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Natural Satsuma Dwarf Virus Infection of Two Woody Plants, *Daphniphyllum teijsmannii* Zoll. ex Kurz. and *Viburnum odoratissimum* Ker-Gaul. var. *awabuki* (K. Koch) Zabel near Citrus Fields

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Abstract

In Japan, the production of Satsuma mandarin (*Citrus unshiu* Marc.) is seriously affected by *Satsuma dwarf virus* (SDV). This study provides evidence of the natural SDV infection of two woody plants, *Daphniphyllum teijsmannii* and *Viburnum odoratissimum* var. *awabuki*, which are common in the warm temperate forests of Japan. Forty-four species of wild woody plants that grew near several SDV-infested fields were surveyed for the presence of SDV using immunochromatography assay (ICA). Five *D. teijsmannii* trees and four trees of *V. odoratissimum* var. *awabuki* tested positive for SDV. Trees of *D. teijsmannii* and *V. odoratissimum* var. *awabuki* that grew far from the citrus fields tested negative for SDV, suggesting that these species are hosts but not the main natural reservoirs of SDV. Comparisons of the partial nucleotide sequences of the larger coat protein gene showed that SDV isolates from closely-grown trees of citrus, *D. teijsmannii*, and *V. odoratissimum* var. *awabuki* were genetically very homogeneous. The results suggested that the natural transmission of SDV occurred among trees of citrus, *D. teijsmannii*, and *V. odoratissimum* var. *awabuki*.

Discipline: Plant disease
Additional key words: Satsuma mandarin, Soil Transmission

Introduction

Dwarfing of satsuma mandarin (*Citrus unshiu* Marc.), a major citrus variety in Japan, was first noted as early as the 1930s (Iwanami 2010). The disease is currently widespread in citrus-producing areas in Japan and in some parts of China (Chi et al. 1991), Korea (Kim et al. 2001) and Turkey (Azeri 1973). The affected trees typically develop boat-, or spoon-shaped leaves with shortened internodes, and the quality and yields of fruit decline significantly (Iwanami & Koizumi 2000). The causal agent is a spherical virus approximately 26 nm in diameter, which is designated *Satsuma dwarf virus* (SDV), in the genus Sadwavirus of the family *Secoviridae* (Sanfacon et al. 2011). Like most of the viruses in this family, the genome consists of two molecules of linear positive-sense single-stranded RNA (Sanfacon et al. 2011). The coat protein is located in the C-terminal region of the polyprotein encoded by RNA-2 (Iwanami et al. 1999). SDV is readily graft-transmitted between citrus plants. Natural spread among citrus trees, apparently through soil, has been observed in many citrus fields of Japan (Iwanami 2010).

SDV infects most citrus and citrus relatives (Iwanami et al. 1993), although few non-citrus woody hosts have been reported. To date, SDV has been detected by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) only once in *Viburnum odoratissimum* Ker-Gaul.

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var. *awabuki* (K. Koch) Zabel (China laurestine), which is a common wild woody plant in warm areas in Japan (Koizumi et al. 1988). Seedlings of *V. odoratissimum* var. *awabuki* are commonly used as ornamental plants and hedges in home gardens, and some citrus growers use it as a windbreak in citrus fields. Because *V. odoratissimum* var. *awabuki* is phylogenetically remote from the family Rutaceae, the presence of SDV in *V. odoratissimum* var. *awabuki* suggested that SDV has a wide host range among woody plants. However, since the brief report by Koizumi et al., it is not known if *V. odoratissimum* var. *awabuki* is the only non-citrus woody host of SDV or whether there are many other woody hosts. To date, no further biological and molecular characterization of SDV from *V. odoratissimum* var. *awabuki* has been made.

