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Prevalence of a vertically transmitted single-stranded DNA virus in spinybacked orbweavers
(*Gasteracantha cancriformis*) from Florida, USA

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Abstract

Spiders (Order Araneae, Class Arachnida) are an important group of predatory arthropods in terrestrial ecosystems that have been recently identified as an untapped reservoir of single-stranded (ss)DNA viruses. Specifically, spiders harbor a diversity of ssDNA viruses encoding a replication-associated protein (Rep) within a circular genome. However, little is known about the ecology of novel circular Rep-encoding ssDNA (CRESS DNA) viruses. Here we investigated two CRESS DNA viruses recently identified in spinybacked orbweavers (Gasteracantha cancriformis), namely spinybacked orbweaver circular virus (SpOrbCV) 1 and 2. SpOrbCV-1 was detected in the majority (>65%) of spider specimens from all life stages, including eggs, spiderlings, and adults, demonstrating that this virus is active within spinybacked orbweavers. In contrast, SpOrbCV-2 was only detected in adults at a lower (36%) prevalence. Since we also detected SpOrbCV-2 in other spider species and this virus has been reported from a dragonfly, we suggest that SpOrbCV-2 is accumulated in these predators through common insect prey. The prevalence of SpOrbCV-1 in collected specimens allowed us to design assays to characterize this virus, which represents a new group of CRESS DNA viruses, the “circularisviruses”. To our knowledge, SpOrbCV-1 is the first example of a vertically transmitted virus in spiders, which may explain its high prevalence in spinybacked orbweavers. Since vertically transmitted viruses infecting insects (Class Insecta) can manipulate their host behavior and physiology, future studies should investigate the ecological role of vertically transmitted viruses in spiders.
Introduction

Viruses encoding a replication-associated protein (Rep) within a covalently-closed, single-stranded (ss)DNA genome have been increasingly identified in organisms spanning the eukaryotic tree of life, from unicellular organisms to major groups of vertebrate and invertebrate metazoans [1]. Current classification schemes for eukaryotic circular Rep-encoding ssDNA (CRESS DNA) viruses, which include six monophyletic families (Geminiviridae, Nanoviridae, Circoviridae, Genomoviridae, Smacoviridae, Bacilladnaviridae) [40], lag behind the discovery rate of these viruses. Many CRESS DNA viral species cannot be accommodated within established taxa and remain unclassified, indicating that the number of CRESS DNA viral families will increase. Given that many CRESS DNA viruses have only been identified through sequencing, little is known about the ecology of a large number of CRESS DNA viruses, including the host species for both classified (e.g., Genomoviridae and Smacoviridae) and unclassified viruses. Terrestrial arthropods have been recently identified as an untapped reservoir of CRESS DNA viral diversity, highlighting the need to further investigate viral prevalence and transmission in this dominant and undersampled group of animals [2].

Spiders (Order Araneae) are an important group of predatory arthropods [3] that have been extensively studied due to their fascinating biology (e.g., [4-6]), potential as natural pest biocontrol agents (e.g., [7, 8]), and the biotechnological potential of their silk and venom [9-11]. Previous studies have investigated the influence of microbes, in particular bacteria, on spider biology [12-15]. Notably, studies investigating vertically transmitted bacteria in spiders indicate that these ‘reproductive parasites’ may lead to sex-ratio bias through behavior manipulation and male-killing [16-18]. Although spiders harbor a diversity of viruses, there is scarce information regarding the prevalence and ecological impact of viral infection in spiders. Endogenous viral sequences within spider chromosomes indicate that these arachnids have been infected by both CRESS DNA viruses and double-stranded DNA polyomaviruses [19, 20]. Studies within the past four years have also revealed a diversity of novel extant viruses in multiple spider species, including more than 20 CRESS DNA [2] and 100 RNA viruses [21-24] encompassing various viral families and unclassified species. Moreover, RNA viruses have been detected in multiple spider organs, including brain, venom glands and silk glands, indicating that viral infection may play a role in spider ecology [21].
The present study investigated two CRESS DNA viruses, namely spinybacked orbweaver circular virus (SpOrbCV) 1 and 2, that were reported from specimens of *Gasteracantha cancriformis*, commonly known as the spinybacked orbweaver [2]. Spinybacked orbweavers are generally considered beneficial spiders since they trap and consume insects considered pests, such as whiteflies, and are typically found in wooded areas where there is abundant foliage and branches [25]. However, spinybacked orbweavers are also commonly found in man-made structures, such as porches and screen pool enclosures, where they may become a nuisance. These conspicuous spiders, which can be easily spotted due to their contrasting colors and spine-like projections around their abdomen, are widely distributed throughout the Caribbean, Central America, and Southern USA [25, 26]. To our knowledge, SpOrbCV-1 and SpOrbCV-2 are the first viruses that have been identified in this tropical spider species.

Here we examined the prevalence of SpOrbCV-1 and -2 in different spider life stages, including eggs, spiderlings, and adults, to explore the association between spinybacked orbweavers and these CRESS DNA viruses. Similar to known CRESS DNA viruses [1], both SpOrbCVs have a small genome (< 2 kb) encoding the Rep and a putative capsid protein. SpOrbCV-1 is an unclassified CRESS DNA virus that has persisted in the Tampa Bay, Florida, USA spinybacked orbweaver population for at least four years (2014 through 2018). We detected SpOrbCV-1 in the majority (>65%) of specimens from all life stages, demonstrating that this virus is active within spinybacked orbweavers. In contrast, the detection of SpOrbCV-2 in adult specimens alone, combined with its presence in various species of spiders (this study) and a dragonfly [27], indicates that this cyclovirus (genus *Cyclovirus*, family *Circoviridae*) may accumulate in predatory arthropods through common insect prey. The prevalence of SpOrbCV-1 in collected specimens allowed us to design assays to characterize this virus, which represents a new cohesive group of CRESS DNA viruses, referred to here as circularisviruses. Finally, the detection of SpOrbCV-1 in eggs represents the first evidence for vertical transmission of viruses in spiders, which may explain its high prevalence in spinybacked orbweavers.
Materials and Methods

