Placeholder title

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Abstract

Potexviruses (Alphaflexiviridae) are positive-sense single-stranded RNA viruses known to infect multiple species across plants, including species of Cactaceae. Cactus Virus X, Zygocactus Virus X, Schlumbergera Virus X, Pitaya Virus X, and Opuntia Virus X are five of the 48 currently described Potexvirus species, which all infect valuable ornamental and crop plants, often causing production losses. The taxonomic naming schemes often employ outdated plant name synonyms, complicating taxonomic assignments. Also, the source of infections in cultivated plants is unclear, as is the distribution of and significance of infections in wild species of cacti. The lack of clarity is partly related to low sampling across the family. Here, we report results of original RNA-seq experiments and archived sequence deposits, aimed at detecting Potexviruses in cacti, assembling whole genomes, estimating their phylogenetic relationships, and delimiting viral species. The data suggests novel modes of transmission, based on expression analyses across tissues, particularly pollen. We also perform molecular evolutionary analyses to detect genomic regions under different modes of selection. Finally, we examine and discuss the implications of our analyses for the taxonomy of Potexviruses across cacti.

Introduction

The plant viriome represents a fundamentally complex and intertwined evolutionary interaction between eukaryotic host and viral vector (Delwart (2007)). This coincides with the rise of faster, cheaper, high-throughput environmental metagenomic techniques (Delwart (2007); Lefeuvre et al. (2019); Schulz et al. (2017)): greenhouse-raised and lab-grown organisms that are commonly analyzed in experiments actually represent a small fraction of living diversity. Metagenomic studies aim to remedy this by sampling hundreds of thousands of genomes, and have vastly expanded both the cellular tree of life (Schulz et al. (2017); Hug et al. (2016)) and the viral tree of life (Gregory et al. (2019); Lefeuvre et al. (2019); Shi et al. (2016)). However, referring to viruses as a unified whole is not entirely correct or representative of the vast morphological diversity and innovative strategies present within the viral realms, many of which infect plants (Delwart (2007); Lefeuvre et al. (2019)). Metagenomic analysis of plants, particularly understudied or non-crop plants have both enriched our genetic knowledge of plants and uncovered novel insights on viral evolution, adaptation, and transmission. A careful study of the plant viriome provides a view into biological realities that are not presently reflected in the literature.

The study of plant viruses often suffers from poor nomenclature due to the erroneous practice of naming a virus based on hosts. The term "plant virus", for example, implies that plant infection is monophyletic rather than homoplastic (Lefeuvre et al. (2019)). There is strong evidence to suggest that viruses frequently have "jumped" from fungal or inverterbrate hosts to their associated plants (Lefeuvre et al. (2019)). Many agricultural plant viruses are named for the common name of a plant, for example, Pitaya Virus X. The common names "pitaya" and "dragonfruit" can both refer to two separate genera within Echinocereeae (Le Bellec and Vaillant (2011)): Selenicereus (which has 31 species) (Korotkova et al. (2017); Guerrero et al. (2019)) and Stenocereus, which has 23 putative species (Guerrero et al. (2019)). Further, the implication that one viral species exclusively infects all plants with the common name "pitaya" is incorrect since there are reports of multiple Potexviruses infecting the same host plant (Li* 2014). These problems persist throughout Potexvirus and are especially prominent with regards to cactus-infecting Potexviruses, underlining the necessity of a more informed taxonomy.

