Australasian Plant Disease Notes (2023) 18:10
https://doi.org/10.1007/s13314-023-00492-4

Detection of multiple viruses and viroid in apple trees in Brazil and their possible association with decline

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Received: 6 December 2022 / Accepted: 14 February 2023

Abstract
In an attempt to provide additional information on the virome in apples in Southern Brazil in face of recurrent events of tree decline, plant samples of regional apple orchards have been submitted to HTS (high-throughput sequencing) analysis. Besides common latent viruses, results showed additionally the presence of apple rubbery wood virus 2 (ARWV 2) and citrus concave gum-associated virus (CCGaV) in orchards with decline history.

Keywords Apple decline · ARWV 2 · CCGaV · HTS

Brazilian apple production, among the ten largest producers worldwide, has advanced in five decades into a relevant segment of the country's economic activity. Latent viruses apple chlorotic leaf spot virus (ACLSV), apple stem grooving virus (ASGV), and apple stem pitting virus (ASPV) cause significant losses to Brazilian apple production. Recently, Nickel et al. (2020) reported apple rubbery wood virus 1 (ARWV 1) and ARWV 2 and citrus concave gum-associated virus (CCGaV) infecting apples in Brazil. Decline of apple trees is of significant concern to growers after recurrent outbreaks occured in the last five decades, generally involving association with multiple infections by latent viruses. To achieve a more comprehensive picture of viromes associated with particular Brazilian apple accessions and determine pathogens associated with apple decline syndromes, high-throughput sequencing (HTS) was performed on an Illumina NextSeq platform (USA).

Analyzed samples were from two 36 years-old apple trees of cultivars (cvs.) Fuji and Gala Standard from an orchard of which approximately 10% of plants had been affected by apple decline (Bom Jesus, RS, 28°39'35" S, 50°21'44" W) and a hybrid apple genotype MR1789 from a breeding program, approx. 30 years old (São Joaquim, SC, 28°16'29" S, 49°56'52" W). Total nucleic acid (TNA) was extracted using the RNeasy Plant Mini kit (Qiagen). The sequencing libraries were prepared using Illumina TruSeq Stranded Total RNA kit, and plant ribosomal RNA was depleted using the Ribo-Zero Plant kit. Raw reads were first processed by Fastp (Chen et al. 2018) and host sequences (*Malus domestica*) were removed by Bowtie 2 program (Langmead and Salzberg 2012). The taxonomic classification of the reads was performed using the Kraken 2 tool (Wood et al. 2019) built on a standard basis of Bacteria, Archaea, Viruses and Fungi. The results were plotted by the Recentrifuge tool (Martí 2019), only for viruses. Metagenomes were assembled by MetaviralSPAdes (Antipov et al. 2020) and MEGAHIT (Li et al. 2016). The resulting contigs were classified by BLASTn (Camacho et al. 2009), first against a RefSeq complete genome database (O'Leary et al. 2016) and then against the standard nucleotide (nt) database for refinement. For each virus species, the best contig (lowest e-value and highest bitscore) of each tool was extracted.

A total of 58, 62 and, 54 millions paired-end reads were obtained by HTS for cvs. Fuji Standard, Gala Standard and, MR1789, respectively. Considering the common latent virus isolates detected, the information of HTS data analysis (number of mapped reads, % mapped and, mean depth of coverage - times) was 242 to 19,991; 0.0014 to 0.1076 and, 21.3 to 259.8 (ACLSV), 6,941 to 31,364; 0.0264 to 0.1688 and, 104.9 to 476.8 (ASGV) and, 1,099 to 54,773; 0.0066 to 0.2948 and, 18.8 to 570.7 (ASPV), respectively. Long contigs of sizes 1,087 to 7,552 nucleotides (ACLSV), 6,482 to 6,487 nt (ASGV) and, 5,693 to 9,265 nt (ASPV) were obtained. Multiple contigs derived from complete