Analysis of viral sequences related to SpOrbCV-1

SpOrbCV-1 (GenBank accession number MH545519) and SpOrbCV-2 (GenBank accession number MH545515) were recently described as a part of a CRESS DNA viral discovery effort in terrestrial arthropods [2]. Since SpOrbCV-1 cannot be accommodated within established CRESS DNA viral families, we further evaluated the characteristics of this virus and other unclassified CRESS DNA viruses. CRESS DNA virus genomes with top BLASTn matches, including reciprocal searches, to the SpOrbCV-1 genome were obtained from GenBank in December 2018. SeqBuilder from the Lasergene software package version 11.2.1 (DNASTAR, Madison, WI) was used to explore genomic features in the identified genomes, including: size, open reading frames (ORFs), genome organization, and putative origin of replication (ori), which is marked by a conserved nonanucleotide motif at the apex of a predicted stem-loop structure [28]. Genome-wide and predicted amino acid sequence pairwise identities were calculated using the Sequence Demarcation Tool version 1.2 [29]. Predicted Rep amino acid sequences from each genome were aligned using the MUSCLE algorithm as implemented in MEGA7 [30, 31]. Alignments were then used to create a graphical representation of conserved residues found within CRESS DNA viral Rep domains, including endonuclease and superfamily 3 (SF3) helicase motifs, in WebLogo 3 [32].

Collection of spinybacked orbweavers to investigate SpOrbCVs

To further investigate SpOrbCVs, spinybacked orbweaver samples were collected from vegetation and man-made structures found outdoors as well as screen pool enclosures in the Tampa Bay area in Florida, USA in 2017 and 2018. The majority of samples, including adults (66%, n = 154), eggs (89%, n = 35) and spiderlings (100%, n = 14), were collected from a single screen pool enclosure that was closely monitored. Based on size and the presence of abdominal spines [26], 99% of sampled adults were female. All eggs were collected by carefully scraping and collecting complete egg sacs from surfaces without damaging the eggs. Spiderling collections only took place in 2018. To do this, egg sacs were monitored until spiderlings emerged (~2 - 5 weeks) and spiderlings were then collected before they dispersed from the silk surrounding the egg sac. All specimens were collected using sterile 15 or 50 ml conical tubes and kept at room temperature until taken to the lab within one hour of collection. Once in the lab, samples from adults were
either immediately stored as whole specimens or two legs were dissected off individual specimens using a sterile scalpel and stored separately. Whole and dissected specimens as well as leg subsamples were stored at -80°C until further processing.

**PCR assays to evaluate the prevalence of SpOrbCVs**

The prevalence of SpOrbCV-1 and SpOrbCV-2 was investigated in specimens representing different spinybacked orbweaver life stages through PCR. The prevalence in adults was initially assessed based on positive PCR products from virions isolated from whole specimens following a method previously described for discovery of CRESS DNA viruses in arthropods (Rosario et al 2012, Rosario et al 2018). Briefly, spiders were rinsed with sterile suspension medium (SM) buffer [0.1 M NaCl, 50 mM Tris/HCl (pH 7.4), 10 mM MgSO₄] to remove debris, followed by homogenization in 1 ml SM buffer through bead beating in a Fisherbrand™ Bead Mill 4 homogenizer (Fisher Scientific, Hampton, NH) for one minute using 1 mm glass beads. Homogenates were then centrifuged at 6,000 x g for 6 min and the supernatants containing viral particles were filtered through a 0.45-μ m Sterivex filter (MilliporeSigma, Burlington, MA) to remove cells. DNA was extracted from 200 μl of the filtrate containing partially purified virus particles using the QIAmp MinElute Virus Spin kit (Qiagen, Hilden, Germany). However, since virus particles isolated from whole specimens may include viruses from dietary content, viral prevalence was further assessed in adult spiders by testing legs. For this purpose, DNA was directly extracted from two legs per specimen using the Extract-N-Amp Tissue PCR Kit (MilliporeSigma, St. Louis, MO) following the manufacturer’s protocol.

Viral prevalence in eggs and spiderlings was assessed on a per egg sac basis rather than individual eggs or spiderlings. For this purpose, the silk surrounding the eggs was removed using sterile pipette tips and scapels and about a third of the eggs or two spiderlings from a given sac were transferred to a 1.5 ml microcentrifuge tube. After rinsing the eggs and spiderlings with SM buffer, DNA was directly extracted from tissues using the Extract-N-Amp Tissue PCR Kit (MilliporeSigma, St. Louis, MO).

To assess the prevalence of each virus, primer pairs SpOrbCV1_F2/R2 and SpOrbCV2_F2/R2 (Table 1) were used for PCR assays targeting partial SpOrbCV-1 and SpOrbCV-2 genomes, respectively. For this purpose, 1 μl of DNA extract from adults, eggs, and spiderlings was amplified through rolling circle amplification (RCA) to enrich for small circular
templates [33], such as CRESS DNA genomes, using the Illustra TempliPhi Amplification kit (GE Healthcare, Chicago, IL). Four microliters of DNA extract or 0.6 µl of RCA product were used as template in 50 µl PCR reactions containing 1.5 mM MgCl₂, 1X Apex NH₄ buffer, 0.5 µM of each primer, 0.2 mM of each dNTP, and 1U Apex Red Taq DNA Polymerase (Genesee Scientific, San Diego, CA). Each PCR proceeded at 94 ºC for 5 min, followed by 35 cycles of 94 ºC for 1 min, appropriate annealing temperature for 45 s (Table 1), and 72 ºC for 2.5 min, followed by a final extension at 72 ºC for 8 min. Additionally, RCA products obtained from virus particles isolated from a collection of 106 spiders from various families (Supplemental Table S1) were tested to evaluate if SpOrbCVs could be detected in other spider species using the same PCR assay. This spider collection included specimens from areas where we identified spinybacked orbweavers positive for SpOrbCVs and specimens originally collected for CRESS DNA virus discovery purposes [2].