In this study, wild woody plants growing near several SDV-infested fields were surveyed for the presence of SDV using an immunochromatography assay (ICA) developed by Kusano et al (Kusano et al. 2007). SDV-infested citrus fields were selected around Kumano city, Mie prefecture, where SDV infection of *V. odoratissimum* var. *awabuki* had originally been found (Koizumi et al. 1988). The first screening of 44 species of woody plants showed that *Daphniphyllum teijsmannii* Zoll. ex Kurz, which is another common wild woody plant in the warm temperate forests, was also positive for SDV. Further field surveys were conducted to estimate the spatial distribution of the SDV-infected trees of *D. teijsmannii* and *V. odoratissimum* var. *awabuki* that grew near and far from citrus fields. Several trees of *D. teijsmannii* and *V. odoratissimum* var. *awabuki* as well as the satsuma mandarin that tested positive for SDV were closely located in the periphery of one citrus field. The nucleotide sequences of the SDV isolates from these citrus and non-citrus trees were determined and compared to estimate SDV movement among the trees of these completely different species.

Materials and methods

1. Primary field survey

Primary field surveys of the presence of SDV were conducted at four locations near SDV-infested citrus fields around Kumano city, Mie prefecture as shown in Table 1 and Fig. 1, where SDV-infection of *V. odoratissimum* var. *awabuki* was originally reported (Koizumi et al. 1988). For the primary screening, young tender leaves were taken from the 44 woody plants in May (Table 1).

Typically, two leaves from each tree were taken and three or more small leaves were used from trees such as *Cryptomeria japonica* (L. fil.) D. Don and *Myrica rubra* Sieb. et Zucc.. The leaves were then macerated by rubber-mashers in a maceration buffer provided by Mizuho Medy, Saga, Japan. SDV was serologically detected by immediately applying the filtrated macerate to ICA test plates for SDV, which is also provided by Mizuho Medy (Kusano et al. 2007). Visual assessments of the positive reaction were made on site within 15 minutes.

2. Sampling and RNA extraction for RT-PCR and sequencing

Young leaves were taken from six satsuma mandarin trees, five *D. teijsmannii* trees and two *V. odoratissimum* var. *awabuki* trees that grew closely to the periphery of one SDV-infested citrus field (Location 4 in Table 1, Figs. 1 and 2). Total RNA was extracted and purified from 0.1 g of infected young, fully-expanded leaves using an RNeasy plant mini kit (QIAGEN, Valencia, CA). For RT-PCR to amplify the fragment of larger component of coat protein of SDV, complementary DNA was synthesized at 50°C for 30 min using the SDR2CPS1M primer (Table 2), and the Prim Script One Step RT-PCR kit (TAKARA, Shiga, Japan), fol-

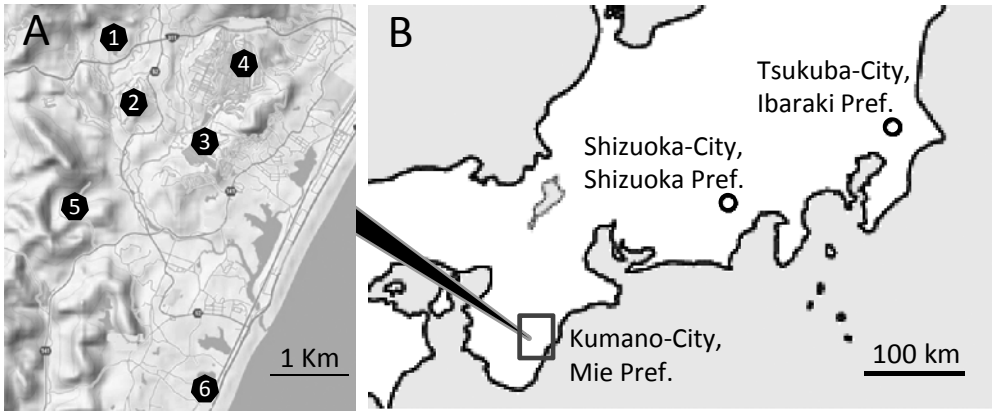

Fig. 1. Locations of the field surveys of *Satsuma dwarf virus* (SDV) infection in woody plants including *Daphniphyllum teijs*mannii and *Viburnum odoratissimum*

Plate A shows an enlarged image of the Kumano area depicted in Plate B. Numbers 1- 4 in Plate A indicate locations 1-4 in Table 1.

