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Characterization of three new viruses of the family *Betaflexiviridae* associated with camellia ringspot disease

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ABSTRACT

Foliar chlorotic and necrotic ringspots of different sizes were observed in many ornamental camellia (*Camellia* spp.) species and cultivars with or without variegation symptoms. In this study, flexuous, filamentous virions of approximately 680–780 nm long were observed by electron microscopy in sap of camellia trees with chlorotic ringspots. Five large viral contigs were identified by high-throughput sequencing technology, and complete genome sequences of them were determined. Sequence analyses show that these five isolates represent three novel viruses, two in the genus *Prunevirus*, one in the genus *Capillovirus*. The genome organization of the two camellia pruneviruses resembles that of pruneviruses but does not contain the nucleic acid-binding protein (NABP) at the 3′-terminal region. They share 66.5–66.8% with each other and 51.9–58.6% with the known pruneviruses at the genome sequence level. The genome of the camellia capillovirus contains an additional NABP at the 3′-terminus when compared to those of *Capillovirus*. The genomes of the two capillovirus variants are 72.7% identical to each other and 42.1–48.4% to the known capilloviruses. Phylogenetic analyses support these viruses are new members of either *Prunevirus* or *Capillovirus*. The two pruneviruses are tentatively named as camellia ringspot associated virus 1 (CRSaV-1) and CRSaV-2, and the capillovirus is named as CRSaV-3. Infections of these viruses were common in camellia species, cultivars and hybrids. The viruses were also detected in seedlings from seeds collected from two camellia trees, indicating that they are seed transmissible.

1. Introduction

Camellias *(Camellia* spp.) are an economically important group of perennial evergreen flowering plants in the family *Theaceae* that includes tea, oil camellia and several ornamental species (Gao et al., 2005). They originated from East and Southeast Asia and have been cultivated in those regions for thousands of years. There are more than 250 species in the family, with *C. japonica*, *C. reticulara*, *C. sasanqua* and their hybrids being popular ornamental plants in many countries. *C. japonica* was first introduced to North America in the late 1700s [2] (Curtis, 1790) where it is a widely grown flowering and landscaping shrub, especially in the southeast region of the United States. It is popular due to its wide range of bloom colors, attractive flower shapes, and varied seasonal blossoming (King, 2012).

Although new camellia cultivars are derived from crosses, most of them are propagated from cuttings, especially ornamental species and cultivars, Therefore, they are prone to accumulating viral infections. Virus-like symptoms that have been reported include mottle, mosaic and ringspots on leaves (Milbrath and McWhorter, 1946; Ahlawat and Sardar, 1973; Gailhofer et al., 1988) and variegation (color breaking) on flowers and leaves (Hildebrand, 1954; Plakidas, 1954; Hiruki, 1985; Ofsoski et al., 1990). The mottling symptoms vary from mild to severe, and their appearances are even, marginal or irregular. Different sizes and shapes of light green, yellowish or necrotic ringspots with surrounding islands of green on the leaves are frequently observed, too. The variegation of flowers is often a desired ornamental characteristic

Abbreviations: NABP, nucleic acid binding protein; CRSaV, camellia ringspot associated virus; HTS, high-throughput sequencing; BLAST, basic local alignment search tool; RT-PCR, reverse transcriptase-polymerase chain reaction; RACE, rapid amplification of cDNA ends; nt, nucleotide; aa, amino acid; CPrV, Caucasus prunus virus; CVA, cherry virus A; CP, coat protein; ORF, open reading frame; PVT, potato virus T; AVCaV, apricot vein clearing associated virus; UTR, untranslated region; RdRp, RNA dependent RNA polymerase; MP, movement protein; ASGV, apple stem grooving virus; CuVA, currant virus A

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of camellia where graft-transmissibility of the trait can create uncertainty about its cause but may suggest viral etiology (Hiruki, 1985; Plakidas, 1954; Sharma and Raychaudhuri, 1972; Inouye, 1982; Dardick, 2005; Valverde et al., 2012). Rod-shaped (bacilliform) viral particles of 120–170 x 30 nm were observed by electron microscopy in *C. japonica* with yellow mottle and flower variegation (Gailhofer et al., 1988; Hiruki, 1985; Inouye and Inouye, 1975) and *C. reticulata* with leaf and stem distortions (Ofsoski et al., 1990), and Hiruki (1985) was unable to transfer the virus to several herbaceous plant species by sap inoculation. The virus was named as camellia leaf yellow mottle virus [6, 8] (Gailhofer et al., 1988; Hiruki, 1985). Similar virus particles were also observed in camellia trees with flower and foliar variegation, and the name camellia infectious variegation virus was proposed (Miličić et al., 1986). However, neither antiserum nor genomic information is available for these viruses, making it difficult to determine their taxonomic position and study them further. It is also uncertain whether the rod-shaped virus caused the yellow mottling and/or variegation diseases.

Viral pathogens associated with variegation and yellow mosaic symptoms of camellias were transmitted to healthy camellia plants by grafting (Hiruki, 1985; Tourje, 1950; Plakidas, 1954; Sharma and Raychaudhuri, 1972; Inouye, 1982). A possible virus pathogen associated with tea rose yellow mosaic symptom was transmitted to *C. japonica* and *C. sinensis* plants by the citrus brown aphid (*Toxoptera aurantii*) and dodder (*Cuscuta reflexa*) (Ahlawat and Sardar, 1973). The pathogen was also transmitted to *Duranta plumieri* plants by dodder (Ahlawat and Sardar, 1973). Plakidas (1991) described the transmission of the flower variegation through root grafting. A virus associated with flower variegation and ringspots was transmitted by an obligatory soil fungus, *Olpidium* spp. (Hiruki and Merz, 2013). We report here the discovery and characterization of three different members of the family *Betaflexiviridae* from a camellia tree with ringspot symptom. Infections of these viruses are common in ornamental camellias, and the viruses are transmitted through seed.

2. Materials and methods

2.1. Virus sources

For electron microscopy, leaves were collected from trees of cultivars CJ5 and Herme. CJ5 was a camellia tree (*C. japonica*, cultivar unknown) in a public garden in Montgomery County Maryland (39.06 °N, 77.03 °W) with foliar chlorotic ringspots of about 2–3 mm in diameter (Fig. 1). The tree produced spring blossoms with red petals. The Herme tree was purchased from a commercial nursery and maintained in an insect-proof greenhouse. It displayed foliar chlorotic ringspots on some leaves and flower variegation. CJ5 was used for high-throughput sequencing (HTS) analysis. For virus detection among various species and cultivars, leaf samples were collected from 23 more trees in the same Maryland public garden along with 15 locally purchased trees maintained in the greenhouse (Table 1).

2.2. Transmission electron microscopy

Leaf sap dips or partially purified viral preparations were stained with 3% uranyl acetate (pH 4.2) and examined with a Hitachi H-7000 transmission electron microscope (Hitachi, Ltd.). Images with virus particles were recorded digitally and a few were measured.

