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A novel emaravirus is associated with redbud yellow ringspot disease

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ABSTRACT

Yellow ringspot is the only virus-like disease reported in redbud (*Cercis* spp.) with symptoms including vein clearing, chlorotic ringspots and oak-leaf pattern. A putative new emaravirus was present in all trees displaying typical yellow ringspot symptoms and the name redbud yellow ringspot associated virus is proposed. The virus genome is composed of at least five RNA segments. Two coding regions were studied to determine isolate diversity with results pointing to a homogeneous virus population. Host range was evaluated using graft transmission and by testing species found in close proximity to infected trees. Mite transmission with *Aculops cercidis*, the predominant species found in redbud trees in the epicenter of the disease, was evaluated but was not found to be a vector of the virus. Based on this study and the accumulated knowledge on emaravirus evolution we propose that speciation is allopatric, with vectors being a major component of the process.

1. Introduction

Redbud (*Cercis* spp.) is a deciduous tree in the family *Fabaceae* found in natural ecosystems in temperate areas worldwide and has become a popular ornamental among horticulturalists and home gardeners because of the attractive pink flowers in early spring and the thick, green foliage later in the season. In North America, the eastern redbud, (*Cercis canadensis* L.) is the prevalent species found south of 45° latitude and east of the Rocky Mountains, a range that overlaps with the cultivation of many economically important legumes.

Little work has been done on redbud viral diseases even though many viruses are known to infect legumes (Aapola et al., 1974; Elbeaino et al., 2015; Hampton et al., 1978; Jones, 2012). In fact, the only published report of a virus or virus-like disease is redbud yellow ringspot (RYRS; Kim and Martin, 1978). RYRS is characterized by the presence of chlorotic ringspots, vein-clearing, and oak-leaf patterns on leaves (Fig. 1); is closely associated with pleomorphic, double membrane-bound particles (Kim and Martin, 1978) and the putative causal agent is transmitted by an, as yet, unidentified eriophyid mite (Kim et al., 2001), attributes that point to a member of the genus *Emaravirus*. Most of the trees in the Kim and Martin study (Kim and Martin, 1978) died a few years after disease onset (Kim et al., 2001), although the role of the putative causal agent was never elucidated.

Here we characterize the putative causal agent of the disease and a potential new member of the genus *Emaravirus*, provisionally named redbud yellow ringspot associated virus (RYRSaV). As there are noticeable differences in symptom expression in the genotypes grown in Arkansas, we studied the virus population structure using two genomic regions to determine the influence of virus diversity in symptom development. A detection protocol was developed and used in host range and transmission studies.

2. Materials and methods

2.1. Virus discovery

Double-stranded RNA (dsRNA) enriched material was extracted from a surviving tree of the Kim et al. (2001) mite-transmission study using the protocol optimized in the characterization of rose rosette virus (RRV; Laney et al., 2011) other than the fact that 120 g of tissue was used in the extractions. Sequencing templates were either degenerate oligonucleotide-primed reverse transcriptase-PCR (DOP RT-PCR; Laney et al., 2011) or RT-PCR amplicons utilizing the emaravirus 5′/3′ end primer PDAP213 (Di Bello et al., 2015; Supplemental Table 1) performed as previously described with a

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Table 1
Redbud yellow ringspot associated virus (RYRSaV) positive samples by variety and symptom expression from across four states.

State	Variety	RYRSaV positive/symptomatic	RYRSaV positive/asymptomatic
Arkansas	Canadensis	25/25	0/1
	Forest Pansy	N/A[a]	2/45
	Oklahoma	23/23	0/2
Missouri	Canadensis	N/A	0/15
Illinois	Canadensis	N/A	0/15
Oklahoma	Canadensis	N/A	0/11
	Oklahoma	N/A	0/5

[a] N/A: Material without yellow ringspot symptoms.

reduction in the extension time to 15 s to enrich for RNAs shorter than 2 kb. Sequencing outputs were analyzed using VirFind (Ho and Tzanetakis, 2014), revealing several hundred orthologs of the previously characterized emaraviruses (data not shown).

Gaps in the virus sequences were filled using virus-specific oligonucleotide primers (Supplementary Table 1) followed by cloning and Sanger sequencing to obtain at least a 3 x genome coverage. The 5′ and 3′ ends of each of the RNAs were verified as described for RRV (Laney et al., 2011). The nucleotide sequence of the virus has been deposited in GenBank under accession numbers JF795479–JF795482 and KU904300.