Table 1. Results of SDV tests on woody plants in the primary field survey[a)]

Species	Location 1	Location 2	Location 3	Location 4
Akebia quinata (Thunb.) Decne.	0[b)]/3[c)]	0/2		
Ampelopsis brevipedunculata (Maxim.) Trautv. var. *heterophylla* (Thunb.) Hara	0/2	0/5	0/2	0/2
Aralia elata (Miq.) Seemann				0/1
Broussonetia kazinoki Sieb.		0/2		
Castanopsis cuspidata (Thunb. ex Murr.) Schottky		0/1		
Celtis sinensis Pers. var. *japonica* (Planch.) Nakai				0/1
Chamaecyparis obtusa (Sieb. et Zucc.) Endl.				0/3
Cinnamomum japonicum Sieb. ex Nakai				0/3
Clerodendrum trichotomum Thunb.	0/3	0/4		0/7
Cryptomeria japonica (L. fil.) D. Don	0/3			
Daphniphyllum teijsmannii Zoll. ex Kurz	0/3			5/26
Dendropanax trifidus (Thunb.) Makino				0/2
Deutzia crenata Sieb. et Zucc.	0/4	0/2		
Elaeagnus glabra Thunb.			0/3	
Elaeagnus pungens Thunb.	0/2			
Elaeocarpus japonicus Sieb. et Zucc.	0/3	0/1	0/1	0/5
Euonymus japonicus Thunb.				0/1
Eurya japonica Thunb.	0/11	0/1	0/1	0/30
Ficus erecta Thunb.	0/4	0/1	0/3	0/1
Ilex rotunda Thunb.				0/1
Kadsura japonica (Thunb.) Dunal	0/5			0/1
Ligustrum japonicum Thunb.				0/4
Litsea coreana Lév.				0/1
Lonicera japonica Thunb.				0/1
Machilus thunbergii Sieb. et Zucc.			0/2	0/5
Mallotus japonicus (Thunb. ex Murr.) Müll.	0/4	0/7	0/2	0/5
Morus australis Poir.		0/1		
Myrica rubra Sieb. et Zucc.		0/1		
Myrsine seguinii Lév.				0/2
Parthenocissus tricuspidata (Sieb. et Zucc.) Planch.				0/1
Pittosporum tobira (Thunb. ex Murr.) Aiton		0/1		0/1
Prunus jamasakura Sieb. ex Koidz.				0/1
Quercus glauca Thunb. ex Murr.	0/2			
Rhododendron reticulatum D. Don				0/1
Rhus succedanea L.		0/1	0/1	0/3
Rosa multiflora Thunb.	0/5		0/2	0/3
Rubus parvifolius L.			0/1	
Rubus sumatranus Miq.	0/1			
Sinomenium acutum (Thunb.) Rehd. et Wils.				0/2
Smilax china L.				0/7
Stephania japonica (Thunb.) Miers.	0/1			
Symplocos glauca (Thunb.) Koidz.	0/1			
Viburnum odoratissimum Ker-Gaul. var. *awabuki* (K. Koch) Zabel		1/5	1/6	2/7
Vitis thunbergii Sieb. et Zucc.			0/1	

a) Reading was made 15 min after applying three drops of fresh leaf extract to the ICA test plate.
b) The number of trees positive for SDV, c) The number of trees tested.