2.3. High-throughput sequencing and data analyses

Total nucleic acids were extracted from CJ5 by a CTAB method (Li et al., 2008) and then used for isolation of total RNAs by RNeasy® Plant Mini Kit according to the manufacturer's instructions (Qigen, Maryland, USA). The RNA sample was subjected to Illumina RNA sequencing (SeqMatic, Fremont, CA). Plant ribosomal RNAs (rRNA) were removed from total RNAs by Epicentre Ribo-Zero Kit, and cDNA library was constructed using SeqMatic TRailorMix Directional RNA Library Preparation Kit. The resulting fragments were sequenced on the Illumina NextSeq 500 sequencing platform with 20-sample bar-coded multiplexing. Analyses of the total RNA reads were performed using CLC Genomics Workbench (version 9.5.2, Qiagen). The raw reads were first filtered to remove failed reads, and qualified reads were then assembled into contigs using *de novo* assembly tool. Contigs were annotated by BLASTx comparisons to two databases including Viruses_NR and Viroid_NT downloaded from NCBI GenBank. A local database containing uquiqe sequences of 40 phytoplasmas were provided by Dr. Yan Zhao of USDA-ARS.

2.4. Analyses of genomic sequences

To obtain the genomic sequence of each virus, specific primers (Table S1) were designed based on alignment of five contig sequences representing three putative new viruses. Each of the overlapping primer pairs was designed at virus/variant-specific region with insertion or deletion or low identity. RT-PCR was conducted using SuperScript™III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA). The 5′-end sequence was obtained using a 5′ RACE System Kit (Invitrogen), and the 3′-terminus was amplified by a virus-specific forward and oligo (dT) primers. RT-PCR reactions were performed in a 25-µl reaction containing 1 µl of total nucleic acid extract, 1.0 µl of each primer (5 µM), 12.5 µl of 2× Reaction Mix, 0.5 µl of Enzyme Mix and 9.0 µl of water. The thermal cycling conditions for RT-PCR were 1 cycle of denaturation at 50 °C for 30 min and 94 °C for 2 min, 35 cycles of amplification at 94 °C for 30 s, 60 °C for 30 s and 68 °C for 2 min and one final cycle of extension at 68 °C for 5 min. PCR products were electrophoresed on 1% agarose gels in TAE buffer and visualized by staining with ethidium bromide (0.5 µg/ml) under UV illumination. Amplicons were isolated and cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), and plasmid DNAs isolated from overnight cultures of selected colonies were sequenced by MCLAB (San Francisco, CA, USA).

Sequence reads were assembled and analyzed by the CLC Genomics Workbench. Pairwise comparisons of the genome and putative protein sequences were conducted by Muscle of EMBL-EBI at https://www.ebi.ac.uk/Tools/msa/muscle/. The conserved domains of putative gene products were searched by Motif Search at http://www.genome.jp/tools/motif/. Phylogenetic relationships of the new viruses/variants and 20 other viruses of the *Betaflexiviridae* family were analyzed using genomic and putative protein sequences by MEGA 7.0 (Kumar et al., 2016). The viral evolutionary history was inferred using the Neighbor-Joining method. Genome sequences of 103 species and tentative species of the *Betaflexiviridae* were aligned by ClustalW of the MEGA 7.0, and aligned sequences were analyzed by RDP 4.83 package (Martin et al., 2015) for recombination event.

2.5. RT-PCR detection

To detect the three new viruses from infected plants, several virus-specific primer pairs were designed to anneal to unique regions of each of them. Primer pairs specific to regions conserved in the two of them were also designed to detect both viruses. Each of the primer pairs was designed for specificity at regions with insertion or deletion (gap). The primer pairs were tested by RT-PCR using CJ5 as the positive control. The negative control was an oil camellia (*C. olerifera*) seedling that was free of the three new viruses. Thirty-eight additional samples were collected from both symptomatic and asymptomatic camellia trees from the public garden and our greenhouse, respectively (Table 1). Total nucleic acids were prepared from leaf tissue of each sample by the CTAB method and used as templates for RT-PCR as described above. Viral amplicons of selected samples of each source were cloned and sequenced.

Fig. 1. Foliar chlorotic ringspots of the camellia tree (CJ5) used for high-throughput RNA sequencing analysis (A), and filamentous virus particles observed by a transmission electron microscope in sap preparation of camellia CJ5 (B) and 'Herme' (C). The number of length is shown above the bar.

2.6. Virus transmission

The following plant species were used for mechanical inoculation: *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana benthamiana*, *N. clevelandii*, *N. occidentalis*, *N. tabacum*. Leaves of the infected plants were triturated in cold 50 mM potassium phosphate buffer, pH 7.0 (1: 10, w/v), containing 2% PVP (10), and the plant sap was gently rubbed onto carborundum-dusted leaves of at least four plants of each testing species. After inoculation, plants were kept in an insect-proof greenhouse at 23–27 °C with 14 h light for up to 8 weeks for observation of symptom development.

The CJ5 tree did not produce any seed, but infected cv. Elaine Lee and hybrid Winter's Star did. The RT-PCR showed that the Elaine Lee tree was infected with at least two of the three viruses, and the Winter's Star tree was infected only with one virus. To investigate the seed transmissibility of the viruses, mature seeds were collected from these two trees in the garden in April 2017. Seeds were also collected from the oil camellia negative control. The seed were first surface sterilized with 10% commercial bleach for 30 min, washed three times with distilled water and then soaked in water overnight before planting in 8-inch pots. Each of the 2-week seedlings was transplanted into a torpedo pot one month later and maintained in the greenhouse. Leaves were tested by RT-PCR for the viruses at 3-, 6-, 9-, 12- and 16-months after planting.

3. Results

3.1. Symptoms

Among the 23 trees grown in the public garden, ten had foliar ringspots of various sizes and numbers. The symptoms usually appeared on the newly expanded leaves as mottle and then as chlorotic ringspots in spring and summer. The chlorotic ringspots turned into necrotic ringspots in winter along with the winter damage (dark reddish leaves) in Maryland condition. However, not all the mottle symptoms developed to ringspots, and not all the ringspots had a defined circular edge. The ringspot symptom occurred with or without other symptoms such as mottle and/or variegation on the same or different leaves of a camellia tree. The ringspots did not appear every year, and the number of leaves with the symptoms varied between years. For examples, many leaves of the CJ5 tree showed defined ringspot symptom in the spring of 2016 (Fig. 1A), but only few leaves had the symptom in the spring of

Table 1
Plants of *Camellia spp.* tested in this study.