All PCRs included a negative process control composed of RCA product from SM buffer DNA extracts processed alongside samples as well as a negative PCR control where sterile water was used as template. DNA from the original spinybacked orbweavers where SpOrbCV-1 and SpOrbCV-2 were detected served as positive controls. All PCR results were confirmed through agarose gel electrophoresis and ethidium bromide staining. Templates resulting in strong, single bands of the correct size relative to the positive control in agarose gels were considered positive, whereas products producing weak bands were confirmed through Sanger sequencing with the relevant PCR primers.

**Egg surface decontamination to evaluate SpOrbCV-1 vertical transmission**

Since eggs were positive for SpOrbCV-1, we evaluated if the virus could be detected in individual eggs after surface decontamination. To test this, a total of 24 eggs from four egg sacs known to be positive for SpOrbCV-1 were transferred individually to 1.5 ml microcentrifuge tubes and processed through one out of four treatments. Each treatment was performed on three eggs from each of two egg sacs for a total of six replicates per treatment. Individual eggs were rinsed with SM buffer (untreated control) or decontaminated by submerging each egg in either 50 µl of 2% bleach for 5 min or 0.1% bleach for 30 mins [34]. For the remaining decontamination treatment, each egg was submerged in 50 µl of 70% ethanol followed by 10% bleach for 1 min each [35]. After bleach exposure, eggs were rinsed four times with 50 µl of SM buffer. The last
SM buffer wash was removed and DNA was immediately extracted from the rinsed egg using the
Extract-N-Amp Tissue PCR Kit (MilliporeSigma, St. Louis, MO). RCA products from each egg
were then tested using the PCR assay described above with SpOrbCV-1_F2/R2 primers. A
representative DNA extract from each treatment was spiked with a positive SpOrbCV-1 DNA
control and subjected to RCA followed by PCR to assess potential inhibition due to the
decontamination treatment.

Characterization of virion and tissue associated SpOrbCV-1 DNA

The single-stranded nature and polarity of the encapsidated SpOrbCV-1 DNA was
evaluated using approaches similar to those previously used to describe novel circular ssDNA
viruses [36]. All specimens selected for genome characterization assays were known to be positive
for SpOrbCV-1 based on leg subsamples. For virion strand or encapsidated DNA characterization
assays, virus particles were first purified from adult spinybacked orbweavers (n = 5) homogenized
in SM buffer. Virus particles were purified by filtering homogenates through a 0.22 µm Sterivex
filter and incubating 170 µl of filtrate at 37°C for 2 hrs with a nuclease cocktail [37, 38] containing
1X Turbo DNAse Buffer (Invitrogen, Carlsbad, CA), 21U Turbo DNAse (Invitrogen, Carlsbad,
CA), 4.5U of Baseline-ZERO DNase (Epicentre, Madison, WI), and 112.5U Benzonase
(MilliporeSigma, Burlington, MA) to remove unencapsidated DNA. DNA was extracted from
200 µl of nuclease-treated viral particles using the QIAmp MinElute Virus Spin kit (Qiagen,
Hilden, Germany). The single-stranded nature of encapsidated SpOrbCV-1 DNA was then
assessed based on the susceptibility of the extracted DNA to S1 nuclease and restriction enzyme
digestion, which degrade single- and double-stranded DNA, respectively.

A controlled experiment using commercially available single- and double-stranded forms
of bacteriophage phiX174 circular DNA (New England Biolabs, Ipswich, MA) was conducted to
determine appropriate concentrations and incubation times for S1 nuclease treatment. Two PCR
assays with primer pairs, phiX174_F1/R1 and phiX174_F3/R3 (Table 1), were used to confirm
digestion. A total of 24U of S1 nuclease (Promega, Madison, WI) and incubation at 37°C for 1 hr
was determined suitable to digest 120 ng of phiX174 ssDNA without completely degrading 120
ng of dsDNA. After appropriate S1 treatment conditions were determined, DNA extracted from
virus particles purified from spinybacked orbweavers were split into two 12 µl aliquots. One
aliquot was treated with 24U of S1 nuclease and incubated at 37°C for 1 hr, while the other aliquot
was treated with 1 µl FastDigest EcoRI (Thermo Fisher Scientific, Waltham, MA) in 30 µl reactions following manufacturer’s instructions. Products from the S1 nuclease treatment were cleaned with the DNA Clean & Concentrator kit (Zymo Research, Irvine, CA) and eluted with 12 µl of sterile water. Cleaned digested products (2.5 µl) were used as template for a 25 µl PCR reaction using SpOrbCV-1_F3/R3 primers (Table 1), which flank an EcoRI restriction site in the SpOrbCV-1 genome, to confirm nuclease digestions.

A very similar nuclease treatment approach was used to determine if a dsDNA replicative form (RF) of the SpOrbCV-1 genome could be detected in spinybacked orbweaver tissues. To do this, the hard, shell-like abdomen was removed from five adult spinybacked orbweavers and DNA was directly extracted from the cephalothorax. Spiderlings from six egg sacs were also investigated for the presence of a RF. Total DNA was extracted from individual adults and twenty spiderlings per egg sac using the ZR Tissue & Insect DNA Kit (Zymo Research, Irvine, CA) following the manufacturer’s protocol. DNA extracts were treated with 24U of S1 nuclease in 30 µl reactions and incubated at 37ºC for 1 hr. S1 nuclease digested products were cleaned using the DNA Clean & Concentrator kit (Zymo Research, Irvine, CA) and eluted with 12 µl of sterile water. Clean S1 digested products (2.5 µl) were then treated with 1 µl FastDigest EcoRI following manufacturer’s instructions in 10 µl reactions. Cleaned 10-fold diluted S1 digested products and products treated with both S1 and EcoRI were used as template (2.5 µl) for PCR using SpOrbCV-1_F3/R3 primers (Table 1) in 25 µl reactions.

