MOLECULAR CHARACTERIZATION AND DETECTION OF A TRIPARTITE CRYPTIC VIRUS FROM ROSE

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SUMMARY

Three dsRNA molecules with estimated molecular weights of c. 1.7, 1.5 and 1.4 kbp were extracted from a symptomless hybrid tea rose cv. Sheer Bliss, and further characterized. Analysis of complete sequences showed that they represent genome segments of a tripartite cryptic virus with the proposed name Rose cryptic virus 1 (RCV-1). All three segments were monocistronic, with the dsRNA-1 encoding a protein containing conserved motifs of RNA-dependent RNA polymerases. The two proteins encoded by dsRNA 2 and 3 shared c. 20% common amino acids and are probably involved in the make up of the viral capsid. Phylogenetic analysis based on the viral RdRp gene showed closest relationships of RCV-1 with Fragaria chiloensis cryptic virus (FCCV) and Raphanus sativus cryptic virus 2 (RsCV-2), both recently described tripartite cryptoviruses. Interestingly, this group of viruses appears to be phylogenetically closer to mycoviruses belonging in the genus *Partitivirus* than to plant-infecting cryptoviruses. The virus was detected by RT-PCR in *ca.* 30% of the tested samples belonging to different cultivars/hybrids, indicating that it may be quite common in rose germplasm.

Key words: Rose, cryptic virus, dsRNA, sequences, phylogeny, RT-PCR.

INTRODUCTION

Plant cryptoviruses are associated with latent infections of their hosts and have some peculiar properties that distinguish them from other plant viruses, i.e. they are not graft or mechanically transmissible and do not have known natural vectors. Pollen/seed transmission is the only ascertained means of virus dispersal. In addition, it appears that there is no cell-to-cell movement or transport of the virus within the plant except when cell division takes place (Boccardo *et al.*, 1987).

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Cryptoviruses are apparently widespread in nature and have been reported from mono- and dicotyledonous plant species. Virions are isometric, *ca.* 30-40 nm in diameter, with or without the presence of prominent surface structures. The genome usually consists of two molecules of linear dsRNA, believed to be separately encapsidated, and coding for RNA-dependent RNA polymerase (dsRNA-1) and coat protein (dsRNA-2), respectively. Less frequently, three distinct dsRNA molecules have been found associated with this type of virus, i.e. *Radish yellow edge virus* (RYEV; Natsuaki, 1985) and *Carnation cryptic virus* 1 (CCV-1; Lisa *et al.*, 1986). Recently, complete genomic sequence data on tripartite cryptoviruses were reported by Chen *et al.* (2006b) and Tzanetakis *et al.* (2008).

Plant cryptoviruses are currently grouped into two genera, *Alphacryptovirus* and *Betacryptovirus* within the family *Partitiviridae.* The family *Partitiviridae* also embraces the genus *Partitivirus* that contains dsRNA viruses with similar properties, but isolated from fungi (Ghabrial *et al.*, 2005).

During a survey on rose viruses carried out in 2005 and 2006, symptomless accessions of cv. Sheer Bliss used as controls showed the presence of multiple dsRNA bands with molecular weights in the range of those described for cryptoviruses. Similar dsRNA patterns were found in *Rosa multiflora* Thunb. affected by rose rosette disease of (Di *et al.*, 1990), but these dsRNAs were never further characterized.

Thus, as reported in the present paper, it was decided to clone and sequence dsRNAs from cv. Sheer Bliss and to investigate their presence in rose germplasm.

MATERIALS AND METHODS

Virus source and transmission experiments. The original virus sources were two infected rose accessions of cv. Sheer Bliss grafted onto unknown rootstocks and designated ShB-1 and ShB-2. Leaf extracts from each of the two accessions were crushed separately in 0.1 M phosphate buffer pH 7.2 containing 2% nicotine and rubbed onto cellite-dusted leaves of eight herbaceous species belonging to the families *Solanaceae, Chenopodi-*

aceae and *Leguminosae.* Each plant species was represented by four specimens. Plants were kept in a greenhouse at 20-22°C and observed daily over a period of 4 weeks for symptom expression.

