade, including Crangon crangon flavivirus, Gammarus chevreuxi flavivirus, and Gammarus pulex flavivirus, was also found outside the clade of SAYAV, MPFV, the *Orthoflavivirus* (*s.s.*). The cISF clade was further outside the crustacean flavivirus clade (Fig. 3). The results of the phylogenetic analysis including NGOV, for which only partial sequences were available, also showed the same topology as described above (*SI Appendix*, Fig. S8). These phylogenetic analyses revealed that SAYAV, MPFV, and NGOV are unique flaviviruses with evolutionary lineages that differ from those of traditional flaviviruses of the genus *Orthoflavivirus*.

Discussion

Recent extensive viral genome studies employing NGS have uncovered that arthropods host an extraordinarily diverse array of viruses (47). Blood-feeding arthropods like ticks and mosquitoes, known for transmitting various pathogens to vertebrates, have also been found to harbor a multitude of viruses (48, 49). Consequently, humans and other vertebrates might encounter previously unclassified viruses, potentially posing as pathogens. However, the majority of viruses identified through NGS remain unisolated and uncultured, leaving their detailed viral characteristics uncharted (5, 6). Certain features of human pathogenic viruses have previously been examined through the production of pseudotyped viruses from nonisolated and uncultured viruses. For instance, Bas-Congo

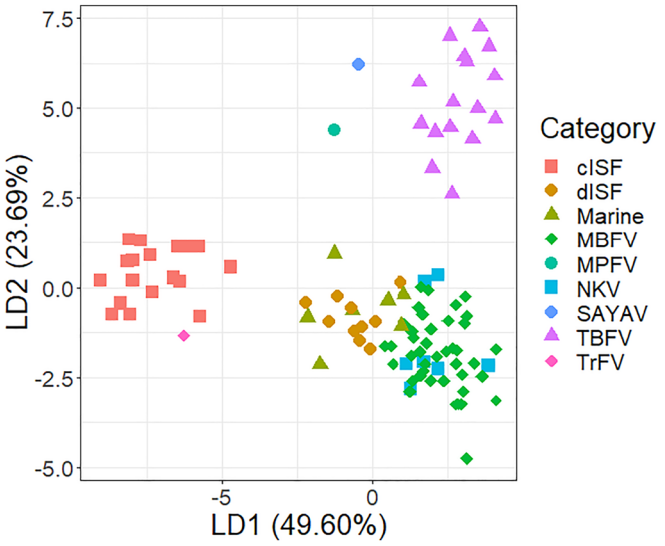

Fig. 2. Score plot of a LDA. Scatter plot of the two discriminant scores of SAYAV and other known flaviviruses from LDA (49.60% and 23.69% for LD1 and LD2, respectively). Each virus used in this analysis is indicated by the legend on the *Right*, whereas SAYAV is indicated by a blue diamond. Abbreviations for the viral categories in the legend are as follows: cISF, classical insect-specific flavivirus; dISF, dual-host-associated insect-specific flavivirus; Marine, flavivirus from marine organisms; MBFV, mosquito-borne flavivirus; MPFV, Mpulungu flavivirus; NKV, no-known-vector flavivirus; TBFV, tick-borne flavivirus; TrFV, Tabanus rufidens flavivirus. The viruses belonging to each category used in this analysis are listed in *SI Appendix*, Table S11.

virus (BASV), a previously unknown member of the *Rhabdoviridae* family, was identified in the sera of patients with acute hemorrhagic fever in the Democratic Republic of Congo using NGS (50). Despite unsuccessful isolation attempts, a pseudotyped virus was crafted based on the glycoprotein sequence of BASV using a vesicular stomatitis virus-based system (50). This methodology facilitated the analysis of viral entry mechanisms into various cells and the conduct of seroepidemiological studies, thereby shedding light on fundamental properties (50, 51). This approach mirrors the current study, where the generation and analysis of pseudotyped viruses have enhanced our comprehension of viruses that remain unisolated and uncultured. Nonetheless, the insights gleaned from pseudotyped viruses have their limitations, and the true pathogenic nature of BASV, particularly its role in causing acute hemorrhagic fever symptoms in humans, remains contentious due to the inability to fulfill Koch's postulates in the absence of virus isolation and culture (52). Adopting a similar research methodology, the current study has also integrated the use of SRIP, a proficient artificial particle production technique for flaviviruses (28, 29). To our knowledge, this study is the pioneer in employing SRIP to analyze a previously unknown, unisolated flavivirus. Beyond the detection of viral RNA, our research has detected neutralizing antibodies against the previously unidentified flavivirus in wild animals, further substantiating the virus's prevalence, notwithstanding its challenging isolation and culture. Although these results strongly suggest the infectivity and replication capacity of SAYAV in some vertebrates, further confirmation of the viral properties will require histopathological and immunohistochemical analyses of tissues from wild animals in which viral RNA was detected. In addition, if SAYAV is detected in diseased humans or animals, isolation and culture of the virus will be required to conclusively establish a causal relationship with its pathogenicity.

