1 **A novel emaravirus is associated with redbud yellow ringspot disease**

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10 **ABSTRACT**

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

22
23 **Keywords:** *Emaravirus*, next generation sequencing, diversity, redbud, legume virus
24

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 reduction in the extension time to 15 sec 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 3x 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).

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., Di Bello et al.,

2011; Supplemental Table 1) and then digested with 2.5 units RNase H (Fermentas) for 1h 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 3x 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'-CCAAGAACAATATCGCAAAT-3') were optimized to amplify a 419-base region of the viral RNA 1 (Fig. 2). The PCR program initiated at 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, annealing at 56°C for 10 sec, and extension at 72°C for 30 sec; this was followed by a final extension of 10 min at 72°C. More than twenty amplicons were sequenced and all were virus-specific, validating the protocol.

2.5 Transmission

Mechanical transmission has been challenging for the virus (Laney, 2010). For this reason cleft grafting was chosen to determine whether the virus is systemic in its primary host, prerequisite knowledge before the initiation of any vector study. All grafted plants were set in a

mist chamber for at least one week before being transferred to an insect-free greenhouse with a 14h photoperiod and daytime temperatures of 22-27⁰C. Two sets of four RT-PCR negative 'Canadensis' redbud seedlings were grafted with 'Canadensis' RYRSaV-infected material. Eight RYRSaV RT-PCR negative seedlings served as controls.

Eriophyid mite transmission was also evaluated. Thirty-eight (38) trees were surveyed in Fayetteville, AR between April and July 2013 and about 50 mites/tree were collected and mounted on microscope slides following standard protocol (Amrine and Manson, 1996; Amrine Jr et al., 2003; De Lillo et al., 2010). Fifty (50) adult mites were transferred from RYRSaV-infected trees to each of 24 RYRSaV RT-PCR negative redbud seedlings (four sets of six plants each). Seedlings were cut back, overwintered and tested monthly by RT-PCR for 6 months after emergence of new growth.

2.6 Alternative hosts

Glycine max (30), *Phaseolus vulgaris* cv. Kentucky Wonder (24), *Vigna unguiculata* cv. Monarch (15), a *Robinia pseudoacacia* seedling, *Solanum lycopersicum* cv. Beefsteak (20) and *Nicotiana benthamiana* (24) were cleft-grafted using 'Canadensis' RYRSaV-infected tissue (Table 2). In addition, a survey of potential alternative hosts in areas of high disease incidence was performed throughout the growing season using the RT-PCR detection test (Supplemental table 4).

3. Results

3.1 Virus discovery and genome analysis

There are five negative-sense RNA species identified as part of the RYRSaV genome. RNA 1 is 7,049 nucleotides (nt) (Fig. 3) with the ORF found between CAU₇₀₀₇₋₇₀₀₉-UUA₁₁₀₋₁₁₂, encoding a putative 2,299 amino acid (aa) of 267 kDa protein (p1). P1 is an RNA-dependent RNA polymerase (RdRp) with bunyavirus RdRp motifs mapped between aa_{648-1,404}. Motif A is conserved across all sequenced emaraviruses as DASKWS₁₁₃₂₋₁₁₃₇ and motif C (SDD₁₂₅₈₋₁₂₆₀), part of the palm domain, are involved in divalent-metal cation binding (Bruenn, 2003; Lukashevich et al., 1997). Motif B (QGNXNX_{2S2 1217-1225}) is also conserved in all sequenced emaraviruses (Supplemental Fig. 1) and thought to bind RNA as the Gly₁₂₁₈ promotes motility of the peptide. Motif D (KK₁₃₀₅₋₁₃₀₆) is thought to have catalytic activity through its Lys residues through its tertiary proximity to motif A. Motif E (EFLST₁₃₁₅₋₁₃₁₉) was found to be identical to all other sequenced emaraviruses except RLBV where Thr₁₃₁₉ is substituted for a Ser (Supplemental Fig. 1). RYRSaV as well as all other emaraviruses have the *Bunyaviridae* endonuclease domain motif RHD₉₈₋₁₀₀X₃₇PD₁₃₇₋₁₃₈X₁₃EXK₁₅₂₋₁₅₄, which is likely involved in cap snatching (Reguera et al., 2010; Walia and Falk, 2012). The protein shares between 32-54% aa sequence identity to other emaravirus orthologs (Supplemental Tables 5) with the highest sequence identity to the European mountain ash ringspot associated virus (EMARaV) p1.