Purification. Attempts to purify and observe virions were made using the modified protocol described for *White clover cryptic virus 1* (Milne *et al*., 2005). For this purpose, 60 g of rose leaves were crushed in the presence of 0.1 M phosphate buffer (PO_4 buffer) pH 7.0 at ratios of 5 and 10 ml buffer/g of tissue, filtered through four layers of cheesecloth and emulsified with an equal volume of chloroform. The emulsion was then centrifuged at 8,000 g for 10 min. After the phase separation, 8% polyethylene glycol (PEG; molecular weight 8,000) and 1% sodium chloride were added to the supernatant, stirred for 2.5 h and precipitated by low speed centrifugation. The pellets were resuspended in 0.02 M PO_4 buffer pH 7.0 and submitted to one cycle of low- (10 min at 4,000 g) and high-speed (120,000 g for 45 min) centrifugation. The final pellets were resuspended in the same buffer and used for electron microscope observations.

dsRNA analysis. Double stranded RNAs were isolated from both original Sheer Bliss accessions using double phenol-chloroform extraction prior to CF-11 column chromatography (Dodds, 1993). Healthy rose plants and *Peanut stunt virus*-infected tobacco plants were used as controls. The extracted dsRNAs were submitted to sequential selective enzymatic digestions with RNAse-free DNase, DNAse-free RNAse in high-salt conditions (2X SSC: 300 mM Sodium chloride and 30 mM Sodium citrate, pH 7.0) and Proteinase K as described by Saldarelli *et al.* (1994). Once purified, dsRNAs were analyzed in 1.2% agarose and/or 6% polyacrylamide gel electrophoresis (PAGE) and stained with ethidium bromide. Approximate molecular weights of the dsRNAs were estimated by comparison with replicative forms of *Peanut stunt virus* (PSV) extracted from infected tobacco plants and used for reference.

Cloning and sequencing. Five hundred micrograms of purified dsRNA preparations from ShB-1 were random-primed and reverse-transcribed using Thermoscript Reverse Transcriptase (Invitrogen, USA). Generated cDNAs were then amplified by Degenerate Oligonucleotide Primers-Polymerase Chain Reaction (DOP-PCR) using a commercial kit (Roche Applied Science, USA). PCR products were cloned into pGEM-T Easy plasmid (Promega, USA) and the resulting recombinant plasmids were transferred to *Escherichia coli* Top 10 competent cells. DNAs of plasmids from 40 selected colonies were sequenced at the DNA Sequencing Facility, Life Science Biotechnology Institute of Mississippi State University. After computer-assisted analysis of the initial sequence data, specific primers were designed in order to fill the gaps between adjacent clones by PCR. Viral ends were generated by protocols described by Lambden *et al.* (1992), involving ligation of 5'phosphorylated/3'amino-blocked oligonucleotides to target dsRNAs prior to cDNA synthesis, or by using 5'/3'RACE kit (Roche Applied Science, USA). Sequence data were assembled and analyzed by Lasergene software (DNASTar Inc, USA) and compared with sequences available in the NCBI DataBank with a BLAST (Altschul *et al*., 1997). Pairwise comparisons and phylogenetic analyses were performed with ClustalW2 (Larkin *et al*., 2007) using default parameters. Phylogenetic trees were visualized with the TreeView program (Page, 1996).