lowing the manufacturer's instruction. Subsequently, PCR was conducted to amplify the synthesized cDNA; with 35 cycles of initial denaturation at 95°C for 1 min, annealing at 52°C for 1 min, extension at 68°C for 2 min, and followed by a final extension at 72°C for 10 min, using the specific SDR2MP1P and SDR2CPS1M primers (underlined in Table 2). These primers were designed to amplify part of RNA-2, including the larger coat protein gene (Iwanami et al. 1999). Direct cycle sequencing of the RT-PCR product (approx. 1.5k bp) was performed with the sequencing primers shown in Table 2 and the BigDye Terminator v3.1 cycle sequencing kit (Life Technologies Corporation, Carlsbad, CA), following separation by electrophoresis in agarose gel and elution and purification from the gel using the QIAquick gel

Table 2. Primers used for RT-PCR amplification and sequence analysis of the SDV coat protein gene

Primer	Polarity	Nucleotide sequence (5' to 3')	Expected annealing site[a)]
SDR2MP1P[b)]	+	TCAACGCTGAAATCCCGACAA	2961-2981
SDR2CPS1M[b)]	−	GTATGGTCTCATCCAAGCTGAG	4524-4544
SDR2CPL2P [c)]	+	CACTAAGGTCTCCTTATTATCT	3364-3385
SDR2CPL3P [c)]	+	GTACATATCTGTGTGTCCAGC	3742-3762
SDR2CPL4P [c)]	+	CTACAACTAGAACTGGCGGT	4128-4147
SDR2CPL2M [c)]	−	AAGGATAGGGGTCACAAGTC	3297-3316
SDR2CPL3M [c)]	−	CCAACGGTAGAAGAGGATTTA	3712-3732
SDR2CPL4M [c)]	−	AACAATTCGGAATATCACTCTTA	4158-4180

a) The numbers indicate the nucleotide positions in the RNA-2 sequence of the SDV isolate S-58 (AB009959).
b) Primers used for RT-PCR were underlined.
c) Used for sequencing only.

extraction kit (QIAGEN). Sequences were determined using an ABI 3130xl automated sequencer. The sequence data was compiled to complete sequences of the larger coat protein gene with the Genetyx ver. 10 program package, which is commercially available from Genetyx Corporation, Tokyo, Japan.

3. Phylogenetic analysis

Multiple alignments of the sequences were performed with the CLUSTAL X program (Thompson et al. 1997). Phylogenetic and molecular evolutionary analyses were also conducted following the neighbor-joining method using version 3.1 of the MEGA5 program, (Tamura et al. 2011) after bootstrapping 1,000 replicates.

Results

1. Primary field survey

Five *D. teijsmannii* trees and four *V. odoratissimum* var. *awabuki* trees tested positive for SDV in ICA (Table 1). All five SDV-positive *D. teijsmannii* trees grew naturally at the border of the citrus field that was designated at Location 4 (Table 1 and Fig. 1). The other species of wild woody plants from the four locations tested negative for SDV (Table 1).

2. Spatial distribution of SDV-infected *D. teijsmannii* and *V. odoratissimum* var. *awabuki* trees

The spatial distribution of SDV-infected *D. teijsmannii* and *V. odoratissimum* trees as well as satsuma mandarin trees in the Location 4 was further examined. All SDV positive trees of *D. teijsmanni* and *V. odoratissimum* var. *awabuki* grew at the periphery of the field where SDV-positive satsuma mandarin trees were clustered (Fig. 2).

Furthermore, two and 14 *D. teijsmannii* trees that grew naturally in the mountain area (Location 5) and coastal forest (location 6), respectively, which were far from citrus fields, also tested negative in ICA (Fig. 1). When ICA was performed on four *D. teijsmannii* trees planted ornamentally near our research institute at Tsukuba city, Ibaraki prefecture, they were also negative (Fig. 1). Similarly, two trees of *V. odoratissimum* var. *awabuki* in another SDV-infested field in Shizuoka prefecture tested positive while several