RNA 2 is 2,220 nt long with an ORF between CAU₂₁₆₈₋₂₁₇₀-UUA₂₄₂₋₂₄₄, coding a putative glycoprotein precursor of 642 aa (74 kDa). The predicted protein structure (Kelley and Sternberg, 2009) is similar to the emaravirus and tospovirus orthologs (data not shown). Two potential cleavage sites were predicted at Ala₂₇ and Glu₁₉₁; however, both cleavage sites had low probability scores (Bendtsen et al., 2004). The N-terminus of p2 contains a secretion signal suggesting that p2 is secreted to the endoplasmic reticulum where the virus probably acquires

150 the particle membranes (Emanuelsson et al., 2000). Four transmembrane helices (Leu₄-Ala₂₆;
151 Try₉₆-Val₁₁₇; Leu₁₇₁-Val₁₈₈; Ile₅₈₅-Ile₆₀₂) were predicted (Sonnhammer et al., 1998). The protein
152 was predicted to form homodimers (Mukherjee and Zhang, 2011) and it has four putative N-
153 glycosylation sites (Asn₆₃, Asn₂₄₄, Asn₃₅₀, and Asn₃₅₄) with Asn₂₄₄ showing the highest
154 glycosylation probability along with a *Phlebovirus* glycoprotein motif (GCX₂CX₂G₄₇₇₋₄₈₄;
155 Supplemental Fig. 1). It shares 41% aa sequence identity (60% similarity) to EMARaV p2 and 21-
156 39% aa sequence identity to other emaravirus glycoproteins (Supplemental Table 6).

157 RNA 3 is 1,414 nt long with an ORF between CAU₁₃₂₃₋₁₃₂₅-UCA₃₈₄₋₃₈₆, coding the 313 aa
158 (35 kDa) putative nucleocapsid protein. The size matches that of the orthologs of wheat mosaic
159 virus (WMoV) and pigeon pea sterility mosaic virus-1 (PPSMV-1). It shares 44% aa sequence
160 identity (66% similarity) to EMARaV, and 18-39% aa identity to other emaravirus p3s
161 (Supplemental Table 7). The protein contains large stretches of residues that probably bind RNA
162 (Wang and Brown, 2006) and is predicted to form homodimers (Mukherjee and Zhang, 2011).
163 No enzymatic motifs were found in p3, though some conserved regions were identified when
164 orthologs were aligned including the two previously reported conserved regions
165 (NAV_{SX₂RX₂A}₁₃₈₋₁₄₇; NKLA₁₈₆₋₁₈₉) (Elbeaino et al., 2009) (Supplemental Fig. 1).

166 RNA 4 is 1,513 nt with an ORF between CAU₁₄₁₂₋₁₄₁₄-CUA₂₈₄₋₂₈₆, coding for the 376 aa
167 (42 kDa) movement protein based on orthology to fig mosaic virus (FMV) and raspberry leaf
168 blotch virus (RLBV) p4s (Ishikawa et al., 2013; Yu et al., 2013). The protein shares core
169 secondary structure to the 30K movement protein superfamily but no detectable sequence

homology as is the case with its orthologs (Yu et al., 2013) whereas its aa identity to its orthologs ranges between 20%-33% (Supplemental Table 8).

RNA 5 is 1272 nt long and contains an ORF (CAU₁₁₈₄₋₁₁₈₆-UCA₅₁₅₋₅₁₇) encoding a putative 26 kDa protein (p5). The p5 protein is most similar to EMARaV p4 than other emaravirus p5 proteins in the databases. In fact, EMARaV p4 does not share this secondary structure or sequence homology with emaravirus movement proteins, instead it likely is a ortholog of FMV p6 and RRV p6 based on secondary structural analysis (Di Bello et al., 2015; Yu et al., 2013). An alignment between RYRSaV p5, EMARaV p4 and FMV, RRV, pigeon pea sterility mosaic virus-2 (PPSMV-2) p6s showed only 15-18% identity (36-38% similarity; Altschul et al., 1997; Supplemental Table 9). Yet, conservation is supported by the similar secondary structure of all five proteins (Supplemental Fig. 2; Buchan et al., 2013). *In-silico* predictions of the protein function indicated its involvement in the secretory pathway (Emanuelsson et al., 2000) having two *O*-glycosylation sites (Ser_{205, 209}) and potentially interacts with p2 (Mukherjee and Zhang, 2011). The discovery of RYRSaV p5 provides an answer as to why EMARaV p4 lacks homology to the movement proteins (Yu et al., 2013) identified in other emaraviruses.