In this study, SAYAV was not isolated from mammalian (BHK-21 and Vero), tick (ISE6), or mosquito (C6/36) cell cultures. Similarly, NGOV and MPFV have not been isolated by cell culture systems and intracerebral inoculation in newborn mice (32, 33). The source of NGOV was hard ticks (*Rhipicephalus evertsi evertsi* and *Rhipicephalus guilhoni*) collected from sheep and goats. In addition, a previous study on MPFV has not described whether MPFV-positive ticks are engorged (33), and it is unclear whether the virus is derived from ticks or animal blood. In the present study, SAYAV was detected in *H. flava* ticks collected from vegetation in a nonblood-fed state while questing for their host animals, suggesting that this viral sequence was unlikely to have originated from the tick's host mammalian blood and may have originated from a virus propagated within the tick body. Consequently, it is plausible that SAYAV detected in the adult male and nymphal stages of the *H. flava* tick was acquired during blood feeding in their preceding developmental stages, namely the nymphal and larval stages, respectively. This leads to the suggestion that transstadial transmission, the transmission of the virus between different developmental stages of the same tick, is a viable transmission mode for SAYAV. Additionally, it is known that several tick-borne orthoflaviviruses are transmitted from virus-infected adult female ticks to their offspring via eggs (transovarial transmission) (53). Since ticks are blood feeders at all developmental stages, verifying vertical transmission of the virus in ticks is challenging with field samples unless the virus is detected in unfed larvae (54). This transmission mode can also be observed under controlled experimental conditions (55), but such an investigation necessitates an isolated and cultured virus.

Virus screening was performed on several tick species in this study; however, SAYAV was detected only in *H. flava* ticks. *H. flava* is a common species distributed throughout Japan that infests a variety of animals, including sika deer and wild boars (56). The peak occurrence of *H. flava* adults and nymphs in Japan is in spring and fall, whereas that of larvae is in summer, although regional differences exist (57, 58). A previous study conducted in the Yamaguchi Prefecture, where SAYAV was detected in this study, confirmed that adult *H. flava* ticks parasitize sika deer throughout the year, with peak infestation in October (59). Therefore, the detection of SAYAV RNA in the sera of wild boars and deer captured in January, August, and October in this study was consistent with SAYAV transmission in these wild animals, considering that *H. flava* was active during these months. Several flaviviruses belonging to the *Orthoflavivirus* (*s.s.*) (JEV, TBEV, Yokose virus, Apoi virus, and YGV) are distributed in Japan (41, 42, 44, 60, 61). Among them, only JEV, TBEV, and YGV infect sika deer and/or wild boar in Japan (39, 41, 62, 63). The SAYAV SRIP generated in this study was not recognized by antibodies induced by JEV or LGTV (a close relative of YGV). Animals with antibodies specific to SAYAV detected using SRIP were found to inhabit a wide area of Japan, which considerably overlapped with the *H. flava* tick habitat. These findings further indicate that this tick species transmits SAYAV to wild boars and sika deer, which may be justified considering the ecological conditions described above. Moreover, analysis of the viral genome also revealed that SAYAV shares a dinucleotide composition akin to that of the tick-borne orthoflaviviruses, bolstering the notion that SAYAV is likely an infectious virus transmitted to mammals by *H. flava* ticks.

The phylogenetic analysis performed in this study revealed that SAYAV, together with MPFV and NGOV, forms a distinct outer clade of the known *Orthoflavivirus* (*s.s.*). Thus, SAYAV, MPFV, and NGOV evolved independently of *Orthoflavivirus* (*s.s.*), diverging from it long ago. Previously, NGOV and MPFV were exclusively identified in ticks, but SAYAV represents the inaugural detection of this virus group in mammals. These viruses differ greatly in their distribution areas, and the tick species detected. This suggests that