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Published online: 17 March 2023

or partial genomes and ORF contigs corresponding to six viral/viroidal pathogens were obtained from three cvs. as follows, cv. Fuji Standard: ASPV (GenBank accession number: OP535342), ASGV (OP535345), ACLSV (OP547330), ARWV 2 (segments L: OP547332, Ma: OP547334, Mb: OP547335, Sa: OP547333, Sb: OP547336) and, CCGaV (RNA 1: OP547337, RNA 2: OP556109); cv. Gala Standard: ASPV (complete RNA-dependent RNA polymerase, RdRp: OP535343), ASGV (OP535346), ACLSV (complete coat protein gene, CP: OP547331), CCGaV (partial RdRp, RNA 1: OP820578, RNA 2: OP820577) and, apple hammerhead viroid, AHVd (OP820576) and, finally, cv. MR1789: ASGV (OP535347) and, AHVd (OP535344). All obtained sequences of Brazilian isolates were submitted to BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide identities ranged from 80% to 98% (ASPV), 83% to 99% (ASGV), 86% to 92% (ACLSV), 98%, 97-98%, 96-99%, 97-99% and, 98-99% (ARWV 2, segments L, Ma, Mb, Sa and, Sb, respectively), 97-99% and, 97-99% (CCGaV, RNA segments 1 and 2, respectively) and, 85% to 99% (AHVd) with the homologous virus/viroid retrieved from GenBank database. Query cover was greater than 80%.