Phylogenetic analysis using the RdRp aa sequence of RYRSaV and related viruses clearly places the virus in the *Emaravirus* genus, being most closely related to EMARaV (Fig. 4). An interesting feature of the phylogram is *Emaravirus* species infecting hosts belonging to the same botanical family (e.g. RYRSaV, PPSMV-1 and PPSMV-2) evolving from different lineages.

3.2 Population structure

Diversity was studied in the two areas commonly used to examine emaravirus population structure (Bargen et al., 2013; Danesh-Amuz et al., 2014; Dong et al., 2016; Kallinen et al., 2009; Laney et al., 2011; Stewart, 2016; Walia et al., 2014; Supplemental Tables 2 and 3). Nucleotide and aa sequence identity ranged from 98-99% and 99-100% respectively, for the ORFs evaluated. A noteworthy polymorphism involved the stop codon of p3. The majority of isolates had an ochre stop codon (UAA) whereas two, including the isolate used to sequence the virus genome, which had an opal stop codon (UGA).

3.3 Detection

RYRSaV was found in all trees showing typical RYRS symptoms; however, two asymptomatic 'Forest Pansy' trees also tested positive for the virus (Table 1). Several positives from each cultivar were sequenced to verify the presence of the virus, and the sequences were over 99% identical to the type isolate.

3.4 Transmission

One of the eight grafted redbud seedlings tested positive for RYRSaV and developed typical RYRS symptoms. Almost all of the over 2,500 eriophyid mites mounted were identified as *Aculops cercidis* (Hall, 1967). Only three mites from those mounted belonged to different species, two *Dichopelmus* sp. and one *Acaralox* sp, none of which have been reported in redbud before. None of 24 redbud seedlings in the *A. cercidis* transmission study developed symptoms and tested negative for the virus.

3.5 Alternative hosts

210 Seventeen *G. max*, one *P. vulgaris*, one *R. pseudoacacia* and two *V. unguiculata* plants tested
211 positive for the virus and developed symptoms after grafting (Fig. 5) whereas the grafted
212 solanaceous hosts, *S. lycopersicum* and *N. benthamiana*, were asymptomatic and tested
213 RYRSaV-free. All field-collected samples of potential alternative hosts tested negative for
214 RYRSaV (Supplemental Table 4).

215 4. Discussion

216 RYRS has only been documented in northwest Arkansas possibly because no other research has
217 been done on redbud viruses other than the Kim and Martin report (Kim and Martin, 1978). We
218 characterized several aspects of RYRSaV, the putative causal agent of the disease including
219 genome and population structure and transmission to redbud and other species.

220 Known emaraviruses have between four and eight genome segments (Mielke and
221 Muehlbach, 2007; Tatineni et al., 2014; Lu et al., 2015). We identified five segments in RYRSaV
222 using different sequencing approaches and applying the models of the Di Bello et al. (2015)
223 study which identified seven segments in the RRV genome. This possibly does not represent
224 the complete genome of RYRSaV. Yet, the recent discovery that emaravirus segment numbers
225 fluctuate within species may also point to the fact that RYRSaV is a smaller virus coding for all
226 essential genes for virus replication, movement and transmission (Stewart, 2016).

227 Forty-five (45) 'Forest Pansy' trees were tested in areas of high disease incidence but
228 only two were positive for RYRSaV. These trees were inspected for three seasons and did not
229 display symptoms. Trees were tested at several times during the growing periods, using both
230 leaf and petiole tissue inspected under a stereoscope with the assayed areas being free of

231 eriophyid mites. Based on field observations, symptoms were present in mature 'Canadensis'
232 leaves only when temperatures were below 25°C for several days whereas they persisted in
233 'Oklahoma' all season long. Given the minimal diversity of the RYRSaV isolates studied, the
234 observed differences in symptoms are probably plant genotype-dependent reactions.

235 The virus can move systemically, proven by graft transmission to both 'Canadensis' and
236 several legume species. Yet, this may not be the case for 'Forest Pansy' where the virus may not
237 move outside the initial infection loci.