The polarity of the encapsidated SpOrbCV-1 DNA was determined using strand-specific, linear amplification with either SpOrbCV-1_F3 or SpOrbCV-1_R3 primers adapting a method previously used to characterize circular ssDNA viral genomes [39]. For this purpose, 2.5 µl of 100-fold diluted DNA extracted from virus particles purified from adult spinybacked orbweavers (n = 2) was linearly amplified for 50 cycles in 25 µl reactions containing 1.5 mM MgCl2, 1X Buffer II, 0.4 µM of either primer, 0.2 mM of each dNTP, and 0.6U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). A mock reaction containing template and all the reagents, except for primer, was also included alongside strand-specific reactions to account for background. Each amplification reaction proceeded at 94ºC for 5 min, followed by 50 cycles of 94ºC for 45 s, 52ºC for 45 s (Table 1), and 72ºC for 1 min, then a final extension at 72ºC for 8 min. One microliter of linearly amplified products and mock reactions were then exponentially amplified in 25 µl PCR reactions using primer pairs SpOrbCV1_F3n1/R3n1 and SpOrbCV1_F3n2/R3n2 (Table 1), which
are nested relative to strand-specific primers. PCR reactions contained 1.5 mM MgCl₂, 1X Buffer II, 0.4 µM of each primer, 0.2 mM of each dNTP, and 0.6U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). Each reaction proceeded for 25 cycles using the same thermocycling conditions listed for strand-specific linear amplification. Note that the encapsidated viral DNA that is complementary to a given strand-specific primer will produce a strong signal after exponential amplification.

**Transmission electron microscopy analysis of SpOrbCV-1 positive specimens**

Transmission electron microscopy (TEM) was used to observe virus-like particles (VLPs) within spinybacked orbweaver tissues. For this purpose, legs were dissected from newly collected adult spinybacked orbweavers and immediately fixed in a mixture of 4% glutaraldehyde and 2% formaldehyde in 0.1M sodium cacodylate buffer, pH=7.3 using a mild vacuum. In addition, two leg subsamples from each specimen were left unfixed and processed with the Extract-N-Amp Tissue PCR Kit (MilliporeSigma, St. Louis, MO) to screen for SpOrbCV-1 through PCR using methods outlined above. Legs from five positive specimens were chosen for TEM analysis. For this purpose, fixed legs were rinsed for two hours with 0.1M sodium cacodylate buffer, pH=7.3 three times followed by an overnight rinse in the refrigerator and a final water rinse for 1 hour. Rinsed samples were secondarily fixed in 2% aqueous OsO₄ for 2 hours and rinsed in water for 30 minutes followed by overnight storage in water in the refrigerator. Tissue was enbloc stained in 0.5% aqueous uranyl acetate in the dark for 2 hours and then rinsed in water three times for 30 minutes each. Samples were dehydrated in an ethanol series, including 70% and 95% ethanol, for 15 minutes each, then two changes in 100% ethanol for 15 minutes each. A final dehydration involved two 30 minute changes in 100% acetone. Leg tissues were embedded in Spurr’s resin and cured in the oven. Ultrathin sections were cut and placed on copper grids for observation on a Hitachi 7100 TEM at 100 kV. A Gatan Orius high-resolution digital camera was used to record images.
Results and Discussion

*SpOrbCV-1 represents a novel group of CRESS DNA viruses*

SpOrbCV-1 and SpOrbCV-2 represent two distinct CRESS DNA viral groups identified in spinybacked orbweavers. SpOrbCV-1 cannot be classified within established taxa, whereas SpOrbCV-2 is an isolate of a species classified within the family *Circoviridae* (genus *Cyclovirus*) known as dragonfly associated cyclovirus 3 (DfACyV-3; accession number JX185424) [2]. Since SpOrbCV-1 shares similar genomic characteristics with unclassified viruses reported from other spider species, dragonflies, and bat feces [2], we further explored the genomic sequences of circularisviruses, named after the first virus reported from this group (dragonfly circularisvirus) [27], and identified common features among this novel group of CRESS DNA viruses. Reciprocal BLAST searches led to the identification of panicum ecklonii-associated virus (accession number MH425571), a virus reported from the leaf tissue of a perennial grass [41], as an additional circularisvirus. Therefore, circularisviruses include viruses identified in terrestrial arthropods, animal feces, and plants (Table 2).

Circularisviruses contain small (~1.9 – 2 kb), circular genomes with two major ORFs encoding Rep and capsid proteins in the same strand (Fig. 1). This genomic architecture was deemed novel back in 2009 when metagenomic analyses uncovered small, circular ssDNA viral genomes with gene arrangements that had only been observed in linear ssDNA viruses [42]. Although similar genome organizations have been observed in CRESS DNA viruses reported from various environments and organisms since then [28, 43-46], circularisviruses are the first phylogenetic group whose members contain genomes with Rep and capsid protein coding sequences on the same strand. Circularisviruses form a monophylogenetic clade when comparing CRESS DNA viruses based on the Rep [2] and share > 57% genome-wide pairwise identity (PI) among each other (Fig. 1), which is congruent with the lower limit of PIs reported for established CRESS DNA viral families [47-49]. Similar to most CRESS DNA viral groups, the circularisvirus Rep is more conserved than the putative capsid based on amino acid pairwise identities (Fig. 1). Based on the lowest species demarcation threshold (75% genome-wide pairwise identity) for established CRESS DNA viral families [50], currently there are eight distinct circularisvirus species sharing <70% genome-wide PI and two genomes representing variants of SpOrbCV-1, which share 91% genome-wide PI (Fig. 1). The distribution of genome-wide PIs among
Circularisviruses should be evaluated as more representative genomes are discovered, which may lead to the creation of a new CRESS DNA family.