Virus detection. A set of virus-specific primers (RCV-1F 5'GATCAGAAAGCCGCTGTTGG3' and RCV-1R 5'TTGGGAATACAGGTTGGGGT3') was designed to amplify a 610 bp-long portion of viral dsRNA-2. These were used in RT-PCR to investigate the relative incidence of the virus in different rose samples. For this purpose, total RNAs were extracted by Plant RNeasy kit (Qiagen, USA) from 82 samples belonging to different rose cultivars/hybrids and species. Extracted RNAs were random-primed and reverse-transcribed by MMLV Reverse Transcriptase (Promega Corporation, USA) for 1 h at 39°C. The cDNA was amplified by PCR in a My-Cycler thermal cycler (Bio-Rad, USA) for 40 cycles (94°C for 30 sec, 51°C for 35 sec and 72°C for 45 sec) after an initial denaturation for 2 min at 94°C. Cycling was followed by a final elongation step at 72°C for 10 min. Ten-microliter aliquots of the PCR product were analyzed by electrophoresis in 1.5% agarose gel in TAE buffer (Sambrook *et al*., 1989), stained with ethidium bromide, and visualized on a UV transilluminator.

RESULTS

dsRNA isolation and analysis. Identical dsRNA patterns were observed in both tested accessions of cv. Sheer Bliss. The dsRNA pattern appeared to consist of two bands when examined in 1.2% agarose gel in TAE (not shown). A 4 hour-long electrophoretic run in 6.5% PAGE, showed that the faster migrating band observed in agarose gels was composed of two distinct molecules of similar size (Fig. 1). The same pattern, always consisting of three dsRNA species, was extracted later in this work from seven randomly chosen RT-PCR positive samples.

Transmission and purification. None of the mechanically inoculated herbaceous plants developed symptoms or contained the virus when checked for the presence of dsRNAs. The absence of the virus was confirmed by

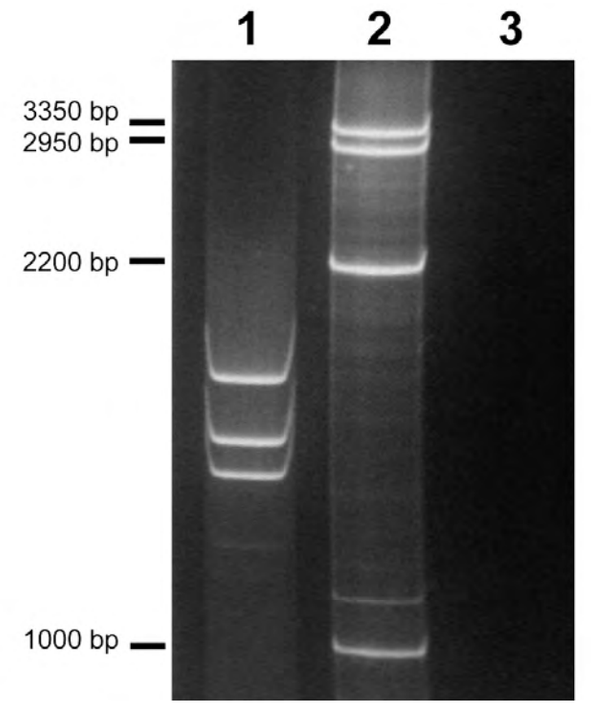

Fig. 1. Electrophoretic profiles of dsRNAs extracted from rose accession ShB-1 (lane 1). Replicative forms of *Peanut stunt virus* (lane 2) are used as reference markers and their size is indicated. No detectable dsRNAs were observed in healthy controls (lane 3).

RT-PCR using RCV-1 specific primers developed in this work. Purification attempts failed to recover virions associated with detected dsRNAs.

Sequence data. Sequences totaled 4,680 bp, divided into three dsRNA segments with an overall adenine and uracil (A+U) content of 55.3% (GenBank accession numbers: EU413666, EU413667, EU413668). Segments 1 and 2 had identical 22 nt-long 5'-proximal sequences, whereas the first 10 nucleotides were conserved in all three dsRNAs. In addition, the three dsRNAs shared *ca.* 90% conserved residues at the 5'end with corresponding genomic segments of Fragaria chiloensis cryptic virus (FCCV; Tzanetakis *et al*., 2008) (Fig. 2A). While the 5' non-coding regions (NCRs) of the three molecules were relatively conserved in length (182, 189 and 172 nts respectively), the 3' NCRs of dsRNA-2 and -3 (249 and 233 nts) were almost double the size of that in dsRNA-1 (127 nt).