Study system

Cactus Virus X, Zygocactus Virus X, Schlumbergera Virus X, Pitaya Virus X, and Opuntia Virus X are all Potexviruses (family Alphaflexiviridae) that are grouped broadly by their infections of certain cacti: Selenicereus undatus and S. polyrhizus (Li et al. (2015); Peng et al. (2016)); Opuntia spp. especially tuna (Koenig et al. (2004); Duarte et al. (2008)) and monacantha (Attathom et al. (1978) Sammons 1961 Duarte 2008); Schlumbergera (previously Zygocactus) truncata and S. bridgesii (Duarte et al. (2008); Koenig et al. (2004)), Parodia (previously Notocactus) leninghausii (Park et al. (2018)), Echinopsis chamaecereus f. cristata, E. pectinatus f. cristata, E. jusbertii, and E. macrogona (Maliarenko and Mudrak (2013)); Mammillaria elongata f. cristata (Maliarenko and Mudrak (2013)); and multiple other species within many genera (Evallo et al. (2021)). Of these viruses, only CVX has been reported on wild Ferocactus cylindraceus (previously Ferrocactus acanthodes) (Attathom et al. (1978)) although this report predates DNA records confirming viral identity. Additionally, although they are originally found on cacti, the viruses are frequently manipulated with serological experiments and have been found to produce lesions (which indicate infection) on: Chenopodium murale L. (Maliarenko and Mudrak (2013)) and C. quinoa (Attathom (1978); Attathom et al. (1978); Brandes and Bercks (1963); Nicotiana alata Link el. Otto (Maliarenko and Mudrak (2013)); Four species of Amaranthaceae (Attathom (1978)); Escobaria vivipara (Attathom (1978)); and other cactaceae (Attathom (1978)). All cactus-infecting potexviruses consist of roughly 6,600 bp of positive-sense single-stranded RNA. They have similar rod-shaped filamentous virions and share the same division of five primary open reading frames (ORFs): Replicase (Rep), Triple gene block (TGB), Coat protein (CP), coded in the 5' direction as well as two smaller overlapping ORFs coded in the 3' direction: ORF6 and ORF7

(Evallo et al. (2021); Liou et al. (2004); Martelli et al. (2007)). They are closely related to other Potexviruses such as Alternantha Mosaic Virus and Papaya Mosaic Virus (Martelli et al. (2007); Park et al. (2018); Liou et al. (2004)). These viruses produce a wide range of symptomatic and damaging infections in cacti. Reports of symptomatic plants range from 5.5 percent of wild Ferocactus cylindraceus (Attathom et al. (1978)) up to 44 percent of crop plants on Hainan Island, China (Peng et al. (2016)). However, many infected plants do not show external signs of viral infection (Liou et al. (2004); Bos (1977)). The most commonly recognized symptoms of disease are mosaic, mottling, stunted growth and distortion (Maliarenko and Mudrak (2013); Peng et al. (2016); Attathom et al. (1978)). It is unclear what the method of transmission from infected plant to new host is. Some reports specify that cactus-infecting Potexviruses can only be transmitted through grafting (Duarte et al. (2008); Martelli et al. (2007)) but most agree that transmission can occur through other mechanical contact such as sap inoculation (Liou et al. (2004); Maliarenko and Mudrak (2013); Park et al. (2018)) and external tissue contact. Grafting is a primary means of propagation among crop cacti (Park et al. (2018)), and Selenicereus is a commonly chosen graft stock. However, there are reports of other members within Alphaflexiviridae transmitting via insect and seed vectors (Martelli et al. (2007)), and pre-DNA studies suggest that CVX may transmit via pollen in the wild (Attathom et al. (1978)). Knowledge about cactus-infecting potexviruses contribute to a growing yet biased study of plant viruses. The pursuit of wild cactus-infecting potexviruses serves to expand our evolutionary knowledge of viral evolution, host selection, and transmission mechanics.

Specific objectives and questions/summary

In this study we present the largest to date phylogeny of cactus-infecting Potexviruses. We attempt to use this expanded phylogeny to answer relevant questions about Potexvirus evolutionary relationships as well as revisiting the utility of decades-old taxonomy in current virus research.

Materials and Methods

Study Organism

RNA Sequencing

Tissues were collected and immediately submerged in 1.5 ml of RNA*later*™ solution (Invitrogen). Submerged samples were generally held at room temperature for thirty minutes and then stored at -80°C. Approximately 100 mg of tissue was ground with a cooled mortar in 1.5 ml tubes. Total RNA was isolated using Total RNA Mini Kit (Plant kit; IBI Scientific, Cat. No. IB47341) following manufacturer's instructions. We assessed RNA concentration and purity with a NanoDrop™ Lite Spectrophotometer (Thermo Scientific). The twenty-three samples used in this study were sequenced as part of a larger sequencing effort which consisted of four separate sequencing runs and included additional samples from other plant species. Sequencing libraries were prepared using the KAPA Stranded mRNA-Seq (Roche), and these libraries were sequenced on a single lane of Illumina HiSeq 4000 or Illumina NovaSeq 6000 platform (paired-end 150 bp reads) at the Duke University Center for Genomic and Computational Biology. The number of resulting read pairs (for the twenty-three samples presented here) ranged from 4,148,932 to 9,618,084 with a median of 6,363,556 and average of 6,293,553 (Table S1).