To confirm the single-stranded nature of the SpOrbCV-1 genome, we took advantage of S1 nuclease and EcoRI restriction endonuclease, which degrade ssDNA templates and dsDNA at a specific site, respectively (Fig. 2). DNA extracts from purified virions were susceptible to S1 nuclease treatment but resistant to EcoRI digestion, confirming that SpOrbCV-1 virions encapsidate circular ssDNA. Based on the putative ori of many CRESS DNA viruses, which contains a conserved nonanucleotide motif (‘NANTATTAC’) at the apex of a stem-loop structure [28], it was predicted that the protein-encoding strand of SpOrbCV-1 is the virion strand. A strand-specific amplification assay on DNA extracted from virions purified from two individual spinybacked orbweavers confirmed that the Rep- and capsid-encoding strand containing the nonanucleotide motif ‘CAGTATTAC’ is indeed the virion strand of this circularisvirus. The majority of circularisvirus genomes contain a putative ori in the protein-coding strand with the conserved nonanucleotide motif ‘NANTATTAC’ (Fig. 1). However, two genomes, one reported from a spider (accession number MH545522) and the other from grass (accession number MH425571), have a substitution in the nonamer, namely ‘NANTGTTC’ and ‘NANTACTAC’, respectively. Similar nonanucleotide variants have been observed in other CRESS DNA viruses, such as members of the family Smacoviridae and some members of the family Genomoviridae [47, 48].

The circularisvirus Rep contains the two-domain organization that is distinctive of eukaryotic CRESS DNA viruses, including endonuclease and superfamily 3 (SF3) helicase domains that are important for rolling circle replication (RCR) (Fig. 1) [51, 52]. RCR motifs found within the endonuclease domain are important for RCR initiation and termination, whereas the helicase domain may be involved in DNA unwinding during elongation (reviewed by [28]). The circularisvirus Rep endonuclease domain contains conserved residues observed in other CRESS DNA viral Reps, including an arginine finger motif found within the SF3 helicase domain of most CRESS DNA viral groups [53]. However, the Walker-A motif within the SF3 helicase domain contains a unique substitution in comparison to all other CRESS DNA viral groups that is consistently found in all circularisvirus Reps. The Walker-A motif, which may be involved in substrate binding [54], is characterized by the sequence ‘GxxxxGK(S/T)’ in most CRESS DNA viral groups, with the exception of members of the families Smacoviridae and Nanoviridae which
contain the sequence ‘GxxGK(S/T)’ [53]. However, the circularisvirus Walker-A motif contains the sequence ‘GxxxxGKD’ (Fig. 1). The biochemical implications of this acidic amino acid substitution within the Walker-A motif are unknown, but it seems to be a distinguishing feature of circularisvirus Reps. Interestingly, BLAST analysis revealed an endogenous circularisvirus-like SF3 helicase sequence in a genome scaffold from Parasteatoda tepidariorum (accession XP_015919340), known as the common house spider. Since SF3 helices have been mainly identified in small DNA and RNA viruses [54, 55] and the P. tepidariorum has significant matches (BLASTp, e-value < 10^-50) to the helicase domain of circularisvirus Reps, including the ‘GxxxxGKD’ domain, it is possible that this spider genome acquired the helicase from a CRESS DNA virus.

SpOrbCV-1 is highly prevalent in spinybacked orbweavers

SpOrbCV-1 and SpOrbCV-2 were originally identified in spinybacked orbweavers [2]; however, SpOrbCV-2 shares 99% genome-wide pairwise identity with DfACyV-3, a cyclovirus species originally discovered in dragonflies collected in Florida in 2010 [27]. Since spiders and dragonflies are top insect predators, we hypothesized that SpOrbCV-2 is of dietary origin whereas SpOrbCV-1 is specifically associated with spinybacked orbweavers. To further explore the association between these CRESS DNA viruses and spinybacked orbweavers, we compared the prevalence of SpOrbCV-1 and SpOrbCV-2 in specimens collected in 2017 and 2018 through PCR.

PCR assays indicated that SpOrbCV-1 was more prevalent in spinybacked orbweavers than SpOrbCV-2 (Fig. 3). Notably, SpOrbV-1 was detected in at least 65% of specimens representing various spinybacked orbweaver life stages, including adults, eggs, and spiderlings. In contrast, SpOrbCV-2 was only detected in adults and the highest prevalence (36%) was detected among samples composed of virus particles isolated from whole specimens compared to 2% based on leg tissues alone. The vast majority (85%) of samples that were positive for SpOrbCV-2 (n = 13) were also positive for SpOrbCV-1. In addition, SpOrbCV-1 was more abundant in leg samples than SpOrbCV-2 given that the former was detected without RCA pre-amplification, whereas SpOrbCV-2 was only detected in PCR reactions containing RCA product as template. A similar trend was observed for virus particles isolated from whole bodies since 40% and 20% of SpOrbCV-1 and SpOrbCV-2 positive samples, respectively, were directly identified from DNA extracts, rather than RCA products.
Although most specimens were collected from a single screen pool enclosure, both SpOrbCV-1 and SpOrbCV-2 were also detected in samples collected outside of this closely monitored area. While SpOrbCV-2 was only detected in spiders from two screen pool enclosures, SpOrbCV-1 was detected in samples collected from all tested sites, including six pool enclosures and three outdoor areas. Therefore, SpOrbCV-1 is more widely associated with spinybacked orbweavers than SpOrbCV-2.

Despite the high prevalence of SpOrbCV-1 in spinybacked orbweavers, including SpOrbCV-2 positive specimens, this virus was not detected in any of the 106 specimens representing various species from at least twelve families of spiders (Supplemental Table S1). However, SpOrbCV-2 was detected in two specimens representing distinct spider species. One of the SpOrbCV-2 positive specimens was collected within the closely monitored area where some spinybacked orbweavers were also positive for this virus. Therefore, the PCR data support that SpOrbCV-1 is highly prevalent and specifically associated with spinybacked orbweavers, whereas SpOrbCV-2 is less prevalent and can be detected in different species of predatory arthropods including insects (dragonflies) and at least three species of spiders. Although these data suggest that SpOrbCV-2 is likely associated with dietary contents, at this time, we cannot rule out the possibility that SpOrbCV-2 infects spinybacked orbweavers. For example, it is possible that SpOrbCV-2 has different tissue tropism than SpOrbCV-1 and, thus, cannot be readily detected in legs. Additionally, little is known about latent infections, a type of persistent infection where virions are not produced, in arthropods [56]. PCR assays used to target viruses associated with whole spiders only tested virus particles and, thus, prevalence data based on whole bodies did not account for potential latent infections.