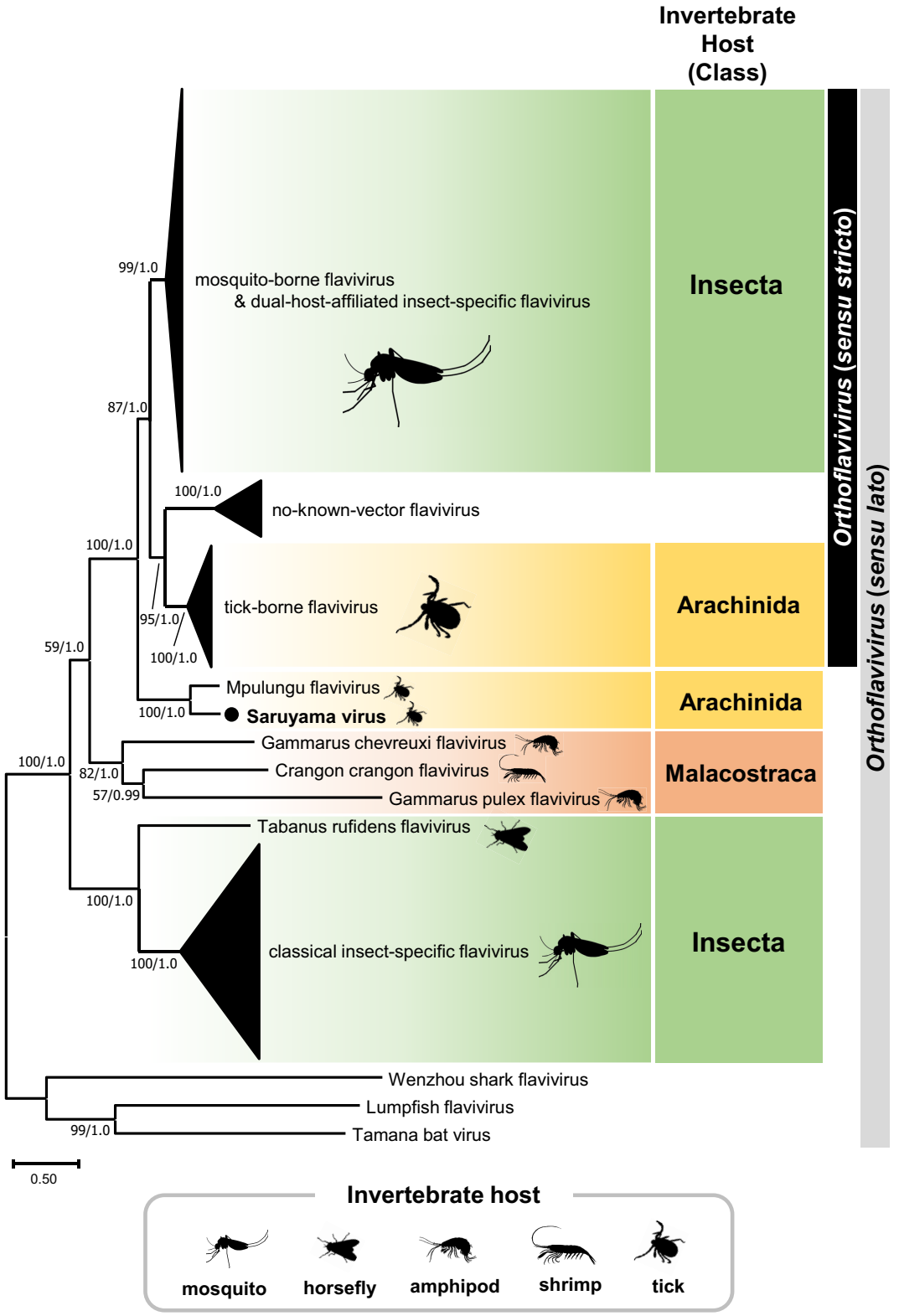

Fig. 3. Phylogenetic dendrogram constructed using the ML method for the polyprotein sequences of SAYAV and other known flaviviruses. In the dendrogram, the numbers on the nodes represent the bootstrap values of ML and the Bayesian posterior probability. SAYAV detected in this study is indicated in boldface and a filled circle. The invertebrate host of each virus is shown as an illustration, and the taxon (Class) of the invertebrate host is shown in the box on the *Right*. The accession numbers of the viruses used in this analysis are shown in the *SI Appendix*, Table S12. The original trees constructed using ML and BI are provided in *SI Appendix*, Figs. S6 and S7, respectively.

unique flaviviruses, such as SAYAV, are widely distributed worldwide. These unique flaviviruses may have been unidentified previously because they are difficult to isolate using conventional virus surveillance methods, such as cell culture systems or intracerebral inoculation of suckling mice. Recently, a previously unknown flavivirus, Batu Kawa virus (BKWV), has been detected in Malaysian rodents (64). BKWV was phylogenetically closely related to a group of viruses previously thought to be insect-specific [dual-host

affiliated insect-specific flaviviruses (18)], but this virus, like SAYAV and others, has not been isolated in cultured cells (64). These results suggest the existence of unknown flaviviruses that cannot be captured by conventional virus isolation methods, as well as unique flaviviruses such as SAYAV. Therefore, a combination of comprehensive viral sequence analysis using NGS and molecular biological analysis methods, such as SRIP, as used in this study, may reveal the existence and properties of diverse flaviviruses in nature.

Materials and Methods

Collection of Ticks. Host-questing ticks were collected from vegetation fields in the cities of Wajima and Kaga, Ishikawa Prefecture, Japan, in May 2018. The Global Positioning System coordinates of the collection sites are presented in our previous publication (31). By dragging a flannel sheet (70 × 100 cm) onto the vegetation, ticks were collected, as described previously (31). Next, the collected ticks were classified into pools based on species, developmental stage, and sex, following which pooled tick samples were stored at −80 °C, until further analyses.

Virome Analysis. Pooled tick samples were homogenized in media and filtered. Each sample was mixed in equal quantities up to a volume of 380 μL (31.7 μL/ pool), followed by NGS library preparation as described previously (20, 65). The NGS library was amplified using NEBNext Ultra II Q5 Master Mix (New England Biolabs, Ipswich, MA) and analyzed using the MiniSeq system (Illumina, San Diego, CA) coupled with the MiniSeq Mid Output kit (300 cycles) (Illumina). The resulting reads were subjected to trimming and de novo assembly using default settings on the CLC Genomics Workbench version 21 (QIAGEN, Venlo, Netherlands). Subsequently, possible viral sequences were identified from the contigs using BLASTN and BLASTX searches against standard and nonredundant protein sequence databases, respectively. To identify flavivirus-positive pools, total RNA extracted from each filtrate was RT-PCR-amplified using the primers 18HKR-fla-FW2 and 18HKR-fla-RV2 (*SI Appendix*, Table S3) in addition to the PrimeScript One-Step RT-PCR Kit Ver. 2 (Takara Bio, Shiga, Japan), as previously described (20).