The three dsRNAs extracted from rose shared a high overall content of identical nucleotide residues with corresponding genomic segments of FCCV (81%, 73% and 70%, respectively) and Raphanus sativus cryptic virus 2 (RsCV-2; Chen *et al*., 2006b) (48%, 47% and 46%, respectively) (Table 1).

dsRNA-1 was 1749 bp in length and contained a single ORF (ORF1) potentially encoding a 479 amino acid protein with an estimated M_r of 55.9 kDa (p1). Computer-assisted analysis showed that p1 contained conserved motifs 3-8 characteristic for partitivirus RNA-dependant RNA polymerases (Bruenn, 1993). This protein shared an unusually high homology (87% identity; 94% similarity) with the corresponding protein of Fragaria chiloensis cryptic virus. In addition, it shared 60% identity with RdRps of Raphanus sativus cryptic virus 2, 40% with Pinus sylvestris partitivirus NL-2005 (PinSV; Veliceasa *et al*., 2006), 36% with Pyrus pyrifolia cryptic virus (PpCV, Osaki *et al*., 1998) and 35% with *Beet cryptic virus 3* (BCV-3; Xie *et al*., 1993). Curiously, its overall identity with RdRps of the approved species in the genus *Alphacryptovirus*, *White clover cryptic virus 1* (WCCV-1; Boccardo and Candresse, 2005a,b) and *Vicia cryptic virus* (VCV; Blawid *et al*., 2007), was limited to 11% of common amino acids. At the same time, the conservation of amino acid sequences between VCV and WCCV-1 was almost 85% (see Fig. 2B). Phylogentic analysis of this protein showed that it grouped together with the RdRp of FCCV and RsCV-2 forming a distinct clade within the family *Partitiviridae* and appears distant from alphacryptoviruses (Fig. 3).

dsRNA-2 consisted of 1485 bp and encoded a 348 amino acid putative protein with a predicted M_r of 38.8 kDa (p2). When compared with available protein sequences in NCBI, it matched with products encoded by dsRNAs-2 and -3 of FCCV (69% identical residues with p2 and 23% with p3) as well as that of RsCV- 2 (29% and 27% identity respectively).

dsRNA-3 contained a single ORF (ORF3) encoding a polypeptide (p3) with a similar size (346 amino acids) and M_r (38.6 kDa) as that of dsRNA-2. Their pairwise comparison showed that they are related to each other, sharing *ca.* 20% common amino acids. In addition, as with p2, this protein shared the highest degree of identity with the corresponding proteins encoded by dsRNAs-3 of FCCV and RsCV-2 (65% and 30%, respectively).

Detection. The virus was found in 24 out of 82 samples tested and was detected in hybrid tea roses (cvs

Table 1. Percentage of amino acid and nucleotide identity of Rose cryptic virus 1 (RCV-1) with Fragaria chiloensis cryptic virus (FCCV) and Raphanus sativus cryptic virus 2 (RsCV-2).

	RCV-1					
	dsRNA 1		**dsRNA 2**		**dsRNA 3**	
	nt*	aa**	nt*	aa**	nt*	aa**
FCCV	81	87	73	69	70	65
RsCV-2	48	60	47	29	46	30