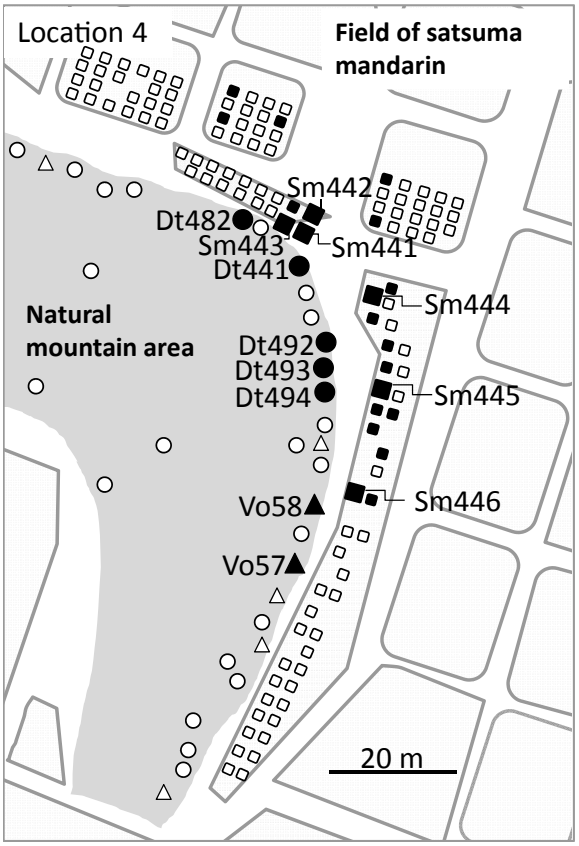

Fig. 2. Distribution of SDV-infected trees in the citrus field (Location 4) in Kumano city, Mie prefecture, Japan
The symbols show the positions of the trees. Open circle, healthy *D. teijsmannii*; filled circle, infected *D. teijsmannii*; open triangle, healthy *V. odoratissimum* var. *awabuki*; filled triangle, infected *V. odoratissimum* var. *awabuki*; Open square, healthy satsuma mandarin; filled square, infected satsuma mandarin. The source trees that were used to analyze the nucleotide sequence for SDV (Fig. 3) are shown with larger symbols.

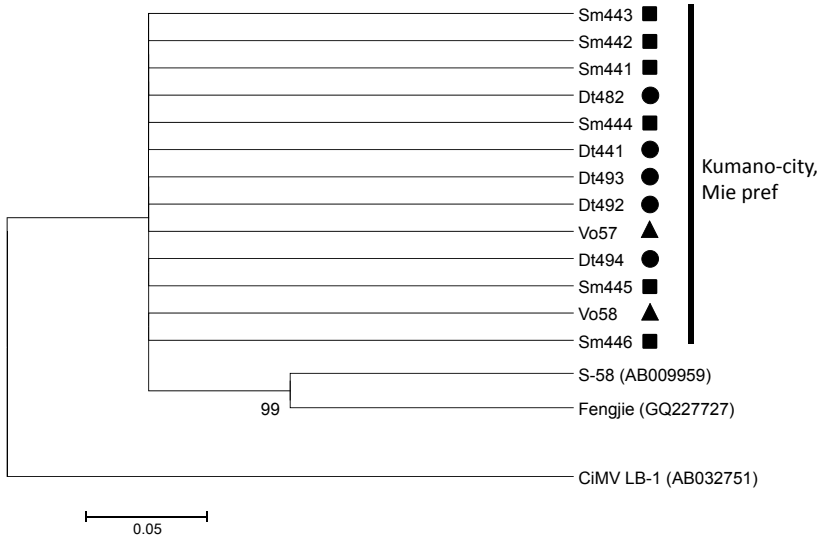

Fig. 3. The phylogenetic tree

The consensus phylogenetic tree was generated from alignments of the nucleotide sequences of the larger coat protein regions (1329 nt long) of SDV isolates from five *D. teijsmannii* trees (Dt), two *V. odoratissimum* var. *awabuki* trees (Vo) and six satsuma mandarin trees (Sm) in Location 4 (Fig. 2) by the neighbor-joining method using the MEGA5 program, version 5.2 (Tamura et al. 2011) after bootstrapping in 1000 replicates. SDV isolate S-58 (AB009959), isolated from satsuma mandarin trees in Shizuoka prefecture, and SDV isolate Fengjie (GQ227727), isolated from Satsuma mandarin trees in China, were incorporated into phylogenic analysis as SDV isolates of other fields. CiMV isolate LB-1 (AB032751) was used as an outer-group. The bar represents a phylogenetic distance of 0.05%. Nodes with low bootstrap values (<70) were collapsed.