Cultivar	Symptoms	Source	Virus[a]
CJ5[b]	Foliar ringspots[c]	Community Park	Prunevirus group, capillovirus
Pink Perfection	No symptoms	Community Park	Prunevirus group, capillovirus
Lady Clare	Foliar ringspots; flower variegation	Community Park	Prunevirus group, capillovirus
Jury's Yellow	Foliar ringspots	Community Park	Prunevirus group, capillovirus[d]
Jacks	Foliar mottle and ringspots	Community Park	Prunevirus group
Tricolor	Flower variegation	Community Park	Prunevirus group, capillovirus
RL Wheeler	Foliar ringspots; flower variegation	Community Park	Prunevirus group, capillovirus
Berenice Boddy	Foliar ringspots; rough vein; flower variegation	Community Park	Prunevirus group, capillovirus
Winter's Star[e]	No symptoms	Community Park	Prunevirus group[d]
Winter's Waterlily	No symptoms	Community Park	None
Winter's Interclude	No symptoms	Community Park	None
Winter's Beauty	No symptoms	Community Park	None
Aston's Pride	Mild ringspots	Community Park	Prunevirus group
Paulette Goddard[e]	Foliar ringspots; foliar and flower variegation	Community Park	Prunevirus group
Spring Cardinal	Foliar mottle	Community Park	Prunevirus group
April Tryst[e]	Foliar mottle and ringspots	Community Park	Capillovirus
April Dawn	Foliar mottle and ringspots	Community Park	Prunevirus group
April Remembered	No symptoms	Community Park	Prunevirus group, capillovirus
Snow Flurry	Foliar ringspots	Community Park	Prunevirus group
Long Island Pink	No symptoms	Community Park	Prunevirus group, capillovirus
Hagoromo	No symptoms	Community Park	Prunevirus group
Tama-no-ura	No symptoms	Community Park	None
Korea Fire[d,e]	Mottle	Community Park	None
Elaine Lee	Foliar ringspots	Community Park	Prunevirus group, capillovirus
Herme[d]	Foliar chlorotic ringspots; flower variegation	Nursery	Prunevirus group, capillovirus
Dad's Pink	Flower variegation	Nursery	None
Dalkagura[e]	Foliar ringspots; flower variegation	Nursery	Prunevirus group, capillovirus
Goggy	Foliar and flower variegation	Nursery	None
Kumasaka[e]	Foliar mottle; foliar and flower variegation	Nursery	None
Lemon Glow[e]	Foliar mottle	Nursery	Prunevirus group
Lady Laura	Flower variegation	Nursery	None
Shibori-Egao[e]	Foliar and flower variegation	Nursery	Capillovirus
Snow Flurry[e]	Foliar mottle, ringspots and distortion	Nursery	Prunevirus group
RL Wheeler	Foliar ringspots; flower variegation	Nursery	Prunevirus group, capillovirus
Lady Clara	Flower variegation	Nursery	None
Winter's Charm	Foliar ringspots	Nursery	Capillovirus
Jerry's Hill	Foliar ringspots	Nursery	Prunevirus group
Adolphe Audusson	Foliar ringspots	Nursery	Prunevirus group, capillovirus
Spring Awakening	Foliar ringspots	Nursery	Prunevirus group, capillovirus
C. oleifera[c]	None	Seedling	None

[a] Prunevirus group = Camellia ringspot associated virus 1 (CRSaV-1) or CRSaV-2 or both. One RT-PCR assay was used to detect both CRSaV-1 and CRSaV-2 so the two pruneviruses could not be distinguished. Therefore, the amplification by RT-PCR for the prunevirus group showed that the tree was infected by either of the two viruses or both. Capillovirus = CRSaV-3.
[b] Camellia plant used for high-throughput sequencing analysis. Cultivar name is not available.
[c] Ringspots vary in size among different cultivars and color between seasons.
[d] Weak reaction was confirmed by another RT-PCR test and sequencing.
[e] Infection and non-infection of the tree by either one or both virus groups were confirmed by sequencing.

2017. Necrotic ringspots appeared on some leaves in 2018 after a cold winter in 2017. The Elaine Lee tree had no foliar ringspots in the spring of 2016 and 2017, but a few leaves showed the symptom in the early spring of 2018. The Korea Fire tree had severe mottling in 2017 and did not show any symptoms in 2018. The RL Wheeler tree had large chlorotic ringspots and flower variegation in 2017, but only flower variegation in 2018. The fifteen trees purchased from the nursery had obvious symptoms at the time of purchase (Table 1). Variegation symptoms, especially the flower variegation, did not show up in the greenhouse every year after the first year on some varieties. The foliar mottle and ringspot symptoms of most cultivars were consistent, although the severity varied between seasons. However, the symptoms disappeared in cvs. Lemon Glow, RL Wheeler, Winter's Charm and Spring Awakening under greenhouse conditions.

3.2. Virus particles

Flexuous, filamentous particles were observed in leaf-dip preparations of both trees (Fig. 1B -C). Virus particles of 660–880 x 12 nm and 465–815 x 12 nm were obtained in negatively stained virus preparation from the CJ5 (33 particles) and Herme (29 particles) trees, respectively.

3.3. Virus identification by high-throughput sequencing

A total of 30,434,100 RNA reads of 76 nucleotides (nt) was obtained from CJ5 by Illumina NextSeq sequencing after removing the failed reads. *De novo* assembly of these reads generated a total of 91,904 contigs (≥ 200 nt). Blastx search of the contigs against the Viruses_NR database revealed the presence of three [7723 nt (CJ5_1176), 7723 nt (CJ5_6003) and 7,053 nt (CJ5_2013)] and two [7,417 nt (CJ5_676) and 7,411 nt (CJ5_807)] contigs with the highest degree of amino acid (aa) sequence identities to the replicase domain of Caucasus prunus virus (CPrV, genus *Prunevirus*) (51.5–65.8%) and cherry virus A (CVA, genus *Capillovirus*) (21.8-30.4%) in the subfamily *Trivirinae* of the family *Betaflexiviridae*, respectively. These contigs represented nearly full-length genomes of at least two viruses. Mapping of these contigs to RNA reads with 0.98 similarity showed coverage of 146-, 234- and 55-fold to the three CPrV-like contigs, and 1,159- and 1,450-fold to the two CVA-like contigs, respectively, supporting the presence of these viruses/variants in this plant. No viroid or phytoplasma was identified by HTS from this plant.

Table 2
Percentage identities[a] of nucleotide (nt) or amino acid (aa) sequences between each of the three new viruses and selected viruses in the family *Betaflexiviridae*.