238 *A. cercidis* was found ubiquitously on redbud in northwest Arkansas, but adults of this
239 species did not transmit RYRSaV during the course of our trials. Further work will determine
240 whether *A. cercidis* nymphs or other, potential cryptic species could transmit the virus. In the
241 case of wheat streak mosaic virus, different genotypes of the mite *Aceria tosichella* transmit the
242 virus at different efficiencies (Wosula et al., 2016). It is plausible that another mites could
243 transmit the virus, such as *Aculops scotti*, a species originally found on *C. canadensis* var.
244 *texensis* in California, (Keifer, 1940), but those were not found during the course of our
245 experiments.

246 The phylogeny of the genera creates an evolutionary conundrum. How did the genus
247 *Emaravirus* arise? Emaraviruses appear polyphyletic in origin showing differences in even the
248 number of segments between isolates (Stewart, 2016), with the evolution of particular species
249 being disconnected from the phylogeny of their hosts. EMARaV, RLBV, and RRV, three
250 rosaceous-infecting emaraviruses all occupy three distinct nodes and are not as closely related
251 to each other as EMARaV is to RYRSaV (Fig. 4). Even PPSMV-1 and PPSMV-2, two viruses

infecting the same legume species do not appear to share a common ancestor (Fig. 4; Elbeaino et al., 2015). The vector phylogeny is also complex. FMV, PPSMV, PPSMV-2 and WMoV are transmitted by mites in the genus *Aceria*, whereas RLBV, and RRV are transmitted by mites in the genus *Phyllocoptes* (Caglayan et al., 2012; Di Bello et al., 2015; Kulkarni et al., 2002; McGavin et al., 2012; Tatineni et al., 2014).

Even though eriophyids are largely considered host-specific (Skoracka et al., 2010), a plausible explanation of emaravirus evolution and speciation in diverse hosts is that vectors move and transmit between species. This has been shown for *A. ficus* transmitting FMV to periwinkle (Caglayan et al., 2012) as well as the *A. tosichella* species complex which can transmit WMoV and survive on a number of grass species (Skoracka et al., 2013). It could be that the allopatric nature of the genus can be explained by such host species changes followed by re-transmission of the virus by eriophyids colonizing that host.

Host range is an important factor to consider as RYRSaV can move systemically and cause symptoms in economically important legumes. As we are yet to identify the virus vector, precaution should be taken so as not to move the virus to RYRSaV-free areas with significant legume production.

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References

- Aapola, A., Knesek, J., Mink, G., 1974. The influence of inoculation procedure on the host range of pea seed-borne mosaic virus. *Phytopathology* 64, 1003–1006.
- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi:10.1093/nar/25.17.3389
- Amrine, J., Manson, D., 1996. 1.6. 3 Preparation, mounting and descriptive study of eriophyoid mites. *World Crop Pests* 6, 383–396.
- Amrine Jr, J., Stasny, T.A., Flechtmann, C.H., others, 2003. Revised keys to world genera of Eriophyoidea (Acari: Prostigmata). Indira Publishing House.
- Bargen, S. von, Arndt, N., Robel, J., Jalkanen, R., Büttner, C., 2013. Detection and genetic variability of European mountain ash ringspot-associated virus (EMARaV) in Sweden. *For. Pathol.* 43, 429–432.
- Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* 340, 783–795.
- Bruenn, J.A., 2003. A structural and primary sequence comparison of the viral RNA-dependent RNA polymerases. *Nucleic Acids Res.* 31, 1821–1829.
- Buchan, D.W.A., Minneci, F., Nugent, T.C.O., Bryson, K., Jones, D.T., 2013. Scalable web services for the PSIPRED Protein Analysis Workbench. *Nucleic Acids Res.* 41, W349–W357. doi:10.1093/nar/gkt381
- Caglayan, K., Elçi, E., Serce, C.U., Kaya, K., Gazel, M., Medina, V., others, 2012. Detection of Fig mosaic virus in viruliferous eriophyid mite *Aceria ficus*. *J. Plant Pathol.* 94, 629–634.
- Danesh-Amuz, S., Rakhshandehroo, F., Rezaee, S., 2014. Prevalence and genetic diversity of fig mosaic virus isolates infecting fig tree in Iran. *Acta Virol* 58, 245–52.
- De Lillo, E., Craemer, C., Amrine Jr, J., Nuzzaci, G., 2010. Recommended procedures and techniques for morphological studies of Eriophyoidea (Acari: Prostigmata), in: *Eriophyoid Mites: Progress and Prognoses*. Springer, pp. 283–307.
- Di Bello, P.L., Ho, T., Tzanetakis, I.E., 2015. The evolution of emaraviruses is becoming more complex: seven segments identified in the causal agent of Rose rosette disease. *Virus Res.* 210, 241 – 244. doi:http://dx.doi.org/10.1016/j.virusres.2015.08.009
- Dong, L., Lemmetty, A., Latvala, S., Samuilova, O., Valkonen, J., 2016. Occurrence and genetic diversity of Raspberry leaf blotch virus (RLBV) infecting cultivated and wild *Rubus* species in Finland. *Ann. Appl. Biol.* 168, 122–132.
- Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797.
- Elbeaino, T., Digiario, M., Martelli, G.P., 2009. Complete nucleotide sequence of four RNA segments of fig mosaic virus. *Arch. Virol.* 154, 1719–1727. doi:10.1007/s00705-009-0509-3
- Elbeaino, T., Digiario, M., Uppala, M., Sudini, H., 2015. Deep sequencing of dsRNAs recovered from mosaic-diseased pigeonpea reveals the presence of a novel emaravirus: pigeonpea sterility mosaic virus 2. *Arch. Virol.* 160, 2019–2029.