RNAseq Assemblies

Raw paired-end Illumina reads were first processed using Rcorrector v1.0.4 **?** to correct for random sequencing errors. Then, reads were trimmed with Trimmomatic v0.39 **?** to remove any read containing bases with Phred scores lower than 20, low quality reads less than 50 bp long, and any adapter or other Illumina-specific sequences that were still present. The remaining reads were filtered with Kraken 2 **?** to remove Small and Large Subunit ribosomal RNA (SILVA database) **?** and contaminating reads (minikraken2_v2 database). Additionally, we used custom-built databases, derived from RefSeq libraries: UniVec_Core, viral, mitochondrion, plastid, plasmid, archaea, bacteria, protozoa, human, and fungi to minimize the number of contam-

inating and non-nuclear reads. Only paired reads were used for transcriptome assemblies. *Schlumbergera truncata* filtered reads were combined across all samples into a single RNA-seq data set. We conducted a *de novo* transcriptome assembly using Trinity v2.8.5 **?** to generate a single reference transcriptome assembly for *Schlumbergera truncata*. The same assembly protocol was followed for the single pistil sample of *Matucana madisoniorum*.

NCBI Data Collection and Compilation

We collected publicly available genomes, complete proteins, gene annotations, and available metadata from Potexviruses (NCBI:txid12176) (NCBI: www.ncbi.nlm.nih.gov/, accession numbers provided in Supplemental Data). The untranslated regions (UTRs) were trimmed from the sequences to provide consistency.

We also searched the NCBI Sequence Read Archive (SRA) database (www.ncbi.nlm.nih.gov/sra) for RNA-sequencing (RNA-seq) data within Caryophyllales (NCBI:txid3524) that had been sequenced using the Illumina library sequencing platform. For each identified SRA run accession (SRR), any viral RNA that matched sample cactus-infecting Potexvirus RNA (accession numbers provided in Supplemental Data) was identified, extracted, and assembled using the kakapo 0.7.3-dev pipeline (http://flightless.one) with Kraken2 viral filters disabled. The .sam files produced through kakapo were loaded through Geneious 11.1.5 along with the Schlumbergera reads. These sequences were annotated using the Geneious 11.1.5 "Find ORFs" function.

The complete dataset comprises: 37 existing Potexvirus genomes and proteins, 4 new viral sequences located within original Schlumbergera truncata RNA-seq data, and 52 viral sequences found within NCBI Caryophyllales RNA-seq data.