Virus[b]	CCRSaV-1				CCRSaV-2-CJ5_6003				CCRSaV-3-CJ5_676				
	Genome (nt)	Replicase (aa)	MP (aa)	CP (aa)	Genome (nt)	Replicase (aa)	MP (aa)	CP (aa)	Genome (nt)	Replicase (aa)	MP (aa)	CP (aa)	NBP (aa)[3]
CCRSaV-2-CJ5_6003	66.8	64.2	75.2	90.5	100.0	100.0	100.0	100.0	45.1	27.2	12.8	25.0	N/A
CCRSaV-2-CJ5_2013	66.5	64.2	75.7	89.6	85.6	91.0	90.5	97.3	45.4	27.2	12.5	25.0	N/A
CCRSaV-3-CJ5_676	44.9	27.1	12.5	25.9	45.1	27.2	12.8	25.0	100.0	100.0	100.0	100.0	100.0
CCRSaV-3-CJ5_807	45.4	29.8	13.5	26.2	44.9	30.5	15.1	25.0	72.7	67.4	80.9	91.3	72.9
AVCaV	51.9	42.4	43.8	32.0	52.3	42.8	44.2	30.6	42.4	24.9	13.9	32.2	16.0
CPrV	57.4	51.5	54.6	64.4	58.6	51.6	54.1	65.8	43.9	26.5	11.4	25.0	17.1
ASGV	45.2	28.6	16.0	25.6	45.2	28.6	16.4	26.5	42.1	23.0	21.3	29.6	N/A
CVA	45.7	22.0	13.2	23.3	45.7	22.1	12.8	22.8	48.4	28.4	21.8	30.4	N/A
CuVA	45.1	22.9	11.6	23.3	45.3	22.8	13.2	22.8	47.5	27.8	23.2	33.2	N/A
CLBV	50.3	39.9	43.4	12.1	50.6	39.1	42.3	10.2	42.0	26.2	13.0	12.1	N/A
ACLSV	45.4	31.1	13.0	25.5	45.7	32.4	14.7	27.0	41.9	25.7	15.3	21.5	N/A
PrVT	45.8	31.1	17.4	25.0	47.0	30.6	17.1	26.0	42.1	25.7	15.5	21.0	N/A
CtCV-1	44.9	30.7	16.1	16.2	45.7	32.5	15.8	16.7	42.1	26.0	20.5	17.2	N/A
DiVA	45.1	29.5	14.4	22.1	45.6	30.0	16.1	22.6	42.5	23.6	17.8	17.5	N/A
GVA	43.6	28.7	12.1	22.5	43.9	28.2	10.9	25.0	39.4	23.9	15.2	28.6	N/A
LNRSV	43.0	28.4	15.5	17.5	45.1	29.4	15.5	17.5	40.0	24.3	5.8	11.3	18.3
ASPV	44.9	29.1	13.8	14.5	46.2	29.5	14.8	15.0	41.2	26.2	11.3	12.5	N/A
CNRSV	44.1	29.3	15.2	11.7	44.4	29.7	16.1	12.1	41.0	26.5	9.1	10.0	N/A

[a] Result of pairwise comparison.

[b] CRSaV – Camellia ringspot associated virus; ACLSV – Apple chlorotic leaf spot virus (NC_001409); AVCaV – Apricot vein clearing associated virus (NC_023295); Caucasus prunus virus – CPrV (KM507061); CLBV – Citrus leaf blotch virus (NC_003877); ASGV – Apple stem grooving virus (NC_001749); CVA – Cherry virus A (NC_003689); CuVA Currant virus A (NC_029301); PrVT – Prunus virus T (NC_024685); CtCV-1 – Carrot Ch virus 1 (NC_025469); DiVA – Diuris virus A (NC_019029); GVA – Grapevine virus A (NC_003604); LNRSV – Ligustrum necrotic ringspot virus (NC_010305); ASPV – Apple stem pitting virus (NC_003462); CNRMV – Cherry necrotic rusty mottle virus (NC_002468).

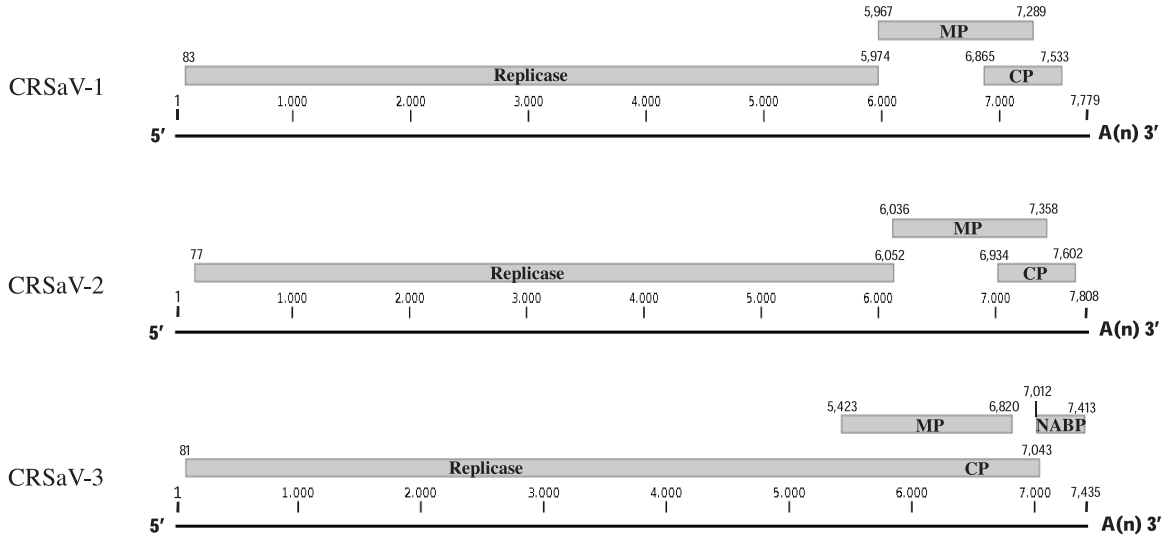

Fig. 2. Genome organization and structure of two camellia ringspot associated viruses (CRSaV). MP, movement protein; CP, coat protein; NABP, nucleic acid-binding protein.

3.4. Analyses of genomic sequences

The complete genomic sequences of three CPrV-like variants and two CVA-like variants were assembled from overlapping RT-PCR amplicons. The genomic sequences of three CPrV-like variants (CJ5_1176, CJ5_6003 and CJ5_2013) were determined to be 7,779 nt (GenBank accession no. MK050792), 7,808 nt (MK050793) and 7,806 nt (MK050794), respectively, excluding the poly(A) tail at the 3′ end. Pairwise comparisons of the genomic sequences of the three CPrV-like variants showed that they shared sequence identities of 66.5–85.6% at whole genome level among each other (Table 2). The genomic sequence identities between CJ5_1776 and the other two variants were 66.5–66.8%, while the identity was 85.6% between CJ5_6003 and CJ5_2013. According to the species demarcation criterion (< 72% identity at genomic sequence) for the family *Betaflaxiviridae* (Adams et al., 2012), the three CPrV-like variants should represent two new viruses, with variant CJ5_1776 belonging to one virus and the other two variants (CJ5_6003 and CJ5_2013) belonging to another virus.