- Emanuelsson, O., Nielsen, H., Brunak, S., von Heijne, G., 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* 300, 1005–1016. doi:10.1006/jmbi.2000.3903
- Gupta, R., Jung, E., Brunak, S., 2004. Prediction of N-glycosylation sites in human proteins. <http://www.cbs.dtu.dk/services/NetNGlyc/>.
- Hall, C., 1967. *The Eriophyoidea of Kansas*, 9th ed. University of Kansas Publications.
- Hampton, R., Beczner, L., Hagedorn, D., Bos, L., Inouye, T., Barnett, O., Musil, M., Meiners, J., 1978. Host reactions of mechanically transmissible legume viruses of the northern temperate zone. *Phytopathology* 68, 989–997.
- Ho, T., Tzanetakis, I.E., 2014. Development of a virus detection and discovery pipeline using next generation sequencing. *Virology* 471, 54–60.
- Huang, X., Madan, A., 1999. CAP3: A DNA Sequence Assembly Program. *Genome Res.* 9, 868–877. doi:10.1101/gr.9.9.868
- Ishikawa, K., Maejima, K., Komatsu, K., Netsu, O., Keima, T., Shiraishi, T., Okano, Y., Hashimoto, M., Yamaji, Y., Namba, S., 2013. Fig mosaic emaravirus p4 protein is involved in cell-to-cell movement. *J. Gen. Virol.* 94, 682–686. doi:10.1099/vir.0.047860-0
- Jones, R.A., 2012. Virus diseases of annual pasture legumes: incidences, losses, epidemiology, and management. *Crop Pasture Sci.* 63, 399–418.
- Kallinen, A., Lindberg, I., Tugume, A., Valkonen, J., 2009. Detection, distribution, and genetic variability of European mountain ash ringspot-associated virus. *Phytopathology* 99, 344–352.
- Keifer, H.H., 1940. *Eriophyid Studies*. Bull. Dep. Agric. State Calif. 8, 21–46.
- Kelley, L.A., Sternberg, M.J.E., 2009. Protein structure prediction on the Web: a case study using the Phyre server. *Nat. Protoc.* 4, 363–371. doi:10.1038/nprot.2009.2
- Kim, K., Ahn, K., Gergelich, R., Kim, S.-B., 2001. Possible etiology of eriophyid mite-borne pathogens associated with double membrane-bound particles. *Virus–Insect–Plant Interact.* KF Harris OP Smith JF Dodds Eds 29–50.
- Kim, K., Martin, E., 1978. Virus-like particles associated with yellow ringspot of redbud. *Phytopathol News* 12, 119.
- Kulkarni, N., Kumar, P.L., Muniyappa, V., Jones, A.T., Reddy, D., 2002. Transmission of Pigeon pea sterility mosaic virus by the eriophyid mite, *Aceria cajani* (Acari: Arthropoda). *Plant Dis.* 86, 1297–1302.
- Laney, A.G., 2010. Characterization of the causal agents of Rose rosette and Redbud yellow ringspot. M.S. Thesis, University of Arkansas.
- Laney, A.G., Keller, K.E., Martin, R.R., Tzanetakis, I.E., 2011. A discovery 70 years in the making: characterization of the Rose rosette virus. *J. Gen. Virol.* 92, 1727–1732. doi:10.1099/vir.0.031146-0
- Lukashevich, I.S., Djavani, M., Shapiro, K., Sanchez, A., Ravkov, E., Nichol, S.T., Salvato, M.S., 1997. The Lassa fever virus L gene: nucleotide sequence, comparison, and precipitation of a predicted 250 kDa protein with monospecific antiserum. *J. Gen. Virol.* 78, 547–551.
- Lu, Y., McGavin, W., Cock, P.J., Schnettler, E., Yan, F., Chen, J., MacFarlane, S. 2015. Newly identified RNAs of raspberry leaf blotch virus encoding a related group of proteins. *J. Gen. Virol.* 96, 3432–3439. doi: 10.1099/jgv.0.000277.