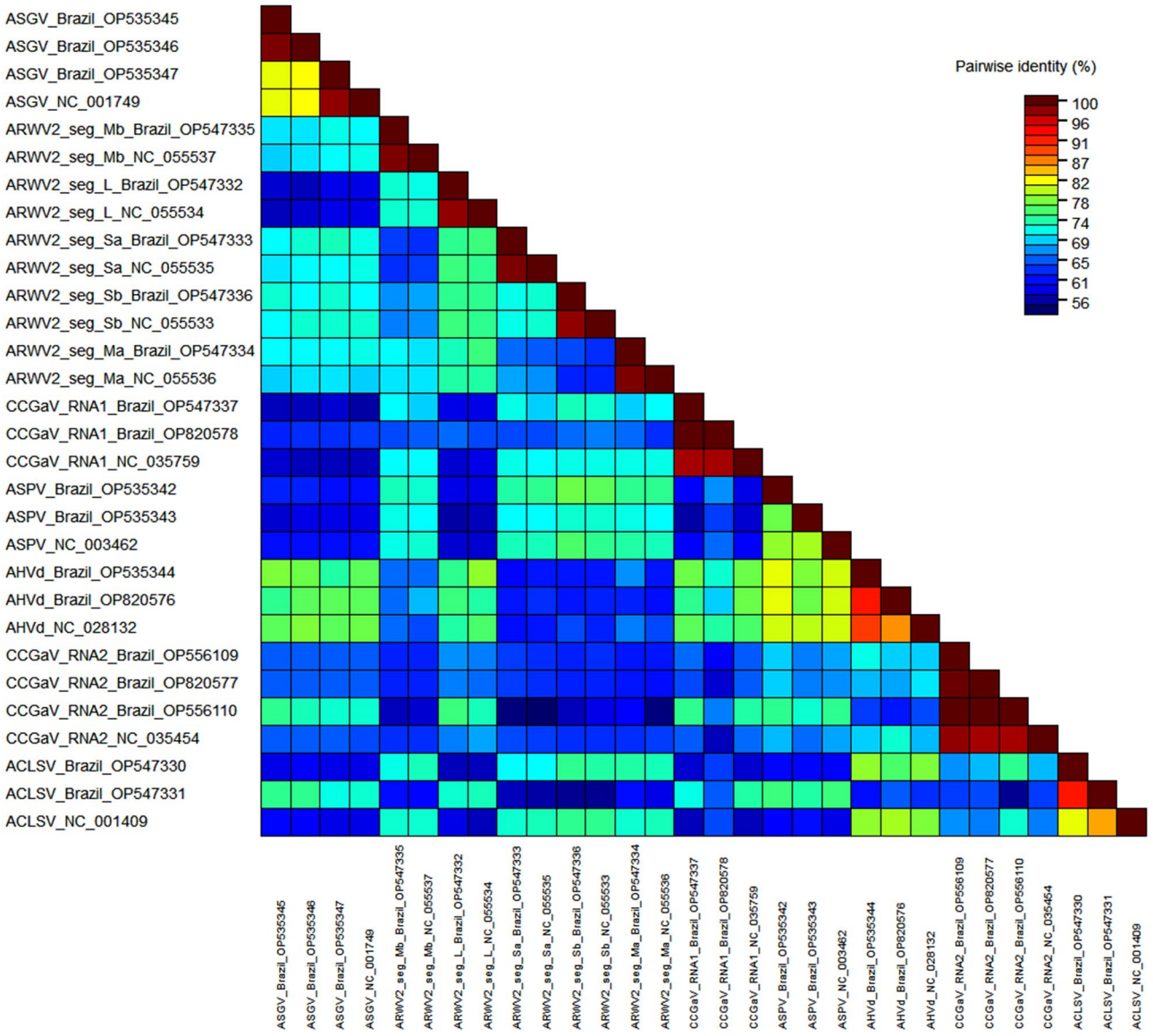

Fig. 1 Pairwise nucleotide sequence identity matrix of 30 isolates (sequences) generated using SDT software: 19 Brazilian sequences characterized in this work, including three isolates of apple stem grooving virus (ASGV), five sequences (L, Ma, Mb, Sa and Sb segments) of apple rubbery wood virus 2 (ARWV 2), five sequences (two RNA 1 and three RNA 2) of citrus concave gum-associated virus (CCGaV), two isolates of apple chlorotic leaf spot virus (ACLSV), two isolates of apple stem pitting virus (ASPV), and two isolates of apple hammerhead viroid (AHVd), and 11 reference sequences (RefSeq, identified as NC_) of homologous viruses and viroid retrieved from GenBank. GenBank accession codes are mentioned after virus/viroid names

Multiple nucleotide sequence alignments were also performed using Muscle alignment program and a pairwise nucleotide sequence identity matrix was generated using Sequence Demarcation Tool version 1.2 (SDT v1.2). The nucleotide identities among 19 obtained sequences and 11 reference sequences of homologous virus and viroid are shown in Fig. 1, thus allowing confirmation of the identity of the six pathogens present in the infected samples.

Long contigs of sizes 1,326 to 7,376 nucleotides (ARWV 2) and, 1,305 to 6,665 nt (CCGaV) were obtained for apple rubbery wood virus 2 (ARWV 2) and, citrus concave gum-associated virus (CCGaV) (order *Bunyavirales*, family *Phenuiviridae*) infecting cvs. Fuji Standard (both pathogens) and Gala Standard (CCGaV) and, contigs of 433-434 nt corresponding to the complete genome of apple hammerhead viroid (AHVd) infecting cvs. Gala Standard and MR1789. Considering the bunya-like virus and viroid isolates detected, the information of HTS data analysis (number of mapped reads, % mapped and, mean depth of coverage - times) was 4,398 to 81,744; 0.0236 to 0.4401 and, 287 to 5,564 (ARWV 2), 352 to 101,807; 0.0021 to 0.5481 and, 10 to 3,578 (CCGaV), and, 342,925 to 548,490; 2.0624 to 3.8690 and, 61,301 to 97,936 (AHVd), respectively.

CCGaV, recently described in citrus, and phylogenetically related to phlebo-like viruses (Navarro et al. 2018) is possibly associated with an apple decline (Wright et al. 2018). Apple rubbery wood disease (ARWD) damaged apple orchards in the 1970s through to the beginning of 2000 in Brazil. Declining plants usually show low vigor, lack of terminal growth, rubbery condition and die back of central leaders, small fruits, yield reduction, absence of feeder roots and tree decline (Souza and Parish 1992). Degradation of the vascular system, scrappy canopies and tree decline on sensitive rootstocks associated with latent virus infections are frequently observed (Souza et al. 2017). Rott et al. (2018) reported ARWV 1 and ARWV 2 in ARWD-affected apple plants from Germany, Canada and Japan associated with rubbery wood disease. Minutolo et al. (2021) suggested that CCGaV could have a wider host range (including citrus and apple trees) and geographic distribution than the one hitherto known. It is also important to highlight the detection by HTS of two plants infected with AHVd. To validate the HTS-based results in apples, RT-PCR assays were performed on TNA of three resampled original source plants using specific primers (Nickel et al. 2020). Amplicons of the expected sizes for each virus and viroid were detected in the analyzed plants. Additionally, ARWV 2 and CCGaV were also detected by RT-PCR using specific primers in TNA extracts of cvs. Belgolden, Fuji JVZ, Braeburn and, Royal Gala (ARWV 2), and Belgolden, Mishima, Fuji Standard, Fuji JVZ and Braeburn (CCGaV) in samples collected in Brazilian apple growing regions in recent years.

Analysis of HTS revealed the complete or near complete genomic sequences of the coinfecting agents. In three old apple trees several common latent viruses (ASPV, ASGV and, ACLSV) and one viroid were simultaneously detected, besides two highly relevant viruses (ARWV 2 and CCGaV), which have been possibly associated with apple declines (Nickel et al. 2020) in Brazil recently. Additional studies on the prevalence and the actual association of these viruses and viroid with apple plant decline are necessary.

Acknowledgements Authors thank Embrapa Uva e Vinho for financial support of Project nr. 20.18.03.036.00.00, and Marcos F. Vanni for technical support.

Data availability Data supporting findings of this study are openly available in NCBI public databases (GenBank).

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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