Virus Isolation. Virus isolation was attempted using the filtered tick homogenate with BHK-21 cells [Syrian hamster kidney; Japan Health Science Research Resources Bank (JCRB)] according to the method described by Kobayashi et al. (66). Moreover, to verify virus isolation, blind-passaged supernatants were harvested and subsequent NGS analysis was conducted as described previously (20). In this study, additional cell lines were utilized for virus isolation: the African green monkey kidney cell line (Vero; Department of Veterinary Science, National Institute of Infectious Diseases, Japan), the tick cell line [ISE6; American Type Culture Collection (ATCC)], and the mosquito cell line (C6/36; European Collection of Authenticated Cell Cultures) were used for virus isolation. A virus RNA-positive filtrated tick homogenate was inoculated into these cell lines, following which the culture supernatants were harvested at different time points. Specially for Vero cells, cells inoculated with the sample were also collected. The total RNAs extracted from both the supernatants and the cells were subjected to RT-PCR-based virus RNA detection using the PrimeScript One-Step RT-PCR Kit Ver. 2 using a viral gene-specific primer set (18HKR-fla-FW3 and 18HKR-fla-RV3 or 18HKR-fla-FW3 and 18HKR-fla-RV3; *SI Appendix*, Table S3).

Examination of the EVE. DNA extracted from the SAYAV-positive tick pool was PCR-amplified using four viral genome-specific primer sets (18HKR-fla-FW2 and 18HKR-fla-RV2; 18HKR-fla-FW5 and 18HKR-fla-RV5; 18HKR-fla-FW6 and 18HKR-fla-RV6; and 18HKR-fla-FW9 and 18HKR-fla-RV3; *SI Appendix*, Table S3), as previously described (31). The 18S ribosomal RNA gene was used as an internal positive control for this reaction using the primers T18S-F and T18S-R designed in our previous study (65).

Determination of the Viral Genome Sequence. A SAYAV-positive pool (No. 18HKR14) was used to determine the complete viral genome sequence. Subsequent RT-PCR was used to fill the sequence gaps in each contig (*SI Appendix*, Table S2) using primer sets specific to the viral sequences (*SI Appendix*, Table S3). The resulting amplicons were subjected to direct Sanger sequencing using an ABI 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA) as previously described (31). Subsequently, to determine viral terminal sequences, the 5′- and 3′-RACE were performed, as described previously (36, 37).

Characterization of the Viral Genome. The ORF of the SAYAV genome and its encoded amino acid sequence were determined using Genetyx version 14 software (Genetyx, Tokyo, Japan). The encoded viral proteins were then analyzed, and proteolytic cleavage sites in the viral polyprotein were predicted, as described previously (20). The identities of the nucleotide and amino acid sequences between SAYAV and other flaviviruses were subsequently determined using sequence alignment based on Lipman–Pearson's method (67) using Genetyx software version 14.

Screening of Viral RNA in Japanese Field Samples of Ticks and Wild Animals. For RT-PCR-based screening of SAYAV infection, RNA samples were extracted from pooled ticks and the sera of wild animals using a QIAGEN Viral RNA Mini Kit (Qiagen). A total of 3,690 ticks (174 pools) collected from the Yamaguchi and Wakayama Prefectures, Japan, between 2013 and 2014 were used for this analysis. Serum samples from wild boars and sika deer collected from several prefectures in Japan were also used for viral RNA screening. The sampling prefectures and quantities are as follows: wild boar samples were from Chiba (30 individuals), Ehime (38 individuals), Gifu (one individual), Gunma (five individuals), Kagawa (20 individuals), Toyama (26 individuals), and Yamaguchi (51 individuals); sika deer samples were from Chiba (21 individuals), Ehime (nine individuals), Gifu (five individuals), Kagawa (nine individuals), and Yamaguchi (79 individuals) (*SI Appendix*, Fig. S4). Serum samples from Yamaguchi Prefecture were collected in 2013 and 2014, whereas samples from other areas were collected in 2018. Viral RNA detection was subsequently conducted using the QIAGEN OneStep RT-PCR Kit (Qiagen) with flaviviral consensus primers targeting the NS5 gene, and the resultant amplicons were sequenced using the BigDye Terminator Cycle Sequencing Kit v3.1 (Thermo Fisher Scientific, Waltham, MA), as described previously (41). Furthermore, 701 *H. flava* ticks, collected from September to November 2022 at sites in Toyama Prefecture where wild boars with SAYAV antibodies were found, were grouped into 111 pools by collection site and developmental stage (*SI Appendix*, Table S10). RNA extraction from these samples was performed using ISOGEN II (Nippon Gene, Tokyo, Japan), and viral RNA detection was conducted using the PrimerScript one-step RT-PCR kit ver. 2 with the same flaviviral consensus primers (41) and SAYAV-specific primers (*SI Appendix*, Table S3).