356 Marchler-Bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He,
 357 J., Gwadz, M., Hurwitz, D.I., Lanczycki, C.J., Lu, F., Marchler, G.H., Song, J.S., Thanki, N.,
 358 Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Bryant, S.H., 2015. CDD: NCBI's
 359 conserved domain database. *Nucleic Acids Res.* 43, D222–D226.
 360 McGavin, W.J., Mitchell, C., Cock, P.J.A., Wright, K.M., MacFarlane, S.A., 2012. Raspberry leaf
 361 blotch virus, a putative new member of the genus Emaravirus, encodes a novel genomic
 362 RNA. *J. Gen. Virol.* 93, 430–437. doi:10.1099/vir.0.037937-0
 363 Mielke, N., Muehlbach, H.-P., 2007. A novel, multipartite, negative-strand RNA virus is
 364 associated with the ringspot disease of European mountain ash (*Sorbus aucuparia* L.). *J.*
 365 *Gen. Virol.* 88, 1337–1346, <http://dx.doi.org/10.1099/vir.0.82715-0>.
 366 Mukherjee, S., Zhang, Y., 2011. Protein-protein complex structure predictions by multimeric
 367 threading and template recombination. *Structure* 19, 955–966.
 368 doi:10.1016/j.str.2011.04.006
 369 Reguera, J., Weber, F., Cusack, S., 2010. Bunyaviridae RNA polymerases (L-protein) have an N-
 370 terminal, influenza-like endonuclease domain, essential for viral cap-dependent
 371 transcription. *PLoS Pathog* 6, e1001101.
 372 Skoracka, A., Kuczyński, L., Szydło, W., Rector, B., 2013. The wheat curl mite *Aceria tosichella*
 373 (*Acari: Eriophyoidea*) is a complex of cryptic lineages with divergent host ranges:
 374 evidence from molecular and plant bioassay data. *Biol. J. Linn. Soc.* 109, 165–180.
 375 Skoracka, A., Smith, L., Oldfield, G., Cristofaro, M., Amrine, J.W., 2010. Host-plant specificity and
 376 specialization in eriophyoid mites and their importance for the use of eriophyoid mites
 377 as biocontrol agents of weeds. *Exp. Appl. Acarol.* 51, 93–113.
 378 Sonnhammer, E.L., Von Heijne, G., Krogh, A., others, 1998. A hidden Markov model for
 379 predicting transmembrane helices in protein sequences. In *Proc. Int. Conf. Intell. Syst.*
 380 *Mol. Biol.* 6, 175–182.
 381 Stewart, L.R., 2016. Sequence diversity of wheat mosaic virus isolates. *Virus Res.* 213, 299–303.
 382 Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular
 383 evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729.
 384 Tatineni, S., McMechan, A.J., Wosula, E.N., Wegulo, S.N., Graybosch, R.A., French, R., Hein, G.L.,
 385 2014. An eriophyid mite-transmitted plant virus contains eight genomic RNA segments
 386 with unusual heterogeneity in the nucleocapsid protein. *J. Virol.* 88, 11834–11845.
 387 Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of
 388 progressive multiple sequence alignment through sequence weighting, position-specific
 389 gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
 390 doi:10.1093/nar/22.22.4673
 391 Tzanetakis, I.E., Halgren, A., Mosier, N., Martin, R.R., 2007. Identification and characterization of
 392 Raspberry mottle virus, a novel member of the Closteroviridae. *Virus Res.* 127, 26–33.
 393 Walia, J.J., Falk, B.W., 2012. Fig mosaic virus mRNAs show generation by cap-snatching. *Virology*
 394 426, 162–166. doi:10.1016/j.virol.2012.01.035
 395 Walia, J.J., Willemsen, A., Elci, E., Caglayan, K., Falk, B.W., Rubio, L., 2014. Genetic variation and
 396 possible mechanisms driving the evolution of worldwide Fig mosaic virus isolates.
 397 *Phytopathology* 104, 108–114.