**Similar VLPs are found in SpOrbCV-1 positive specimens**

TEM was used to demonstrate the presence of VLPs within spider leg tissues that were positive for SpOrbCV-1 by PCR. Similar icosahedral VLPs, 25 nm in diameter, were observed in leg muscle tissue from each of the five specimens analyzed (Fig. 4). Although this morphology and VLP size resembles that of well-characterized CRESS DNA viruses, such as circoviruses [57], we cannot conclude that the observed VLPs represent SpOrbCV-1 without further research. It should be noted that one of the specimens also contained a second type of VLPs with icosahedral
morphology, averaging 66 nm in diameter, indicating that some spinybacked orbweavers may be co-infected with multiple viruses (Supplemental Fig. S1).

**SpOrbCV-1 is active within spinybacked orbweavers and is vertically transmitted**

Since SpOrbCV-1 was prevalent in spinybacked orbweavers we set out to determine if the virus was replicating within spiders by screening tissues for a dsDNA replicative form (RF) form. S1 nuclease treatment followed by EcoRI digestion of total DNA extracted from spider tissues was used to detect SpOrbCV-1 dsDNA, keeping in mind that dsDNA will be resistant to S1 treatment but not EcoRI digestions (Fig. 2). The five total DNA extracts from adult specimens subjected to the enzyme treatments resulted in the detection of dsDNA. The dsDNA RF was also detected in total DNA extracts from spiderlings, albeit with a lower detection rate (two out of six). These findings confirm that SpOrbCV-1 replicates within spinybacked orbweavers, including spiderling and adult life stages.

The prevalence of SpOrbCV-1 in eggs and spiderlings that had not emerged from the egg sac indicates the vertical transmission of this virus among spinybacked orbweavers. To distinguish between a transovum route of transmission, where contaminated egg surfaces lead to infected offspring, and a transmission pathway where viruses are passed to offspring within eggs [34, 56], we evaluated if the virus could still be detected after decontaminating egg surfaces. In at least five out of six replicates, SpOrbCV-1 was detected in individual eggs after decontaminating the egg surfaces using strategies from insect rearing studies, including 0.1% and 2% bleach treatments (Kukan 1999). SpOrbCV-1 was also detected in three out of six eggs treated with 70% ethanol followed by 10% bleach, which has been used as an effective surface decontamination strategy for molecular analysis [35]. However, this may underestimate the number of positive eggs since PCR inhibition was documented in the failed amplification of positive controls spiked into DNA extracts from samples treated with 10% bleach. Nevertheless, the detection of SpOrbCV-1 in eggs regardless of surface decontamination strategy indicates that this virus can be transmitted within eggs. Based on the high prevalence of SpOrbCV-1 in female spinybacked orbweavers, we speculate that this virus is maternally transmitted, which is a common form of parasite vertical transmission in arthropods [58], through a transovarial route. However, the role of male spiders in virus transmission cannot be excluded since the vast majority of specimens in this study were female.
Elucidating viral transmission strategies is critical to understand how a given virus persists in natural populations [59, 60]. To our knowledge, SpOrbCV-1 is the first virus found to be vertically transmitted in spiders, since most studies investigating viruses in spiders have focused on adults [21]. Moreover, although CRESS DNA viruses have been identified in all major lineages of terrestrial arthropods [2], SpOrbCV-1 is the first CRESS DNA virus shown to be vertically transmitted outside of fungal [61] and plant [62] viruses that are known to be vertically transmitted within their insect vector population. Vertically transmitted viruses are expected to have low virulence given that the fitness of the virus is tightly linked to that of its host [58, 60]. The high prevalence of SpOrbCV-1 in seemingly healthy spinybacked orbweavers seems to follow this general notion of low virulence. However, it remains to be determined if SpOrbCV-1 can also be transmitted horizontally. Several DNA and RNA viruses have mixed-modes of transmission, where the prevalence of either a vertical or horizontal route may provide a selective advantage depending on host densities [56, 59]. Considering that vertically transmitted viruses may play a major role in insect biology by manipulating their host’s physiology, behavior, and offspring sex-ratios to increase transmission [59, 63-65], future work should investigate the ecological role of vertically transmitted viruses in spiders.
References


**Author Statements**

**Authors and contributions**
KR and MB designed study. KR, KAM, AMG, and MB collected specimens. KR and KAM performed experimental work and data analysis. AMG performed transmission electron microscopy analysis. KR wrote the manuscript and prepared figures. KAM and MB reviewed and edited manuscript. All authors approved final draft.

**Conflicts of interest**
The authors declare that there are no conflicts of interest.

**Funding information**
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**Tables and Figure Legends**

**Table 1.** Primers used for PCR assays performed during this study.

**Table 2.** Summary of viral isolates representing circularisviruses.

**Figure 1.** Genome organization (A), conserved motifs (B) and pairwise sequence comparisons (C) for circularisviruses. Panel A shows a genome schematic of major open reading frames found within circularisvirus genomes, including replication-associated (Rep, red arrow) and capsid (Cp, grey arrow) protein coding regions, and the nonanucleotide motif marking the putative origin of replication within a predicted stem-loop structure. Lower case letters within the nonanucleotide motif indicate nucleotides that were observed in a single genome sequence and ‘n’ represents any nucleotide. Panel B depicts conserved motifs found within the Rep through amino acid sequence logos generated in WebLogo 3. The endonuclease domain sequence logo, including rolling circle replication (RCR) motifs I through III, is shown above the superfamily 3 (SF3) helicase domain logo, which includes Walker-A, Walker-B, Motif C, and arginine (R) finger motifs. Panel C shows pairwise identities calculated using complete genomes (left) or predicted amino acid sequences (right) for the Rep (dark grey) and Cp (light grey). The information for each panel was extracted from the nine genome sequences representing members of the circularisviruses, namely dragonfly circularisvirus (DfCir), two isolates of spinybacked orbweaver circular virus 1 (SpOrbCV-1A and SpOrbCV-1B), golden silk orbweaver associated circular virus 1 (GoSOrbACV-1), longjawed orbweaver circular virus 1 (LjOrbCV-1), cybaeus spider associated circular virus 1 (CySACV-1), pacific flying fox feces associated circular DNA virus 6 (PfffACV-6), and panicum ecklonii-associated virus (PeaV).