Sequence Alignment and Phylogenetic Analyses

Sequence alignments were performed through MAFFT v7.429 (Katoh (2002)) using the full dataset. The aligned sequences were divided by ORF using the annotations to produce five partial sequence alignments corresponding to each ORF to accompany the full-sequence alignment. The individual proteins were exported to .FASTA files, then gaps at the start of the sequence and stop codons were removed manually. Phylogenetic relationships and bootstrap values were inferred using IQtree v1.6.12 (Nguyen et al. (2015)), ModelFinder (Kalyaanamoorthy et al. (2017)), and UFBoot (Hoang et al. (2018)) for both the individual gene/protein alignments and the full sequence alignment. Trees were visualized in R version 4.0.3 using ggtree v2.4.2 (Yu et al. (2017)). Host information was obtained through reported metadata and mapped onto the phylogeny. Species groupings were determined using the existing species boundaries when compared to the phylogenetic branch lengths within the Potexvirus genus. This was generally consistent with most recent branch lengths over 0.1 subs/site and this value was therefore used as a cutoff. Pairwise distance analysis was conducted on the sequence alignments in R using the ape v5.5 dist.dna() function with a raw model. For each defined clade, nonzero pairwise distances between each possible combination of tips was averaged. Expanded phylogenetic trees and individual gene/protein trees are available in the Supplementary Data. Pistils (without ovaries), pollen, leaf, and root tissues were collected and immediately submerged in 1.5 ml of RNA*later*™ solution (Invitrogen). Submerged samples were held at room temperature for thirty minutes and then moved to a -80 C freezer for storage. Approximately 100 mg of tissue was ground to a fine powder in 1.5 ml tubes submerged in liquid nitrogen. Total RNA was isolated using Total RNA Mini Kit (Plant kit; IBI Scientific, Cat. No. IB47341) following manufacturer's instructions. We assessed RNA concentration and purity with a NanoDrop™ Lite Spectrophotometer (Thermo Scientific). The XX samples used in this study were sequenced as part of a larger sequencing effort which consisted of XXX separate sequencing runs and included additional samples from other plant species. Sequencing libraries were prepared using the KAPA Stranded mRNA-Seq (Roche), and these libraries were sequenced on a single lane of Illumina HiSeq 4000 or Illumina NovaSeq 6000 platform (paired-end 150 bp reads) at the Duke University Center for Genomic and Computational Biology. The number of resulting read pairs (for the XX samples presented here) ranged from

X,XXX,XXX to X,XXX,XXX with a median of X,XXX,XXX and average of X,XXX,XXX (Table S1).

Data Accessibility

Results

Phylogenetic analysis

The phylogenetic tree places the new viral sequences from Schlumbergera and Selenicereus near existing viral species within Potexvirus (Figure 1).

The S. truncata samples were located within the Cactus Virus X clade and appear to represent the only known discovery of Cactus Virus X on members of Schlumbergera. The publicly available data which was collected from NCBI produced 52 new viral sequences which were dispersed among viral species. These samples were exclusively representative of Selenicereus undatus and Selenicereus polyrhizus hosts. The Cactus Virus X species clade is expanded by a factor of 8. The only putative species that was not expanded by either the Schlumbergera data or the Selenicereus data was Opuntia Virus X, which appears to be an outgroup to the other cactus-infecting Potexviruses.

Divisions within species

The five species within the phylogeny appear to be generally characterized by long (*) branch lengths separating clusters of closely related (short branch lengths) tips. Cactus Virus X, as a putative species, appears to have two time-separated evolutionary distant sets of tips. These have been marked as different colors in Figure 1. The other group that has outliers is Zygocactus Virus X, which appears to have a longer evolutionary distance between the tip "Zygocactus virus X KM288845.1" and the rest of the samples either identified as Zygocactus Virus X or identified as closely related to ZyVX. The relationship and its bootstrap value have both been marked with different colors in Figure 1.

Pairwise distances between species were calculated for six groups, with a value of 0 indicating identical sequences and a value of 1 indicating completely divergent sequences (Figure 2). The average nonzero pairwise distance between all included potexvirus tips was 0.256, maximum = 0.4925. When the outgroup (including Plantago asiatica MV, Alternantha MV, Papaya MV, etc.) was excluded from pairwise analysis, the average nonzero pairwise distance value was 0.177, maximum = 0.326. When these cactus-infecting potexviruses were subdivided into six groups of relatively recent diversification, the average nonzero pairwise distance for full-genome sequences was always above 0.015.

For the genes RNA-dependent RNA polymerase (RdRp) and Coat protein (CP), which the ICTV recommends be analyzed for species delimitation, the average nonzero pairwise distance was always above 0.02 (Figure 2). This correlates to roughly greater than 97.5 and 98 nucleotide identity.

Gene phylogenies

The assembled phylogenies for each of the five genes are available in their fully annotated versions in the Supplemental Data. The gene trees generally supported the species groupings of the full-genome tree and do not change the interpretation of the "main" tree.

Host range

The host from attached metadata for each species is noted in Figure 1. There are two unique samples collected from NCBI that report non-plant hosts of cactus-infecting potexviruses: "Cactus virus X SCM51431", and "Mytcor Virus 1 MG210801". These have the hosts Diptera and Bivalve, respectively.

Discussion

Discussion text.

Acknowledgments

Acknowledgments text.

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Figures