*overall nucleotide identity (%) of each dsRNA segment

**overall amino acid identity (%) of related proteins

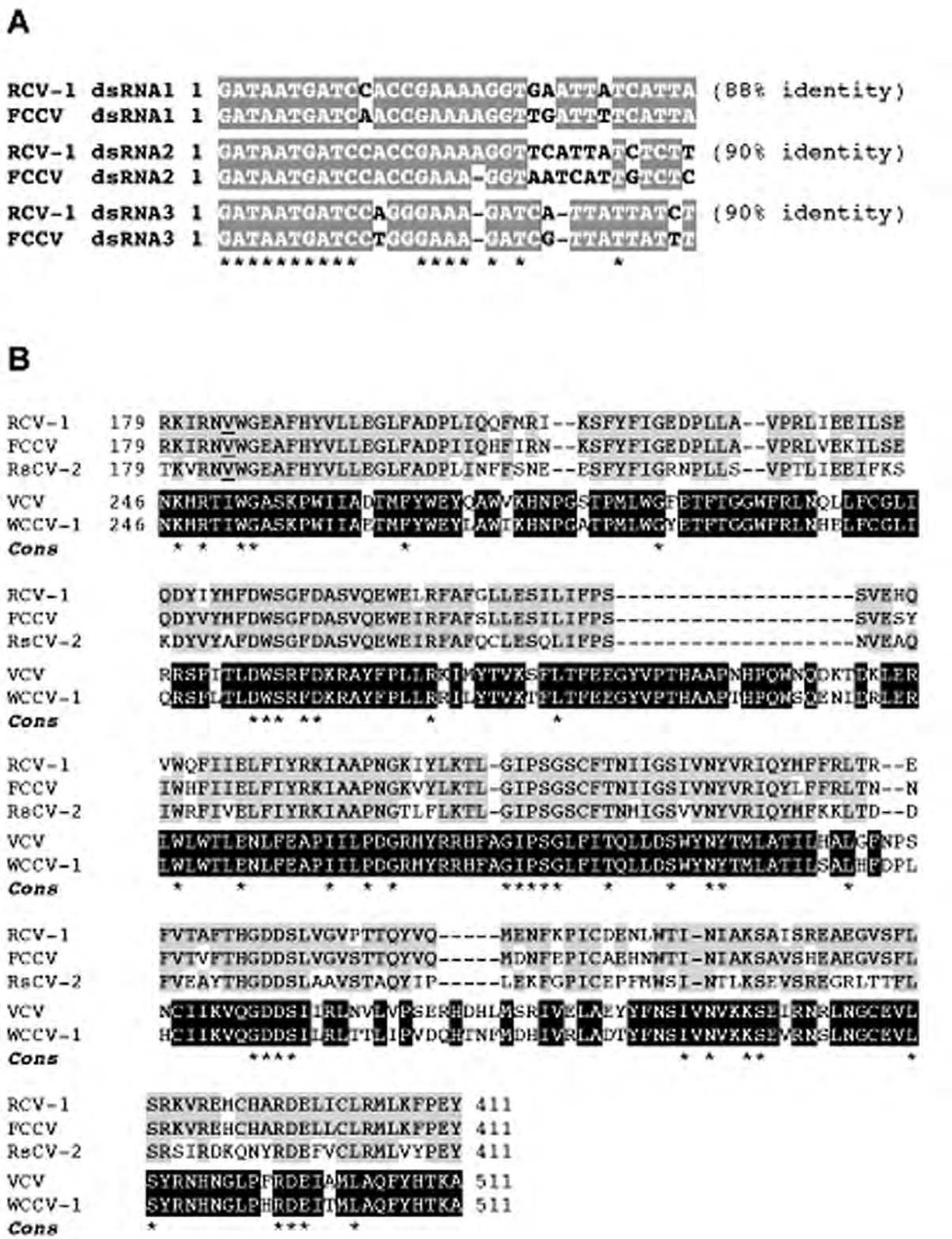

Fig. 2. A. Alignments of the 5' non-coding regions of dsRNAs 1-3 of Rose cryptic virus 1 and Fragaria chiloensis cryptic virus (FCCV). Conserved residues are shaded. Asterisks denote residues conserved in all six molecules. Percentages of identities are indicated. **B.** Comparison of conserved regions of the viral RdRps of tripartite cryptic viruses (RCV-1, FCCV and RsCV-2; upper subgroup) with the corresponding proteins of alphacryptoviruses (WCCV-1 and VCV; lower subgroup). Asterisks in the consensus (cons) sequence denote identical residues in all proteins used in analysis. Identical residues among the members of each subgroup are shaded. Note high conservation of a number of amino acids within subgroups (232 and 265 aa, respectively).