Wang, L., Brown, S.J., 2006. BindN: a web-based tool for efficient prediction of DNA and RNA binding sites in amino acid sequences. *Nucleic Acids Res.* 34, W243–W248. doi:10.1093/nar/gkl298

Wosula, E., McMechan, A., Oliveira-Hofman, C., Wegulo, S., Hein, G., 2016. Differential Transmission of Two Isolates of Wheat streak mosaic virus by Five Wheat Curl Mite Populations. *Plant Dis.* 100, 154–158.

Yu, C., Karlin, D.G., Lu, Y., Wright, K., Chen, J., MacFarlane, S., 2013. Experimental and bioinformatic evidence that raspberry leaf blotch emaravirus P4 is a movement protein of the 30K superfamily. *J. Gen. Virol.* 94, 2117–2128. doi:10.1099/vir.0.053256-0

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 ¹	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

¹N/A: Material without yellow ringspot symptoms

Table 2: Species cleft-grafted with redbud yellow ringspot associated virus (RYRSaV)-infected material.

Species	RYRSaV positive /total
<i>Cercis canadensis</i>	1/8
<i>Glycine max</i>	17/30
<i>Nicotiana benthamiana</i>	0/24
<i>Phaseolus vulgaris</i> cv Kentucky Wonder	1/24
<i>Robinia pseudoacacia</i>	1/1
<i>Solanum lycopersicum</i> cv Beefsteak	0/20
<i>Vigna unguiculata</i> cv Monarch	2/15

Figure 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.

Figure 2

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

Figure 3

Redbud yellow ringspot associated virus genome organization with coordinates of the predicted proteins; RNA 1: Bunyaviridae RdRp motifs A–E (Supplemental Fig. 1), RNA 2: the phlebovirus glycoprotein motif (Phl) , the four transmembrane domains (gray boxes), and the putative cleavage site (arrow), RNA 3: emaravirus nucleocapsid motifs 1,2 (Supplemental Fig. 1).

Figure 4

Fig. 4. Redbud yellow ringspot associated virus phylogram (RYRSaV; JF795479) and members of the *Bunyaviridae* using the RdRp amino acid sequence, with species from the genera *Emaravirus*, *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, *Tospovirus*, and *Tenuivirus*. Citrus psorosis virus (CPsV; YP_089661.1), *Ophiovirus*; *Ophioviridae* was used as an outgroup. Species include tomato spotted wilt virus (TSWV; NP_049362), capsicum chlorosis virus (CaCV; YP_717924.1), impatiens necrotic spot virus, (INSV; NP_619710.1), groundnut bud necrosis virus (GBNV; NP_619688.1), tomato zonate spot virus (TZSV; YP_001740047.1), rice stripe virus (RSV; NC_003755), rice grassy stunt virus (RGSV; NC_003755.1), dugbe virus, (DUGV; NP_690576.1), crimean-congo hemorrhagic fever virus (CCHFV; YP_325663.1), bunyamwera virus (BUNV; NP_047211.1), akabane virus (AKAV; YP_001497159.1), la crosse virus, (LACV; NP_671968.1), rift valley fever virus (RVFV; YP_003848704.1), european mountain ash ringspot-associated virus (EMARaV; YP_003104764.1), fig mosaic virus (FMV; CAQ03479.7), pigeon pea sterility mosaic virus (PPSMV1; CCP46989), pigeon pea sterility mosaic virus-2 (PPSMV-2; HF912243), rose rosette virus, (RRV; ADZ54688.1), raspberry leaf blotch virus (RLBV; CBZ42024.1), wheat mosaic virus (WMoV; AIK23031.1), and hantaan virus (HTNV; NP_941982.1). Analysis was performed with MUSCLE in MEGA6, using the neighbor-joining algorithm, and bootstrapping consisting of 1000 pseudoreplicates (Tamura et al. 2013).

Figure 5

Symptoms on hosts grafted with redbud yellow ringspot-infected material and tested positive for redbud yellow ringspot associated virus: A. *Glycine max*; B. *Phaseolus vulgaris* cv. Kentucky Wonder; C. *Vigna unguiculata* cv. Monarch and D. *Robinia pseudoacacia*