Serum Samples for the Serosurveillance of SAYAV Infection. Serum samples were collected from wild boars and sika deer. A total of 85 serum samples were collected from sika deer in Yamaguchi (n = 45) in 2020, Aomori (n = 18) in 2021, and Hokkaido (n = 22) in 2013 (*SI Appendix*, Fig. S5). Fifteen serum samples were collected from wild boars in Toyama in 2021 (*SI Appendix*, Fig. S5). Hunters captured these animals as a countermeasure to the official population control program. All the serum samples were stored at −20 °C until use.

Production of Polyclonal Antibodies against Flavivirus prM-Es. Anti-prM-E rabbit sera were produced by inoculating rabbits with expression plasmids encoding each flavivirus prM-E. The prM-E genes of SAYAV (accession number LC657549), LGTV (accession number AF253420), POWV (accession number NC_003687), and MPFV (accession number LC582740) were artificially synthesized (Azenta, Chelmsford, MA) and cloned into the expression vector pCAGGS (68). JEV prM-E (accession number EF571853) has been previously constructed (27). Each rabbit was inoculated with 400 μg of each purified plasmid DNA six times, at 2-wk intervals, and then rabbit sera were collected 2 wk after final injection. All animal experiments were approved under Permission Number 121180 by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases.

Production of SRIP. SRIP were generated, as described previously (29). In this study, we performed a YFV-based SRIP assay. Briefly, human embryonic kidney 293T cells (HEK293T) (ATCC CRL-3216) were cotransfected using polyethylenimine with the following three plasmids: a YFV-derived replicon plasmid containing the nanoluciferase gene, a YFV capsid-expression plasmid, and each expression plasmid prM-E of SAYAV, JEV, LGTV, TBEV, POWV, and MPFV (69). Culture media were collected 3 d after transfection and used as SRIP. Unfortunately, only the SRIP of MPFV did not have a sufficient titer.

SNT with SRIP. SNTs with SRIP were performed, as previously described (29). Before the test, the complement in the serum was inactivated at 56 °C for 30 min. Serially diluted sera were mixed with each SRIP (approximately 50 to 100

infectious units/well) in a 1:1 ratio. After incubating the mixtures at 37 °C for 2 h, they were added to monolayers of African green monkey kidney cell line, Vero cells (JCRB9013) in 96-well plates (Iwaki, Tokyo, Japan) and incubated at 37 °C for 5 h. After incubation, extra culture medium was added, and the plate was incubated at 37 °C. After 3 d of incubation, the Vero cells were washed once with phosphate-buffered saline (PBS) and lysed with passive lysis buffer (Promega, Madison, WI). Luciferase activity was determined using Nano-Glo® Luciferase Assay System (Promega). Neutralization titers were represented as the serum dilution that reduced SRIP infectivity by 75% (IC75) compared to the nonserum control. IC75 was calculated using CompuSyn software (ComboSyn Inc., Paramus, NJ).

IFA. HEK293T cells were transfected with each prM–E–expressing plasmid using polyethylenimine and incubated at 37 °C for 2 d. After fixation in cold acetone (Sigma-Aldrich, St. Louis, MO) at 4 °C for 30 min, the cells were incubated with serum samples diluted with PBS containing 5% skim milk (Bean Stalk Snow Co., Ltd. Tokyo, Japan) at 37 °C for 30 min. After 3 times PBS washes, the cells were incubated with the second antibody diluted in PBS containing 5% skim milk at 37 °C for 30 min. Fluorescein isothiocyanate (FITC)–labeled goat anti-rabbit IgG (H+L) secondary antibody (Thermo Fisher Scientific) and FITC–labeled protein A/G (Thermo Fisher Scientific) were used to detect antibodies in rabbits, and wild boars, and sika deer, respectively. IFA titers were recorded as the highest serum dilution showing specific fluorescence. Expression plasmids without inserts and pCAGGS–transfected cells were used as negative controls.

Dinucleotide Composition Analysis. For this analysis, we used a dataset of 102 flaviviruses, excluding an incomplete sequence of Watasenia scintillans flavivirus sequence from the data set prepared in a previous study (22). Odds ratios for the dinucleotide motifs of SAYAV and two recently identified flaviviruses, MPFV (33) and Tabanus rufidens flavivirus (20), were calculated as described previously (22). The results were added to the dataset, and LDA was conducted using the MASS package in R Studio.

Phylogenetic Analysis. Multiple sequence alignments were performed using the MAFFT (70). The Gblocks program (version 0.91b, January 2002) (71) was used to remove divergent or ambiguously aligned regions. Subsequently, suitable substitution models were selected using MEGA X (72). A phylogenetic dendrogram was constructed using the ML method and BI. The ML phylogenetic tree was constructed using MEGA X with the LG + G + F model and 1,000 replications of bootstrap replications (73). The BI dendrogram and posterior probabilities were estimated using MrBayes version 3.2.7a (74) using the WAG + G + F model. Markov chain Monte Carlo methods were executed over ten million generations, with tree sampling occurring every 100 generations.