Sheer Bliss, Memorial Day, Golden Celebration, Passionate kisses) as well as in floribunda and shrub roses (Fig. 4). In addition, the virus was detected in two samples of *R. multiflora* collected in the Great Smoky Mountains National Park (GSMNP).

DISCUSSION

Based upon sequence data and phylogenetic analyses, we proved that the three dsRNA species (designated dsRNAs 1-3 in order of size), originally isolated from two accessions of tea hybrid rose cv. Sheer Bliss and later from several additional rose samples, represent the genome of a cryptic virus. Taking into account that rose may host additional related viruses, as reported in several other botanical species (i.e. beet, carrot, white clover, raphanus, etc.) (Ghabrial *et al*., 2005; Chen *et al*., 2006a), we propose for this virus the name Rose cryptic virus 1 (RCV-1).

Since particles of the closely related virus Raphanus sativus cryptic virus 2 were successfully purified (Chen *et al*., 2006b), we assume that RCV-1 forms virions. However, we were unable to observe them, probably because of the recalcitrant nature of roses for virus purification and the relatively low virus titer in host tissue.

During the survey, RCV-1 was found frequently in different types of roses, including the two tested *R. multiflora* samples. The number and size of dsRNAs, as well as the host (*Rosa* spp), indicate that RCV-1 may be the same virus as that reported "associated" with rose rosette symptoms by Di *et al*. in 1990. However, consid-