The genomic organization and structure of CRSaV-1 and CRSaV-2 (only data of one variant is presented) are virtually identical to one another, containing three overlapping open reading frames (ORF) (Fig. 2). This structure resembles that of some members of the subfamily *Trivirinae* such as apple chlorotic leaf spot virus (*Trichovirus*), grapevine berry inner necrosis virus (*Trichovirus*), potato virus T (PVT, *Tepovirus*) and prunus virus T (*Tepovirus*) (Rubino et al., 2012; Adams et al., 2012; Marais et al., 2015a), rather than that of CPrV and apricot vein clearing associated virus (AVCaV) of the genus *Prunevirus* which contain an additional ORF encoding a nucleic acid-binding protein (NABP) (Marais et al., 2015b). The 5′ untranslated regions (UTR) are 82 nt for CRSaV-1 and 76 nt for CRSaV-2, while the 3′-UTRs are 246 nt for CRSaV-1 and 206 nt for CRSaV-2. The sequence identities are 59.2% for the 5′ UTRs and 77.2% for the 3′ UTRs, respectively, between the two viruses. ORF 1 (nt 83–5,974 for CRSaV-1; nt 77-6,052 for CRSaV-2) encodes a putative polyprotein of 1,963 aa residues for CRSaV-1 and 1,990 aa residues for CRSaV-2. This protein contains motifs associated with domains of the viral methyltransferase (aa 44–346 for both CRSaV-1 and CRSaV-2), 2OF-Fe(II) oxygenase 2 (aa 883–972 for CRSaV-1; aa 903–996 for CRSaV-2), peptidase_C23 (aa 992-1,078 for CRSaV-1; aa 1,016-1,100 for CRSaV-2), viral RNA helicase 1 (aa 1,171-1,415 for CRSaV-1; aa 1,195-1,442 for CRSaV-2) and RNA dependent RNA polymerase (RdRp, aa 1,171-1,415 for CRSaV-1; aa 1,591-1,907 for CRSaV-2), indicating it is a putative replicase polyprotein (Rubino et al., 2012; Marais et al., 2015a, b; Martelli et al., 1994). Pairwise comparisons show that the replicase aa sequences of the two pruneviruses are 64.2% identical to each other, but the identities between them and CRSaV-3 were only 27.1–30.5% (Table 2). The aa sequence identities of this protein between the CRSaV-1 and selected betaflexiviruses range from 22.0% (CVA) to 51.5% (CPrV), and a very similar result is obtained for CRSaV-2. ORF 2 (nt 5,967-7,289 for CRSaV-1; nt 6,036-7,358 for CRSaV-2) overlaps with both ORF 1 and ORF 3. It encodes a putative protein of the same size (440 aa residues) for CRSaV-1 and CRSaV-2. A viral movement protein (MP) domain is present at aa 61–191 of this protein. The MP aa sequences of the two pruneviruses are 75.2–75.7% identical, but the identities between them and CRSaV-3 are only 12.5–12.8% (Table 2). Overall, aa sequence identities of 10.9–54.6% are obtained between CRSaV-1/CRSaV-2 and the other viruses. ORF 3 (nt 6,865-7,533 for CRSaV-1; nt 6,934-7,602 for CRSaV-2) encodes a protein consisting of 222 aa residues. It contains a trichovirus CP domain at aa 59–219 for both CRSaV-1 and CRSaV-2. The CP aa sequence identity is as high as 91.3% between them, but the identities are only 25.0–26.2% between the two viruses and CRSaV-3 (Table 2). The overall identities are 10.2–65.8% between the two viruses and other selected viruses based on the CP sequences. The results support that CRSaV-1 and CRSaV-2 are most closely realted to CPrV, with identities as 64.4% and 65.8%.

The complete genomic sequences of the two CVA-like variants, CJ5_676 and CJ5_807, were determined to be 7,435 nt (MK050795) and 7,450 nt (MK050796), respectively, excluding the poly(A) tail at the 3′ end. The two variants were 72.7% identical to one another at the whole genome sequence level (Table 2), which is slightly higher than the species demarcation criterion. The two variants were, therefore, considered to belong to the same virus. These results indicate that three distinct betaflexiviruses are associated with the diseased CJ5 camellia, for which we propose the names camellia ringspot associated virus 1 (CRSaV-1, CJ5_1776), CRSaV-2 (CJ5_6003 and CJ5_2013), CRSaV-3 (CJ5_776 and CJ5_807).

The genomes of two CRSaV-3 variants contain three ORFs, a large ORF 1 (nt 83-7,043 for CJ5_676; nt 84-7,046 for CJ5_807), a nested ORF 2 (nt 5,423-6,820 for CJ5_676; nt 5,438-6,835 for CJ5_807) and a small ORF 3 (nt 7,012-7,413 for CJ5_676; nt 7,027-7,428 for CJ5_807) at the 3′ region (Fig. 2, only CJ5_676 is shown). The 5′ UTRs are 80 nt for CJ5_676 and 83 nt for CJ5_807, and they are 50.0–53.8% identical to CRSaV-1 and 53.9–60.9% identical to CRSaV-2. Unlike other betaflexiviruses, the 3′ UTR of CRSaV-3 is very short (22 nt for both variants), due to the addition of the ORF 3. To confirm the 3′ terminal sequences of the two variants, RT-PCR using a variant-specific primer

and oligo d(T) primer was repeated twice, and the same terminal sequence was obtained both times. The arrangement of ORF 1 and ORF 2 of CRSaV-3 is very similar to three members of the genus *Capillovirus* [apple stem grooving virus (ASGV), CVA and currant virus A (CuVA)] (Adams et al., 2012; Jelkmann, 1995; Petrzik et al., 2016), but the presence of ORF 3 is unique to this virus. ORF 1 encodes a polyprotein of 2,292 aa residues for CJ5_676 and 2,320 aa residues for CJ5_807. The polyprotein consists of a methyltransferase (aa 65–346 for CJ5_676 and aa 46–375 for CJ5_807), a viral RNA helicase (aa 814–902 for CJ5_676 and aa 833–922 for CJ5_807), a RdRp domain (aa 1,220-1,551 for CJ5_676 and aa 1,264-1,577 for CJ5_807) and a trichovirus CP (aa 2,120-2,281 for CJ5_676 and aa 2,154-2,313 for CJ5_807) domains. ORF 2 nested within the ORF 1 encodes a 465-aa protein containing the trichovirus MP domain (aa 19–191) for both variants. Unlike three known members of the genus *Capillovirus*, the genome of CRSaV-3 contains ORF 4 that encodes a protein of 133 aa residues. This protein contains a NABP domain (aa 20–118), suggesting it is an analog of the NABP of some betaflexivirus (Adams et al., 2012; Marais et al., 2015b). This protein is most closely related to some viruses of the genus *Carlavirus* in the subfamily *Quinvirinae* by size and aa sequence identities (Table 2). The two CRSaV-3 variants share aa sequence identities of 67.4% for replicase, 80.9% for MP, 91.3% for CP and 72.9% for NABP. Overall sequence identities between CRSaV-3 and CRSaV-1, CRSaV-2 or selected betaflexiviruses are 39.4–48.4% for genomic sequences, 23.0–28.4% for replicase, 5.8–23.2% for MP and 10.0–33.2% for CP.