Data, Materials, and Software Availability. Nucleotide sequences of viruses obtained in this study data have been deposited in International Nucleotide Sequence Database (DDBJ/EMBL-EBI/NCBI) [LC657549 (75), LC658947–LC658950 (76–79).

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1. W. B. Karesh *et al.*, Ecology of zoonoses: Natural and unnatural histories. *Lancet* **380**, 1936–1945 (2012).
2. R. M. Meganck, R. S. Baric, Developing therapeutic approaches for twenty-first-century emerging infectious viral diseases. *Nat. Med.* **27**, 401–410 (2021).
3. S. Zohdy, T. S. Schwartz, J. R. Oaks, The coevolution effect as a driver of spillover. *Trends Parasitol.* **35**, 399–408 (2019).
4. J. P. de Almeida, E. R. Aguiar, J. N. Armache, R. P. Olmo, J. T. Marques, The virome of vector mosquitoes. *Curr. Opin. Virol.* **49**, 7–12 (2021).
5. R. Tokarz, W. I. Lipkin, Discovery and surveillance of tick-borne pathogens. *J. Med. Entomol.* **58**, 1525–1535 (2021).
6. A. N. Faizah *et al.*, Deciphering the virome of Culex vishnui subgroup mosquitoes, the major vectors of Japanese encephalitis, in Japan. *Viruses* **12**, 264 (2020).
7. L. N. Binn, V. R. Harrison, R. Randall, Patterns of viremia and antibody observed in rhesus monkeys inoculated with chikungunya and other serologically related group A arboviruses. *Am. J. Trop. Med. Hyg.* **16**, 782–785 (1967).
8. L. C. LaMotte Jr., G. T. Crane, R. B. Shriner, L. J. Kirk, Use of adult chickens as arbovirus sentinels. I. Viremia and persistence of antibody in experimentally inoculated adult chickens. *Am. J. Trop. Med. Hyg.* **16**, 348–356 (1967).
9. A. J. Shepherd, P. A. Leman, R. Swanepoel, Viremia and antibody response of small African and laboratory animals to Crimean-Congo hemorrhagic fever virus infection. *Am J. Trop. Med. Hyg.* **40**, 541–547 (1989).
10. N. Komar *et al.*, Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerg. Infect. Dis.* **9**, 311–322 (2003).
11. L. R. Petersen, M. P. Busch, Transfusion-transmitted arboviruses. *Vox Sang.* **98**, 495–503 (2010).
12. F. Kato *et al.*, Evaluation of Macaca radiata as a non-human primate model of Dengue virus infection. *Sci. Rep.* **8**, 3421 (2018).
13. J. Näslund *et al.*, Emerging mosquito-borne viruses linked to Aedes aegypti and Aedes albopictus: Global status and preventive strategies. *Vector Borne Zoonotic Dis.* **21**, 731–746 (2021).
14. S. Madison-Antenucci, L. D. Kramer, L. L. Gebhardt, E. Kauffman, Emerging tick-borne diseases. *Clin. Microbiol. Rev.* **33**, e00083-18 (2020).
15. P. Simmonds *et al.*, ICTV virus taxonomy profile: Flaviviridae. *J. Gen. Virol.* **98**, 2–3 (2017).
16. R. Rico-Hesse *et al.*, The ICTV report on virus classification and taxon nomenclature Flaviviridae Chapter. (2023). https://ictv.global/report/chapter/flaviviridae/flaviviridae. Accessed 18 February 2024.
17. G. Kuno, G. J. Chang, K. R. Tsuchiya, N. Karabatsos, C. B. Cropp, Phylogeny of the genus Flavivirus. *J. Virol.* **72**, 73–83 (1998).
18. B. J. Blitvich, A. E. Firth, Insect-specific flaviviruses: A systematic review of their discovery, host range, mode of transmission, superinfection exclusion potential and genomic organization. *Viruses* **7**, 1927–1959 (2015).
19. C. H. Calisher, S. Higgs, The discovery of arthropod-specific viruses in hematophagous arthropods: An open door to understanding the mechanisms of arbovirus and arthropod evolution? *Annu. Rev. Entomol.* **63**, 87–103 (2018).
20. D. Kobayashi *et al.*, Discovery of a novel Flavivirus (Flaviviridae) from the horse fly, Tabanus rufidens (Diptera: Tabanidae): The possible coevolutionary relationships between the classical insect-specific flaviviruses and host dipteran insects. *J. Med. Entomol.* **58**, 880–890 (2021).
21. M. Shi *et al.*, Divergent viruses discovered in arthropods and vertebrates revise the evolutionary history of the Flaviviridae and related viruses. *J. Virol.* **90**, 659–669 (2016).
22. R. Parry, S. Asgari, Discovery of novel crustacean and cephalopod flaviviruses: Insights into the evolution and circulation of flaviviruses between marine invertebrate and vertebrate hosts. *J. Virol.* **93**, e00432-19 (2019).
23. S. Paraskevopoulou *et al.*, Viromics of extant insect orders unveil the evolution of the flavi-like superfamily. *Virus Evol.* **7**, veab030 (2021).
24. D. A. Sanders, No false start for novel pseudotyped vectors. *Curr. Opin. Biotechnol.* **13**, 437–442 (2002).
25. Q. Li, Q. Liu, W. Huang, X. Li, Y. Wang, Current status on the development of pseudoviruses for enveloped viruses. *Rev. Med. Virol.* **28**, e1963 (2018).
26. E. Cuevas-Juárez, V. Pando-Robles, L. A. Palomares, Flavivirus vaccines: Virus-like particles and single-round infectious particles as promising alternatives. *Vaccine* **39**, 6990–7000 (2021).
27. R. Suzuki *et al.*, Production of single-round infectious chimeric flaviviruses with DNA-based Japanese encephalitis virus replicon. *J. Gen. Virol.* **95**, 60–65 (2014).
28. M. Matsuda *et al.*, High-throughput neutralization assay for multiple flaviviruses based on single-round infectious particles using dengue virus type 1 reporter replicon. *Sci. Rep.* **8**, 16624 (2018).
29. A. Yamanaka *et al.*, Seroprevalence of flavivirus neutralizing antibodies in Thailand by high-throughput neutralization assay: Endemic circulation of zika virus before 2012. *mSphere* **6**, e0033921 (2021).
30. H. Ejiri *et al.*, Isolation and characterization of Kabuto Mountain virus, a new tick-borne phlebovirus from Haemaphysalis flava ticks in Japan. *Virus Res.* **244**, 252–261 (2018).
31. D. Kobayashi *et al.*, RNA virome analysis of questing ticks from Hokuriku District, Japan, and the evolutionary dynamics of tick-borne phleboviruses. *Ticks Tick Borne Dis.* **11**, 101364 (2020).
32. G. Grard *et al.*, Ngoye virus: A novel evolutionary lineage within the genus Flavivirus. *J. Gen. Virol.* **87**, 3273–3277 (2006).
33. H. Harima *et al.*, An African tick flavivirus forming an independent clade exhibits unique exoribonuclease-resistant RNA structures in the genomic 3′-untranslated region. *Sci. Rep.* **11**, 4883 (2021).
34. H. Ejiri *et al.*, Characterization of a novel thogotovirus isolated from Amblyomma testudinarium ticks in Ehime, Japan: A significant phylogenetic relationship to Bourbon virus. *Virus Res.* **249**, 57–65 (2018).