**Figure 2.** Schematic representation of methodological framework used to elucidate the single- or double-stranded nature of spinybacked orbweaver circular virus 1 DNA found within virions and spinybacked orbweaver tissues. Note that S1 nuclease degrades ssDNA templates (dotted line) non-specifically (left panel), whereas EcoRI restriction endonuclease nicks dsDNA at a specific site (shown as a gap) (right panel). PCR was used to confirm endonuclease treatment results. Grey arrows represent primer positions. PCR results for DNA from virions are highlighted in blue, whereas results for DNA from tissues are highlighted in orange. Note that S1 nuclease treated DNA from spinybacked orbweaver tissues was then treated with EcoRI to confirm its double-
stranded nature (indicated by the orange spider). An example gel image is provided in Supplemental Figure S2.

**Figure 3.** Images of spinybacked orbweaver life stages investigated during this study (A) and percentage of specimens from each life stage that were positive for spinybacked orbweaver circular virus (SpOrbCV) 1 and 2 (B). Each specimen category on the x-axis specifies if the data reflects the prevalence for specimens collected during 2017, 2018 or both years and the number (N) of individuals processed. Prevalence in adult specimens based on whole body (WB) and leg (L) samples are distinguished.

**Figure 4.** Transmission electron microscope images of virus-like particles (VLPs) found in leg tissues from spiders that tested positive for spinybacked orbweaver circular virus 1. VLPs had an average diameter of 25 nm and each image originates from a different specimen.

**Supplemental Table and Figure Legends**

**Table S1.** List of spider species tested for spinybacked orbweaver circular viruses (SpOrbCV).

**Figure S1.** Transmission electron microscope images showing a second type of virus-like particles (VLPs) observed within the leg tissues of a spinybacked orbweaver. Observed VLPs averaged ~66 nm in diameter.

**Figure S2.** Example gel image showing results for PCR assays targeting DNA extracted from virions (top) or spider tissues (bottom) after treatment with S1 nuclease or EcoRI restriction enzyme. Samples include an untreated positive control (U), PCR products from virion or tissue samples (S), and a negative control (N).
Table 1. Primers used for PCR assays performed during this study.

<table>
<thead>
<tr>
<th>Targeted virus</th>
<th>Purpose</th>
<th>Primer pair and sequences (5' – 3')</th>
<th>Primer positions¹</th>
<th>Product length²</th>
<th>Annealing temp (°C)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpOrbCV-1</td>
<td>Complete genome recovery (inverse PCR)</td>
<td>SpOrbCV1_F1: CACCACTCTCTGATTTAG SpOrbCV1_R1: CATTAAAGGATTTTTGCTT</td>
<td>377 – 394 395 – 414</td>
<td>2 kb</td>
<td>42</td>
</tr>
<tr>
<td>SpOrbCV-1</td>
<td>Sample screening for prevalence estimates</td>
<td>SpOrbCV1_F2: ACGGATGAAACAGCTGGAT SpOrbCV1_R2: ACGTCGAAGAAGAAGAAACG</td>
<td>455 – 474 1038 – 1058</td>
<td>1.2 kb</td>
<td>50</td>
</tr>
<tr>
<td>SpOrbCV-1</td>
<td>Genome characterization assays</td>
<td>SpOrbCV1_F3: ATCCAGGCTTGTTCATCGG SpOrbCV1_R3: CTTTTCTTCTTCTCGAGGT</td>
<td>455 – 474 1038 – 1058</td>
<td>600 bp</td>
<td>52</td>
</tr>
<tr>
<td>SpOrbCV-1</td>
<td>Nested PCR for genome characterization assays</td>
<td>SpOrbCV1_F3n1: TCTGTATAAGTTCTGAAGTTGAGGA SpOrbCV1_R3n1: AGCCAAACACAAATCGGAGG</td>
<td>496 – 518 673 – 692</td>
<td>200 bp</td>
<td>52</td>
</tr>
<tr>
<td>SpOrbCV-1</td>
<td>Nested PCR for genome characterization assays</td>
<td>SpOrbCV1_F3n2: GTGGAACCTTCGATCGTGTA SpOrbCV1_R3n2: CTGGCGGGGAGAAAAATGAAC</td>
<td>667 – 686 875 – 894</td>
<td>230 bp</td>
<td>52</td>
</tr>
<tr>
<td>SpOrbCV-2</td>
<td>Sample screening for prevalence estimates</td>
<td>SpOrbCV2_F1: ATCTTTACTGGGGCAGATGG SpOrbCV2_R1: AACATTTCCTCTGGGAATCGTTG</td>
<td>24 – 43 639 – 658</td>
<td>1.1 kb</td>
<td>54</td>
</tr>
<tr>
<td>phiX174</td>
<td>S1 nuclease experiment</td>
<td>phiX174_F1: TCAGATATCGACCTGCTGCT phiX174_R1: TCTTTGACGAACCGTCCAA</td>
<td>5034 – 5054 254 – 272</td>
<td>625 bp</td>
<td>54*</td>
</tr>
<tr>
<td>phiX174</td>
<td>S1 nuclease experiment</td>
<td>phiX174_F3: ACGCCGTTCAACCAGATATTG phiX174_R3: GTGGAACACGCATCGGACTC</td>
<td>5226 – 5246 352 – 371</td>
<td>530 bp</td>
<td>53*</td>
</tr>
</tbody>
</table>

¹Genome positions for each primer used to target spinybacked orbweavers circular viruses (SpOrbCV) and bacteriophage phiX174 (GenBank accession number NC_001422).
²Product sizes are listed in kilobases (kb) or base pairs (bp).
³Annealing temperatures highlighted with an asterisk (*) were incrementally decreased by 0.1°C every cycle.
Table 2. Summary of viral isolates representing circularisviruses.