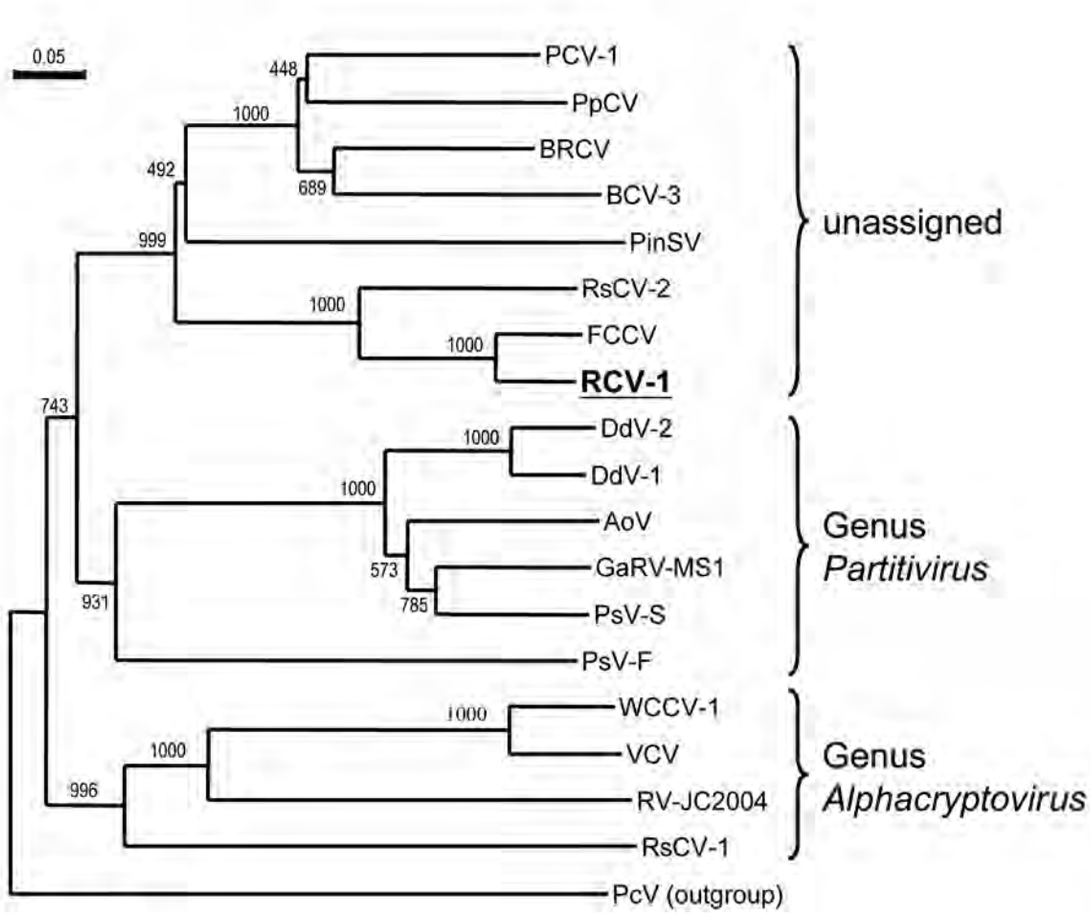

Fig. 3. Phylogenetic relationships of Rose cryptic virus 1 (RCV-1) and some definitive and tentative members of the family *Partitiviridae*. The rooted tree is based on deduced amino acid sequences of the conserved motifs 3-8 of viral RdRps (Bruenn, 1993). Bootstrap values are indicated on the nodes. Sequences used to construct the tree are: *Aspergillus ochraceous virus* (AoV; ABC86749), *Beet cryptic virus 3* (BCV-3; AAB27624), Black raspberry cryptic virus (BRCV; EU082132), *Discula destructiva virus 1* (DdV-1; NC_002797), Discula destructiva virus 2 (DdV-2; NC_003710), Fragaria chiloensis cryptic virus (FCCV, NC_009519), *Gremmeniella abietina RNA virus MS1* (GaRV-MS1, NC_004018*), Penicillium stoloniferum virus F* (PsV-F ; NC_007221); *Penicillium stoloniferum virus S* (PsV-S ; NC_005976); Pepper cryptic virus 1 (PCV-1; DQ361008); Pinus sylvestris partitivirus NL-2005 (PinSV, AY973825), Pyrus pyrifolia cryptic virus (PpCV, AB012616), Radish partitivirus JC-2004 (RV-JC2004, AY748911), Raphanus sativus cryptic virus 1 (RsCV-1; NC_008191), Raphanus sativus cryptic virus 2 (RsCV-2, DQ218036), *Vicia cryptic virus* (VCV, EF173392) and *White clover cryptic virus 1*(WCCV-1, NC_006275). *Penicillium chrysogenum virus* (PcV- gen. *Chrysovirus,* NC_007539) was used as an outgroup.

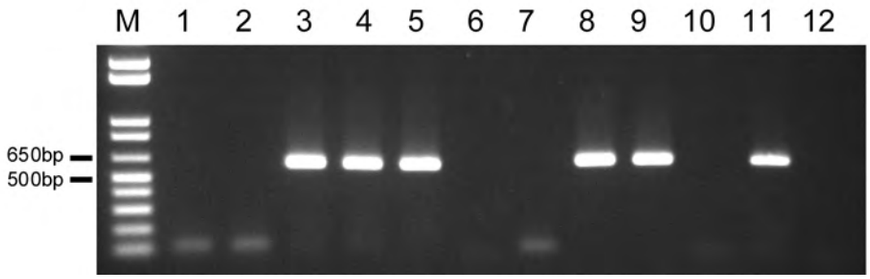

Fig. 4. Detection of RCV-1 in different rose samples. 610 bp-long amplicons were generated from shrub rose cv. Watermelon ice (lanes 3 and 4), *Rosa multiflora* (lane 5), tea hybrid roses cv Memorial Day (lanes 8 and 9). Positive and negative controls are in lanes 11 and 12 respectively. Reference markers (1kbPlus DNA –Invitrogen Corporation, Carlsbad, CA, USA) are in lane M.

ering that the two original sources of cv. Sheer Bliss and the majority of RT-PCR positive samples were symptomless, we cannot confirm the correlation of the virus (dsRNAs) with any particular symdrome.