35. G. Kuno, Host range specificity of flaviviruses: Correlation with in vitro replication. *J. Med. Entomol.* **44**, 93–101 (2007).
36. Z. Li, M. Yu, H. Zhang, H. Y. Wang, L. F. Wang, Improved rapid amplification of cDNA ends (RACE) for mapping both the 5′ and 3′ terminal sequences of paramyxovirus genomes. *J. Virol. Methods* **130**, 154–156 (2005).
37. K. Hoshino *et al.*, Genetic characterization of a new insect flavivirus isolated from Culex pipiens mosquito in Japan. *Virology* **359**, 405–414 (2007).
38. A. Katzourakis, R. J. Gifford, Endogenous viral elements in animal genomes. *PLoS Genet.* **6**, e1001191 (2010).
39. Y. Ohno *et al.*, Detection of antibodies against Japanese encephalitis virus in raccoons, raccoon dogs and wild boars in Japan. *J. Vet. Med. Sci.* **71**, 1035–1039 (2009).
40. I. Sugiyama *et al.*, Serological survey of arthropod-borne viruses among wild boars in Japan. *J. Vet. Med. Sci.* **71**, 1059–1061 (2009).
41. H. Shimoda *et al.*, Detection of a novel tick-borne flavivirus and its serological surveillance. *Ticks Tick Borne Dis.* **10**, 742–748 (2019).
42. I. Takashima *et al.*, A case of tick-borne encephalitis in Japan and isolation of the the virus. *J. Clin. Microbiol.* **35**, 1943–1947 (1997).
43. H. Salje *et al.*, Variability in dengue titer estimates from plaque reduction neutralization tests poses a challenge to epidemiological studies and vaccine development. *PLoS Negl. Trop. Dis.* **8**, e2952 (2014).
44. National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Labour and Welfare, Japanese encephalitis, Japan, 2007–2016. *Infect Agents Surv. Rep.* **38**, 151–152 (2017).
45. J. L. Chaney, P. L. Clark, Roles for synonymous codon usage in protein biogenesis. *Annu. Rev. Biophys.* **44**, 143–166 (2015).
46. A. M. G. Colmant *et al.*, A new clade of insect-specific flaviviruses from Australian Anopheles mosquitoes displays species-specific host restriction. *mSphere* **2**, e00262–17 (2017).
47. E. Harvey, E. C. Holmes, Diversity and evolution of the animal virome. *Nat. Rev. Microbiol.* **20**, 321–334 (2022).
48. W. A. Foster, E. D. Walker, "Mosquitoes (Culicidae)" in *Medical and Veterinary Entomology*, G. R. Mullen, L. A. Durden, Eds. (Academic Press, 2019), pp. 261–325.
49. W. L. Nicholson, D. E. Sonenshine, B. H. Noden, R. N. Brown, "Ticks (Ixodida)" in *Medical and Veterinary Entomology*, G. R. Mullen, L. A. Durden, Eds. (Academic Press, 2019), pp. 603–672.
50. G. Grard *et al.*, A novel rhabdovirus associated with acute hemorrhagic fever in central Africa. *PLoS Pathog.* **8**, e1002924 (2012).
51. I. Steffen *et al.*, Characterization of the Bas-Congo virus glycoprotein and its function in pseudotyped viruses. *J. Virol.* **87**, 9558–9568 (2013).
52. L. M. Branco, R. F. Garry, Bas-Congo virus–not an established pathogen. *Science* (2018). https://www.science.org/doi/full/10.1126/science.aap9072#elettersSection. Accessed 18 February 2024.
53. P. A. Nuttall, "Tick-borne viruses" in *Biology of Ticks*, D. E. Sonenshine, R. M. Roe, Eds. (Oxford University Press, 2014), **vol. 2**, pp. 180–210.
54. D. Kobayashi *et al.*, Detection of jingmenviruses in Japan with evidence of vertical transmission in Ticks. *Viruses* **13**, 2547 (2021).
55. J. Rehacek, Transovarial transmission of tick-borne encephalitis virus by ticks. *Acta Virol.* **6**, 220–226 (1962).
56. N. Takada, H. Fujita, M. Takahashi, M. Natsuaki, "Key to species of mature ticks & specific descriptions" in *Medical Acarology in Japan*, N. Takada, Ed. (Hokuryukan, 2019), pp. 118–147.