ornamental trees of *V. odoratissimum* var. *awabuki* that grew without citrus trees in our research institute tested negative in ICA (Fig. 1). Based on these field surveys, it was concluded that *D. teijsmannii* and *V. odoratissimum* var. *awabuki* did not always harbor SDV but that some *D. teijsmannii* and *V. odoratissimum* var. *awabuki* that grew near SDV-infested citrus field were infected with SDV.

3. Sequence analysis of the larger coat protein gene of SDV from *D. teijsmannii* and *V. odoratissimum* var. *awabuki*, and satsuma mandarin trees

When nucleotide sequences of the larger component of coat protein gene (1329 nt) of SDV from *D. teijsmannii*, *V. odoratissimum* var. *awabuki*, and satsuma mandarin trees were compared, they were remarkably similar, regardless of the host plants. The sequence identities were over 98%. Phylogenetic analysis indicated that SDV isolates of different host plants around the same citrus field in Kumano city, Mie were closely related, yet they were distinct from SDV isolates from Shizuoka (S-58) and Chongqing, China (Fenjiie) (Fig. 3). These results suggested that SDV is transmitted non-selectively between different species of woody plants.

Discussion

SDV infection of *V. odoratissimum* var. *awabuki* had been previously suggested by the results of ELISA, which was further confirmed by RT-PCR and sequencing in this study. In addition, this study found another woody host, *D. teijsmannii*. Unlike satsuma mandarin however, neither tree showed clear foliar symptoms. Obviously, SDV infection of these woody hosts had not been noticed because they were latently infected. Relatively few viruses have been identified in forest trees, and most are caused by viruses already described under some of the other crops. For example, birch line pattern is caused by apple mosaic virus, while elm mosaic is caused by cherry leafroll virus (Agrios 1988). SDV infection of *D. teijsmannii* and *V. odoratissimum* var. *awabuki* is another example of such viral infection of forest trees. SDV infects a wide range of herbaceous hosts (Iwanami, T. 2010). This study confirmed that SDV infects three completely different hosts (satsuma mandarin, *D. teijsmannii* and *V. odoratissimum* var. *awabuki*). Because these three woody plants are not phylogenetically close, the results of this study indicated a wide host range of SDV among woody plants. In addition, transmission among these different spe-

cies of trees supports the hypothesis of SDV soil transmission by some vector, which had been postulated based on field observation.

In this study, all but two of the woody plants in Table 1 tested positive in ICA. Optimization of ICA for these apparently negative woody plants might have made some plants test positive. Further study, including more sensitive RT-PCR, is needed to confirm the negative infection status of these woody plants.

Sequence analyses indicated that SDV transmission occurred primarily between the closely-grown trees of satsuma mandarin, *D. teijsmannii* and *V. odoratissimum* var. *awabuki*. Infected trees of *D. teijsmannii* and *V. odoratissimum* var. *awabuki* were found at the border of one citrus field, but not in the wild forest, apart from citrus trees. It can be surmised from these results that *D. teijsmannii* and *V. odoratissimum* var. *awabuki* are hosts but not main natural reservoirs of SDV.

From a practical perspective, it should be noted that both *D. teijsmannii* and *V. odoratissimum* var. *awabuki* are popular ornamental plants and often grown with citrus in the same nurseries. It is possible that SDV could spread from *D. teijsmannii* and *V. odoratissimum* var. *awabuki* to citrus and vice versa in these nurseries. Thus, care should be taken lest contaminated nursery plants are distributed from these nurseries.

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