3.5. Phylogenetic analyses

Phylogenetic analysis performed using the complete genomic sequences of the three new viruses and selected viruses of different genera of the family *Betaflexivirdae* placed CRSaV-1 and CRSaV-2 in a cluster with two pruneviruses (CPrV and AVCaV) and citrus leaf blotch virus, whereas CRSaV-3 clusters with two capilloviruses (CVA and CuVA) and Diuris virus A in the subfamily *Trivininae* (Fig. 3A). The topologies of the phylogenetic trees changed slightly when the aa sequences of the replicase/replicase domain, MP and CP aa sequences were analyzed but close relationship of CRSaV-1, CRSaV-2 with the two known pruneviruses and CRSaV-3 with the two of the three known capilloviruses were retained (Fig. 3B-D). ASGV, the type species of the genus *Capillovirus*, is separated from CRSaV-3 and the other two capilloviruses in a distinct cluster when the genome (Fig. 3A) and replicase (Fig. 3B) sequences were used in the analyses, suggesting its replicase is more distant from those of other capilloviruses. Recombination analysis shows that no significant recombination events occurred among the CRSaV viruses/variants and other selected betaflexiviruses (data not shown).

3.6. Additional detection of the viruses

In the initial study, RT-PCR using virus-specific primers were tested for the detection of each of the three viruses. However, sequencing of amplicons showed that neither CRSaV-1-specific primers nor CRSaV-2-specific primers tested were virus-specific. Therefore, consensus or degenerate primers were designed based anneal the conserved regions of both viruses and used to detect them in one reaction without differentiation. All three primer pairs worked to amplify the target fragments (data not shown). Based on amplification efficiency, primers CRS12-detF2 and CRS12-detR2 were selected for the detection of CRSaV-1 and CRSaV-2 (414-bp amplicon), while primers CRS3-detF2 and CRS3-detR1 (Table S1) were selected for the detection of CRSaV-3 (723-bp amplicon) in subsequent investigations (Fig. 4; supplementary Fig. 1).

An additional 38 trees were sampled from the public garden (23 cultivars and hybrids) and from nursery plants we purchased and maintained in the greenhouse (15 cultivars) and tested by RT-PCR (partial data, Supplementary Fig. 1). Results showed that 28 trees (73.6%) were infected by the camellia capillovirus (CRSaV-3), prunevirus (CRSaV-1 and/or CRSaV-2) or all three viruses, respectively (Table 1). Three of the trees were infected only by the capillovirus (7.9%), 10 trees were infected only by the prunevirus (26.3%) and 15 trees were infected by both capillovirus and prunevirus (39.5%). The presence of one or more viruses in 7 of these plants were confirmed by sequencing of the amplicons (Table 1). Ten Among the infected trees, five (cvs. Pink Perfection, Winter's Star, April Remembered, Long Island Pink and Hagoromo) were symptomless, four (cvs. Tricolor, Spring Cardinal, Lemon Glow and Shibori-Egao) did not show ringspots, and the remainder of the 19 cultivars showed ringspot symptom in at least one of the three years when the trees were observed. Six of the 19 trees with ringspots also showed variegation. On the other hand, only four of the 10 virus-free trees did not show any obvious symptoms, and they were either hybrids (Winter's Waterlily, Winter's Interclude and Winter's Beauty) or a wild variety (Tama-no-ura). The remaining six trees displayed variegation (5) or mottling (1) symptoms, suggesting that other viruses were present.

3.7. Seed transmission

Either one or both groups of the new viruses were detected in the seedlings of both Elaine Lee and Winter's Star. three 3-month-old Elaine Lee seedlings (10% infection), and the detection rate increased to 50% in the 9-month-old seedlings (data not shown). The infection rates further increased to 73% and 80% at 12 and 16 months, respectively (Fig. S1, supplementary Table 2). Among the 24 infected seedlings, 17 were infected with both capillovirus and prunevirus group, and 7 were infected only with the capillovirus. Among the Winter's Star seedlings, 8 of 16 seedlings were infected with the capillovirus at 16 months after germination. This is consistent with the infection status of the mother tree (Supplementary Table 2). Like their mother plants, the seedlings of neither Elaine Lee nor Winter's Star displayed any obvious symptoms. None of the viruses were detected in 10 seedlings derived from the oil camellia plant that was used as a negative control.

4. Discussion

Foliar ringspot disease has been observed in ornamental camellias for many years (Milbrath and McWhorter, 1946; Plakidas, 1954; Inouve and Inouve, 1975; Gailhofer et al., 1988; Ofsoski et al., 1990), but the symptoms have not been associated with any defined pathogens. In this study, filamentous virions were observed in a camellia tree with foliar ringspot symptom (Fig. 1B), and the HTS analysis of total RNAs from the same tree identified five different virus contigs belonging to the family *Betaflexividae*. The nearly full-length genome of the contigs allowed rapid determination of the complete nucleotide sequences of all variants, which represented three viral species, two (CRSaV-1 and CRSaV-2) in the genus *Prunevirus* and another one (CRSaV-3) in the genus *Capillovirus* (Fig. 3). The viruses were also prevalent in camellias that were sampled from a public garden and that were purchased from a local nursery.

Sequence analysis showed that CRSaV-1 and CRSaV-2 were very similar to one another in genome organization (Fig. 2). Unlike CPrV and AVCaV (Marais et al., 2015b), the CRSaV-1 and CRSaV-2 do not contain ORF 4 that encodes NABP in their genome (Fig. 2). Viruses of the families *Alphaflexiviridae*, *Betaflexiviridae* and *Tymoviridae* might be evolved from a common ancestor by recombination and gene loss (Martelli et al., 2007). The NABP is absent in some members of the genera *Carlavirus* and *Trichovirus* (Martelli et al., 1994; De Souza et al., 2013). The NABP ORF of the two viruses may never have been acquired or may have been deleted in its evolution. Although the CP aa sequence identities of 86.7–90.5% (Table 2) between the two viruses are higher than the species demarcation criterion of 80% for the family *Betaflexiviridae* (Adams et al., 2012), the sequence identities between the two viruses at whole genome (66.5–66.8%), replicase (64.2%) and MP