57. S. Noda, S. Yamamoto, T. Honda, Detection of Japanese spotted fever rickettsiae DNA from ixodid ticks and seasonal occurrence of ixodid tick population on the Osumi Peninsula, Kagoshima Prefecture. *Med. Entomol. Zool.* **55**, 21–28 (2004).
58. M. Sato *et al.*, Diversity and distribution of ticks in Niigata prefecture, Japan (2016–2018): Changes since 1950. *Ticks Tick Borne Dis.* **12**, 101683 (2021).
59. H. Inokuma *et al.*, Tick infestation of sika deer (Cervus nippon) in the western part of Yamaguchi Prefecture, Japan. *J. Vet. Med. Sci.* **64**, 615–617 (2002).
60. F. Billoir *et al.*, Phylogeny of the genus flavivirus using complete coding sequences of arthropod-borne viruses and viruses with no known vector. *J. Gen. Virol.* **81**, 781–790 (2000).
61. S. Tajima, T. Takasaki, S. Matsuno, M. Nakayama, I. Kurane, Genetic characterization of Yokose virus, a flavivirus isolated from the bat in Japan. *Virology* **332**, 38–44 (2005).
62. L. Uchida *et al.*, Survey of tick-borne zoonotic viruses in wild deer in Hokkaido, Japan. *J. Vet. Med. Sci.* **80**, 985–988 (2018).
63. D. Jamsransuren *et al.*, Epidemiological survey of tick-borne encephalitis virus infection in wild animals on Hokkaido and Honshu islands, Japan. *Jpn. J. Vet. Res.* **67**, 163–172 (2019).
64. K. R. Blasdell, J. W. Wynne, D. Perera, C. Firth, First detection of a novel "unknown host" flavivirus in a Malaysian rodent. *Access Microbiol.* **3**, 000223 (2021).
65. D. Kobayashi *et al.*, A novel nyavirus lacking matrix and glycoprotein genes from Argas japonicus ticks. *Virus Res.* **292**, 198254 (2021).
66. D. Kobayashi *et al.*, Detection of a novel putative phlebovirus and first isolation of Dugbe virus from ticks in Accra, Ghana. *Ticks Tick Borne Dis.* **8**, 640–645 (2017).
67. D. J. Lipman, W. R. Pearson, Rapid and sensitive protein similarity searches. *Science* **227**, 1435–1441 (1985).
68. H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199 (1991).
69. O. Boussif *et al.*, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7297–7301 (1995).
70. K. Katoh, J. Rozewicki, K. D. Yamada, MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.* **20**, 1160–1166 (2019).
71. J. Castresana, Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* **17**, 540–552 (2000).
72. S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **35**, 1547–1549 (2018).
73. J. Felsenstein, Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783–791 (1985).
74. F. Ronquist *et al.*, MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **61**, 539–542 (2012).
75. D. Kobayashi *et al.*, Saruyama virus 18HKR14 gene for polyprotein. GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC657549.1. Deposited 29 November 2021.
76. D. Kobayashi *et al.*, Saruyama virus S-18 gene for polyprotein. GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC658947.1. Deposited 29 November 2021.
77. D. Kobayashi *et al.*, Saruyama virus sika13-51 gene for polyprotein. GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC658948.1. Deposited 29 November 2021.
78. D. Kobayashi *et al.*, Saruyama virus WB13-142 gene for polyprotein. GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC658949.1. Deposited 29 November 2021.
79. D. Kobayashi *et al.*, Saruyama virus WB783 gene for polyprotein. GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC658950.1. Deposited 29 November 2021.