The virus shared the same genome organization with Fragaria chiloensis cryptic virus and Raphanus sativus cryptic virus 2, two recently described tripartite cryptic viruses. In all three viruses, dsRNA-1 encodes the viral RdRp, whereas the two smaller genome segments code for related putative proteins of similar size (38.8 and 38.6 kDa). The function of these proteins is still to be ascertained. Considering that cryptic viruses apparently do not move from cell to cell within the plant host and are not vector-transmitted, it is unlikely that p2 and p3 have a transport/dissemination role.

In bipartite cryptic viruses (and in mycoviruses belonging to the genus *Partitivirus*) dsRNA-2 encodes the viral coat protein. Thus, it is likely that p2 of RCV-1 has the same function. Tzanetakis *et al.* (2008) proposed that both polypeptides (p2 and p3) of FCCV may be involved in the build-up of the viral capsid. Analysis of the secondary structures of p2 and p3 in RCV-1 revealed similar folding patterns of the two proteins, as in the case of FCCV, thus supporting this hypothesis. Unfortunately, due to their almost identical molecular weights, p2 and p3 co-migrate in SDS-PAGE analyses, thus making their separation and differentiation by traditional methods very difficult. Specific antisera are being produced against p2 and p3 proteins expressed in *Escherichia coli* to be used in Western blots against partially purified preparations (authors, on-going research) in order to study the possible function of these proteins.

Phylogenetic analysis of the viral RdRp gene placed RCV-1 together with FCCV and RsCV-2 forming a coherent group that appears evolutionarily closer to some unassigned species in the family (i.e. PpCV, PinSV, BCV-3) and to partitiviruses than to alphacryptoviruses (Fig. 3). Accordingly, our data support the polyphyletic nature of cryptovirus evolution with RCV-1, FCCV and RsCV-2 lineage independent from alphacryptoviruses.

Unfortunately, the complete lack of molecular data on viruses belonging to the genus *Betacryptovirus* impedes final conclusions on the taxonomic position of RCV-1 and related viruses within this family. Judging from the available data on betacryptoviruses (number and molecular weights of genomic dsRNAs), it is very unlikely that RCV-1, FCCV and RsCV-2 belong in this taxon. Consequently, it is plausible that they may warrant the establishment of a new genus within the family *Partitiviridae.* In addition, the affiliation of *Beet cryptic virus*, considered an approved species in the genus *Alphacryptovirus* (Ghabrial *et al.*, 2005), with this taxon seems challenged by the phylogenetic analyses (Fig. 3).

In conclusion, based upon data generated in this and similar studies, taxonomic re-examination of viruses belonging to the *Partitiviridae* might soon be necessary, as suggested by Boccardo and Candresse (2005a; 2005b), Veliceasa *et al.* (2006) and Tzanetakis *et al.* (2008).

Note added in proofs

The research work had long been completed and the manuscript submitted for publication when an article by Salem *et al.*, entitled "Complete nucleotide sequences and genome characterization of a novel double-stranded RNA virus infecting *Rosa multiflora*" became available online (*Archives of Virology*, DOI 10.1007/s00705-007-0008-3) describing, under the name Rosa multiflora cryptic virus, a virus seemingly identical to RCV-1

ACKNOWLEDGEMENTS

The authors wish to thank to Dr. A. Henn and Dr. P. Collins (MSU), K. Langdon (GSMNP) and Mrs R. Foil for providing valuable rose material used in this work and A.M. Lawrence for help in electron microscopy. We are indebted to Dr. I. E. Tzanetakis (Oregon State University, USA) for sharing sequence data on Fragaria chiloensis cryptic virus prior to publication. Work on multiflora rose specimens was partially supported by a Discover Life in America Inc grant.

Approved for publication as a Journal Article No J-11281 of the Mississippi Agricultural and Forestry Experiment Station, Mississippi State University.

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Received December 28, 2007
Accepted February 18, 2008