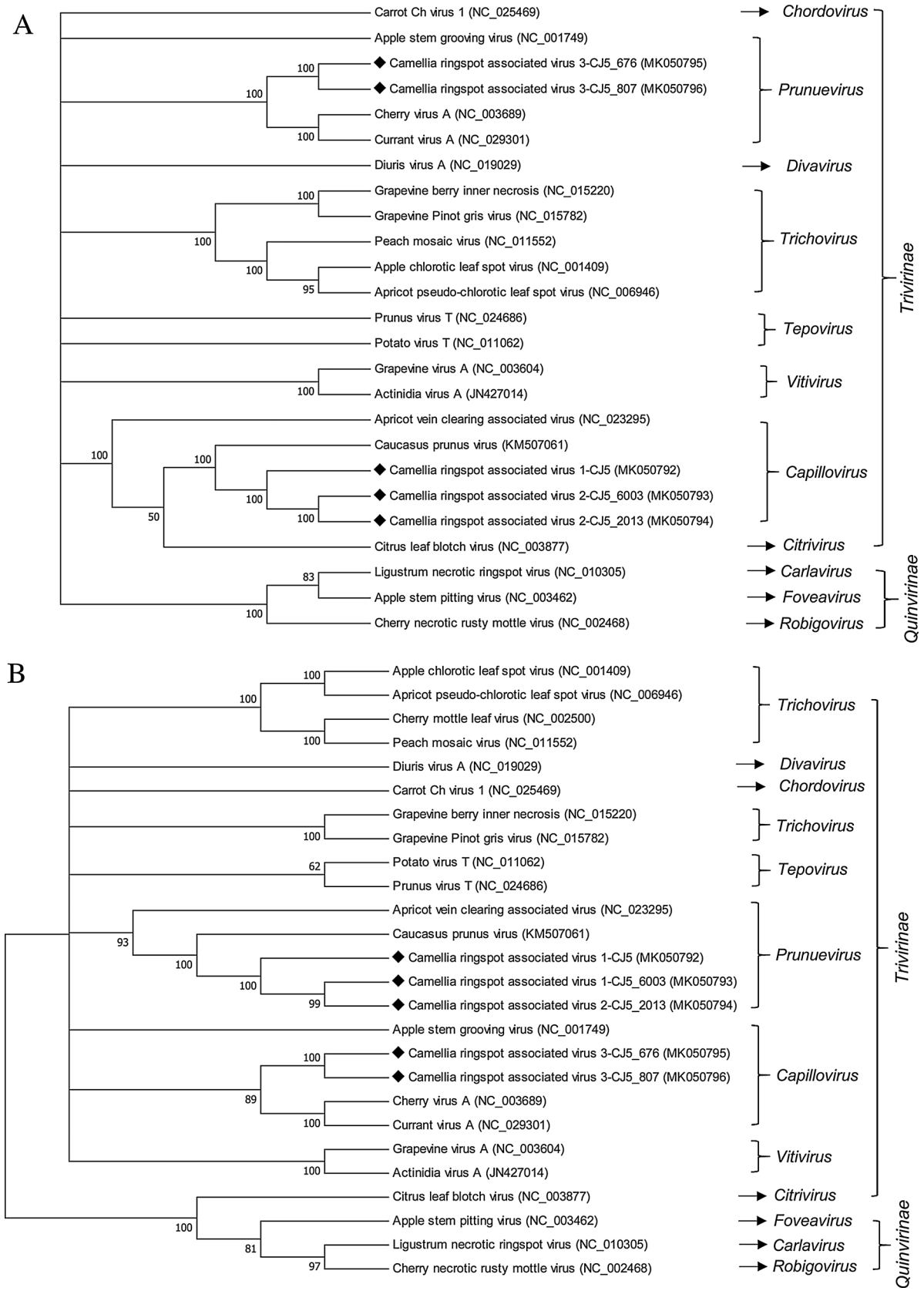

Fig. 3. Neighbor-joining trees derived from complete genomic sequences (A), amino acid sequences of replicase (B), movement protein (C) and coat protein (CP)/CP domain (D) of three Camellia chlorotic ringspot associated viruses and representative members of the family *Betaflaxiviridae*. Only type species of each of three genera in the subfamily *Quinvirinae* is included. Bootstrap analysis was applied using 1000 replicates. Percentage bootstrap support is shown at all branches if ≥50%. Solid diamond indicates the viruses/variants characterized in this study.

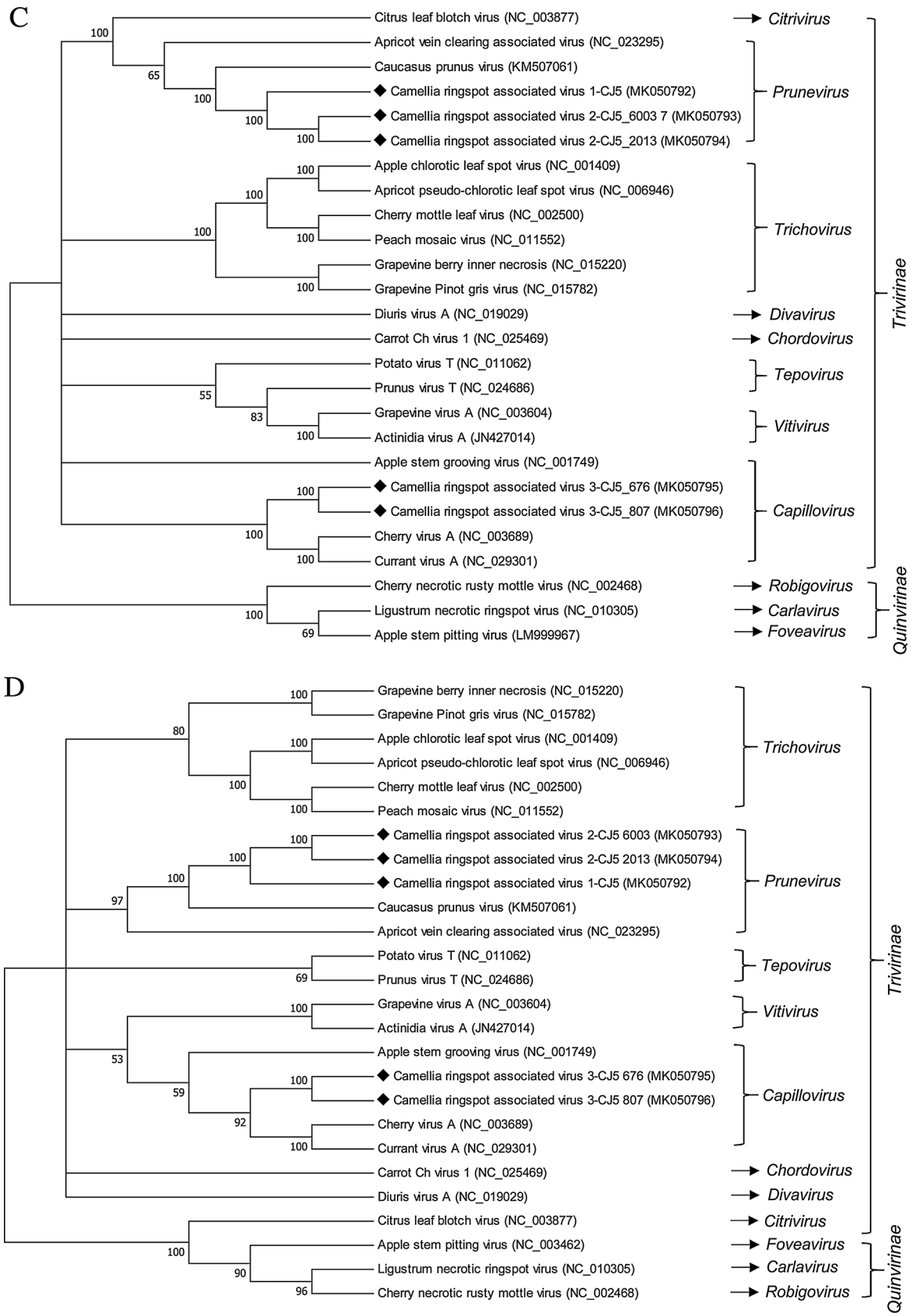

Fig. 3. (*continued*)

(75.2–75.7%) fall below the criteria. A phylogenetic tree of the complete genome also separate them into two distinct groups at species level (Fig. 3A). Phylogenetic analyses by the complete genome, replicase, MP and CP sequences place the CRSaV-1 and CRSaV-2 with CPrV and AVCaV in a cluster, and therefore, CRSaV-1 and CRSaV-2 should be considered as two new members of the genus *Prunevirus*.