2.2. Genome analysis

Structures of the proteins encoded in each RYRSaV RNA species was predicted using PHYRE (Kelley and Sternberg, 2009) and conserved domains were identified with NCBI CDD (Conserved Domain Database; Marchler-Bauer et al., 2015). Glycosylation sites were predicted using NetNGlyc 1.0 (Gupta et al., 2004), trans-membrane helices using TMHMM 2.0 (Sonnhammer et al., 1998), cleavage sites using SignalP (Bendtsen et al., 2004), and protein localization was estimated using TargetP (Emanuelsson et al., 2000). RNA-binding prediction of amino acid residues was done with BindN (Wang and Brown, 2006) and protein–protein interactions were predicted using COTH (Mukherjee and Zhang, 2011). Ortholog comparisons were done using MUSCLE (Edgar, 2004), and secondary structures were predicted with PSIPred (Buchan et al., 2013). Phylogenetic analysis was performed with MUSCLE in MEGA6, using the neighbor-joining algorithm, and bootstrapping consisting of 1000 pseudoreplicates (Tamura et al., 2013).

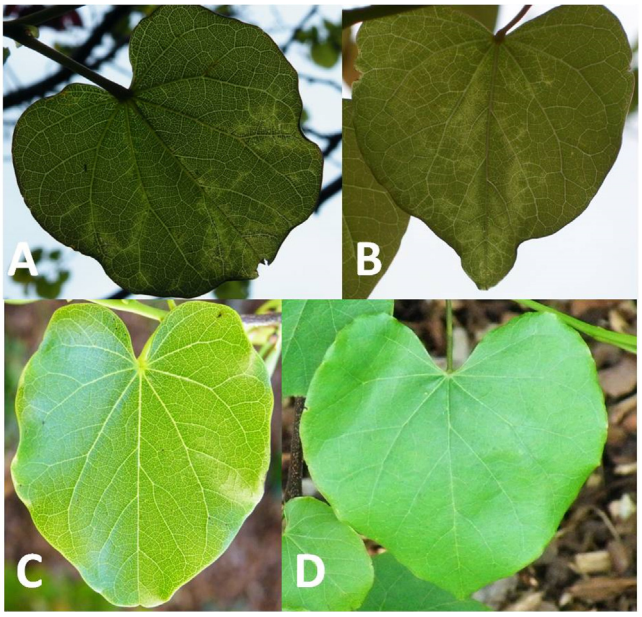

Fig. 1. Redbud yellow ringspot symptoms on cultivar 'Oklahoma' (A) and 'Canadensis' (B). Asymptomatic material are shown in panels C and D for the two cultivars respectively.

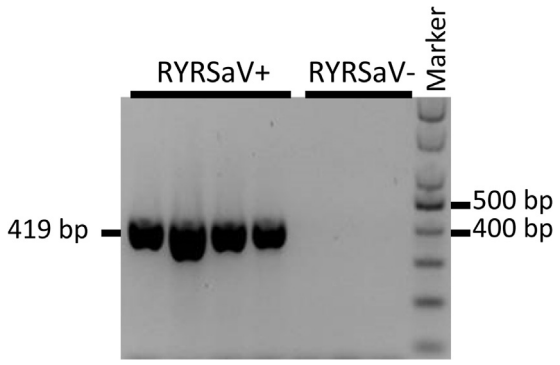

Fig. 2. Detection of redbud yellow ringspot associated virus (RYRSaV). An amplicon of 419 base pairs is only present in virus-infected material.

2.3. Population structure

Tissue from cultivars 'Forest Pansy', 'Canadensis' and 'Oklahoma'; displaying different symptom intensities, ranging from asymptomatic to transient to persistent symptom expression respectively, was collected in northwest Arkansas (Table 1). Asymptomatic redbud trees from Arkansas, Illinois, Missouri and Oklahoma were also used in the study and tested as described above.

The RYRSaV population structure was studied using 18 isolates from the aforementioned cultivars and two genomic regions. Nucleic acids were subjected to reverse transcription as before using the universal emaravirus reverse primer 3′ dT+13 (Laney et al., 2011; Supplemental Table 1) and then digested with 2.5 units RNase H (Fermentas) for 1 h at 37 °C. Thirty amplification cycles were done using 5′/3′ dT+13 primers (Supplemental Table 1). Material was diluted 1:10 and used as a template for nested PCR. Primers flanking the open reading frames (ORF) of RNAs 3 and 4 were selected and the entire ORFs amplified. Internal primers (Supplemental Table 1) were used to directly sequence the PCR products which were repeated at least three times for each segment amplified, for at least a 3 x coverage of the ORFs. Sequences were aligned using CAP3 (Huang and Madan, 1999) trimmed to the ORF for the respective RNA and deposited to GenBank under accession numbers JF795539–JF795574. Alignments were done using ClustalW (Thompson et al., 1994; Supplemental Tables 2 and 3).

2.4. Detection

Total nucleic acids were extracted and reverse-transcribed as previously described (Tzanetakis et al., 2007). Detection primers RYRSaV_F (5′-GCATATGCATATTTAGCTGTG-3′) and RYRSaV_R (5′-