<table>
<thead>
<tr>
<th>Virus1</th>
<th>Accession No.2</th>
<th>Isolate</th>
<th>Source</th>
<th>Scientific name</th>
<th>Sample type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dragonfly circularivirus</td>
<td>JX185415</td>
<td>TO-DF3E-2010</td>
<td>Dragonfly</td>
<td><em>Pantala flavescens</em></td>
<td>Abdomen</td>
<td>[27]</td>
</tr>
<tr>
<td>Cybaeus spider ACV-1</td>
<td>MH545522</td>
<td>BC_I1644C_F12</td>
<td>Spider</td>
<td><em>Cybaeus signifer</em></td>
<td>Whole specimen</td>
<td>[2]</td>
</tr>
<tr>
<td>Golden silk orbweaver ACV-1</td>
<td>MH545520</td>
<td>PR_I0960_F8</td>
<td>Spider</td>
<td><em>Nephila clavipes</em></td>
<td>Whole specimen</td>
<td>[2]</td>
</tr>
<tr>
<td>Longjawed orbweaver CV-1</td>
<td>MH545521</td>
<td>BC_I1601_F12</td>
<td>Spider</td>
<td><em>Leucauge argyra</em></td>
<td>Whole specimen</td>
<td>[2]</td>
</tr>
<tr>
<td>Spinybacked orbweaver CV-1</td>
<td>MH545518</td>
<td>FL_I0831_I69-H8</td>
<td>Spider</td>
<td><em>Gasteracantha cancriformis</em></td>
<td>Whole specimen</td>
<td>[2]</td>
</tr>
<tr>
<td>Spinybacked orbweaver CV-1</td>
<td>MH545519</td>
<td>FL_I1594-I104_E11</td>
<td>Spider</td>
<td><em>Gasteracantha cancriformis</em></td>
<td>Whole specimen</td>
<td>[2]</td>
</tr>
<tr>
<td>Pacific flying fox faeces ACV-6</td>
<td>KT732823</td>
<td>Tbat_H_77994</td>
<td>Bat</td>
<td><em>Pteropus tonganus</em></td>
<td>Feces</td>
<td>[66]</td>
</tr>
<tr>
<td>Panicum ecklonii AV</td>
<td>MH425571</td>
<td>2-82-I</td>
<td>Grass</td>
<td><em>Panicum ecklonii</em></td>
<td>Leaf</td>
<td>[41]</td>
</tr>
</tbody>
</table>

1Associated circular virus (ACV); circular virus (CV); associated virus (AV)
2GenBank accession number
A) Investigated spinybacked orbweaver life stages

B) Viral prevalence in specimens representing each life stage
Table S1. List of spider species tested for spinybacked orbweaver circular viruses (SpOrbCV)

<table>
<thead>
<tr>
<th>Species1</th>
<th>Family</th>
<th>Location2</th>
<th>Number of Specimens</th>
<th>SpOrbCV-1 Positives</th>
<th>SpOrbCV-2 Positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giant house spider (Eratigena atrica)</td>
<td>Agelenidae</td>
<td>BC</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cybaeus signifer</td>
<td>Cybaeidae</td>
<td>BC</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ND</td>
<td>Cybaeidae</td>
<td>BC</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Woodlouse hunter spider (Dysdero crocata)</td>
<td>Dysderidae</td>
<td>BC</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sierra dome spider (Neriene ligiosa)</td>
<td>Linyphiidae</td>
<td>BC</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Running crab spider (Philodromidae sp)</td>
<td>Philodromidae</td>
<td>BC</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pimia alticulata</td>
<td>Pimidae</td>
<td>BC</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zebra jumper spider (Salticus scenicus)</td>
<td>Salticidae</td>
<td>BC</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Segestria pacifica</td>
<td>Segestriidae</td>
<td>BC</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Common house spider (Parasteatoda tepidariorum)</td>
<td>Theridiidae</td>
<td>BC</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Western black widow spider (Latrodectus hesperus)</td>
<td>Theridiidae</td>
<td>BC</td>
<td>3</td>
<td>0</td>
<td>0</td>
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<tr>
<td>False black widow spider (Steatoda grossa)</td>
<td>Theridiidae</td>
<td>BC</td>
<td>3</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Candy stripe spider (Enoplognatha ovata)</td>
<td>Theridiidae</td>
<td>BC</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Goldenrod crab spider (Misumena vatia)</td>
<td>Thomisidae</td>
<td>BC</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leucauge sp</td>
<td>Tetragnathidae</td>
<td>SPE-A, FL</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Multiple (ND)</td>
<td>ND</td>
<td>SPE-A, FL</td>
<td>8</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Cyrtophora sp</td>
<td>Araneidae</td>
<td>SPE-B, FL</td>
<td>8</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Multiple (ND)</td>
<td>ND</td>
<td>SPE-B, FL</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leucauge sp</td>
<td>Tetragnathidae</td>
<td>SPE-C, FL</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Multiple (ND)</td>
<td>ND</td>
<td>SPE-C, FL</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>FL</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leucauge sp</td>
<td>Tetragnathidae</td>
<td>FL</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>FL</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Common and scientific names are listed whenever possible. However, some specimens were not taxonomically identified (ND; not determined). Specimens representing more than one unidentified species collected from the same site were merged into the same field and labeled as ‘multiple’.

2 Specimens were collected from outdoors in British Columbia (BC) and the Tampa Bay area in Florida, USA (FL). Some specimens were collected from three screen pool enclosures (SPE) distinguished by letters A through C.
Figure S1. Transmission electron microscope images showing a second type of virus-like particles (VLPs) observed within the leg tissues of a spinybacked orbweaver. Observed VLPs averaged ~66 nm in diameter.
**Figure S2.** Example gel image showing results for PCR assays targeting DNA extracted from virions (top) or spider tissues (bottom) after treatment with S1 nuclease or EcoRI restriction enzyme. Samples include an untreated positive control (U), PCR products from virion or tissue samples (S), and a negative control (N).