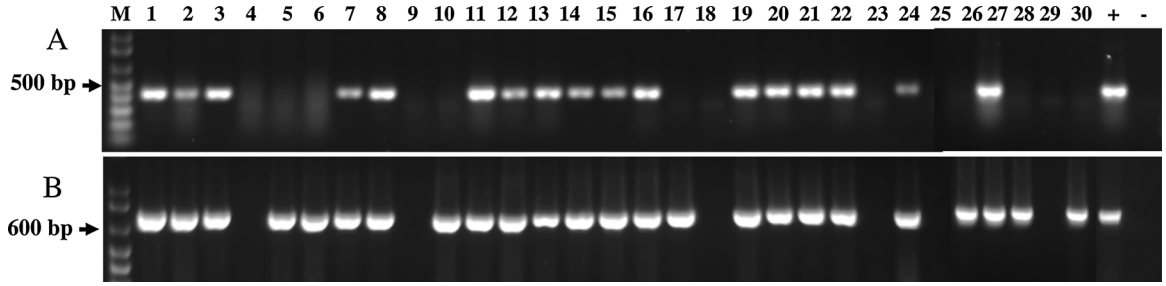

Fig. 4. Detection of Camellia ringspot associated viruses (CRSaV) by camellia prunevirus group (CRSaV-1 and CRSaV-2) consensus and degenerate primers (A) and CRSaV-3-specific primers (B) in 16-month-old seedlings of *Camellia japonica* cv. Elaine Lee. Lanes M) 1 kb plus DNA ladder, 1–30) different seedlings, 23) CJ5 and 24) an oil camellia (*C. oleifero*) seedling. Arrow indicates the size of the marker DNA fragment.

CRSaV-3 is different from CRSaV-1 and CRSaV-2 in both genome organization and sequence identities. The genome organization of CRSaV-3 is very similar to three members of the genus *Capillovirus* (Adams et al., 2012; Petrzik et al., 2016) with the exception of the additional NABP gene (Fig. 2). Addition of the NABP ORF at the 3′ terminus of CRSaV-3 and its short length (22 nt) are unique to this virus. The NABP ORF is observed in both variants of CRSaV-3, supporting its presence. This putative gene product varies in both size and sequence among the viruses of the same genus, and the identity of 72.9% between the two variants at the aa sequence level suggests the same origin. Sequence comparisons showed they should be considered as two distinct variants of the same virus. The two variants are the most closely related to CVA and CuVA, and their genomic sequence identities are 47.5–48.4%. Phylogenetic analyses always group the two CRSaV-3 variants, CVA and CuVA in a distinct cluster (Fig. 3), suggesting that CRSaV-3 is a new member of the genus *Capillovirus*.

The CP of the camellia viruses/variants was very conserved within each genus, whereas the replicase was much more divergent (Table 2). The CP aa sequence identities between CRSaV-1 and CRSaV-2 were as high as 90.5%, but the aa replicase identity between the virus was only 64.2%. The two CRSaV-3 variants were 91.3% identical at the CP aa sequences, whereas the identity was only 67.4% for the replicase, 80.9% for the MP and 72.9% for the NABP, respectively. The different genetic divergence at the replicase, CP and/or NABP ORFs suggests the CP is important in maintaining the virion structure and plays an essential role in the virus life cycle. Comparisons of the aa sequences of the replicase and CP of more than 400 viruses and variants of the family *Betaflexiviridae* showed that such a big difference between the two gene products has only been present for these three new viruses (data not shown).

In this study, seedlings from the two cultivars were tested by RT-PCR for the newly identified viruses, and results showed that the viruses or virus infecting the mother plants were detected from most of seedlings. Although seed transmission is not common for betaflexiviruses, two members, cowpea mild motte virus (genus *Carlavirus*) and PVT of the family *Betaflexiviridae*, have been reported to be seed transmissible (Brunt and Kenten, 1973; Jones, 1982). This is the first report of seed transmission for betaflexiviruses infecting a woody plant.

Mixed infections of plant viruses are common (Read and Taylor, 2001; Syller, 2012), especially in vegetatively propagated crops (Li et al., 2012). This study reports the presence of three different viruses belonging two genera of the family *Betaflexiviridae* in a single camellia tree. The grafting of different rootstock and scion sources might have introduced these viruses and variants into the same plant, and seed transmission may also have contributed to the mixed infections. Intra-host interactions of different viruses or variants may also result in the generation of new variants (Meng and Gonsalves, 2007; Glasa et al., 2017). It is not clear if the large ringspot symptom observed in the CJ5 was caused by mixed infection of the three viruses in the same family since single infection of either CRSaV-1/CRSaV-2 or CRSaV-3 was detected in other cultivars with ringspot symptoms.

Primer pairs were designed based on the conserved regions between them to detect the prunevirus group (Fig. 4A; supplementary Fig. 1). Along with a RT-PCR assay using capillovirus (CRSaV-3)-specific primers, the capillovirus infection was differentially detected from the prunevirus group. A survey of 38 camellia cultivars and hybrids showed that most of them were infected with these viruses, with both single or mixed infection detected (Table 1, supplementary Fig. 1). Not all infected camellia trees had symptoms, but all trees with ringspots harbored at least one of the two groups, suggesting that these viruses are tightly associated with ringspot symptoms. Detection of these viruses in camellia trees without symptoms or with symptoms other than ringspots might be attributed to differences in host genetic background, growth conditions or presence of other unknown viruses such as the rod-shaped viruses observed in camellias with variegation and mottling symptoms (Hiruki, 1985; Miličić et al., 1986).

Identification and characterization of three different viruses from a camellia tree with ringspot symptoms have added new members to the genera *Prunevirus* and *Capillovirus*. The genomic information will facilitate additional studies to determine the etiology of virus-induced camellia diseases and assist in developing reliable detection assays for the pathogens to help produce clean stock camellias.

5. Conclusions

Three new members of the family *Betaflexiviridae* were identified from a camellia tree with ringspot symptom. Complete genome sequences of these viruses, which are tentatively named as CRSaV-1, CRSaV-2 and CRSaV-3, were determined. Sequence analyses showed that CRSaV-1 and CRSaV-2 belong to the genus *Prunevirus* while CRSaV-3 is the new member of the genus *Capillovirus*. Survey of more camellia cultivars and hybrids indicated that these viruses were common and tightly associated with ringspot disease. Testing seedlings obtained from infected trees also showed that the viruses were seed transmissible.

Author contributions

RL designed the study. HL, LW and LZ were involved in conducting experiments. HL, MC and RL analyzed the results. HL and RL prepared the manuscript. All authors read and approved the final version.

Ethical approval

This article does not contain any research involving human or animal participants.

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Declaration of Competing Interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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