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SHORT COMMUNICATION

Biological and molecular characterization of two Brazilian isolates of *Apple stem grooving virus*

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Abstract *Apple stem grooving virus* (ASGV, genus *Capillovirus*) is disseminated worldwide, usually causing a latent infection in most commercial apple cultivars. However, infected scions grafted onto sensitive material display reduction of yield, loss of fruit quality and tree decline. In Brazil ASGV is associated with severe phloem necrosis, xylem pitting and decline of apple trees on Maruba-kaido (*Malus prunifolia* cv. Ringo) rootstocks usually in a complex with other latent viruses. Two Brazilian ASGV isolates from a mixed infection causing differing reactions on apple cv. Fuji on Maruba-kaido rootstocks, i.e., normal growth vs. tree decline, have been completely sequenced. The differing intensity of symptoms they co-induced on several woody indicator species/cultivars are indicative of biological differences between the two inocula. Their nucleotide sequences are 92.2% identical and display between 79.2% and 97.1% identity with other ASGV isolates available in GenBank. The two isolates (M219–3 and M220) shared 92.3% deduced amino acid (daa) sequence identity for ORF1, 95.3% for ORF2 and 93.3% for the CP gene. A high degree of daa identity, 95.8% and 96.6%, was observed between the CPs of M219–3 and M220, respectively, with the Brazilian isolate UV01. Potential recombination events and phylogenetic relationships to major parents were investigated in isolates M219–3 and M220.

Keywords ASGV · Apple virus · Phylogenetic analysis · Recombination

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Apple stem grooving virus (ASGV), the type species of the genus *Capillovirus* (family *Betaflexiviridae,* subfamily *Trivirinae*) (Adams et al. 2012, 2016), is distributed worldwide. No vector is known and the virus is therefore not epidemic. It causes no visible damage to most commercially used apple scions and rootstocks in which a latent infection, usually in complex with other latent viruses, may be detected. However, infected scions grafted onto sensitive material display reduction of yield, loss of fruit quality and tree decline.

In Japan, the virus causes top-working disease, inducing decline of apple trees grafted onto Mitsuba-kaido (*Malus sieboldii*) rootstocks (Yanase 1974). In Brazil ASGV is associated with differing degrees of severe phloem necrosis, xylem pitting and decline of apple trees on Maruba-kaido (*Malus prunifolia* cv. Ringo) rootstocks (Nickel et al. 1999, 2001). Viruses named *Citrus tatter leaf virus* (CTLV) in citrus (Magome et al. 1997) and *Pear necrotic ringspot virus* associated with black necrotic leaf spot disease on pear (Hyekyung et al. 2004) are presently known to be isolates of ASGV. ASGV infects woody, perennial, mono- and dicotyledonous plants (Chavan et al. 2013). Its natural hosts include apple, Japanese and European pear, quince, apricot, cherry, citrus, kiwi, lily and bamboo (Herron and Skaria 2000; Clover et al. 2003; Lovisolo et al. 2003; Tang et al. 2010; Zhong-Bin et al. 2010). *Cotoneaster bullata*, *Pyronia veitchii, M. micromalus*, *M. yunnanensis* and *M. tschonoskei* have been

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reported as sensitive species that react to ASGV infection with a range of foliar and wood symptoms (Van Der Meer 1975; Siebert and Engelbrecht 1981; Howell et al. 1996).

ASGV has flexuous, filamentous particles of approximately 600–700 × 12 nm. The ASGV genome consists of a linear, monopartite, positive-sense ssRNA, capped at the 5′-end, with approximately 6500 nucleotides excluding a poly-A tail at the 3′-end (Yoshikawa and Takahashi 1988; Yanase et al. 1990; Yoshikawa et al. 1992). The genomic RNA consists of two overlapping ORFs named ORF 1 and ORF 2. ORF 1 encodes a 241–242 kDa polyprotein (2105 amino acid residues) containing the replicase domain at its N-terminal region (including consensus motifs of methyltransferase, NTP-binding helicase, papain-like protease and RNA-dependent RNA polymerase) and the coat protein (CP, 27 kDa) at its C-terminal region (Yoshikawa et al. 1992, 1993; Ohira et al. 1995; Hirata et al. 2003). ORF 2, in a different reading frame within ORF 1, encodes the 36 kDa movement protein (MP) (Magome et al. 1997). Two highly variable regions are detected among isolates, a characteristic of capillovirus genome organization. Variable region 1 (VR1) is located between nucleotides (nt) 1239–1993, whereas variable region 2 (VR2) is found between the polymerase and CP coding regions, between nt 4787–5639 (Magome et al. 1997; Hirata et al. 2010; Liebenberg et al. 2012).

To date no ASGV isolate from Brazil has been completely sequenced. Two Brazilian isolates of ASGV, M219–3 and M220, from apple plants expressing different phenotypes are the object of the present report. The study aimed to sequence their complete genomes, comparing the nucleotide sequences of full-length genomes and analyzing phylogenetic relationships between them and other isolates available in Genbank. To determine their origin and to ascertain their relatedness to existing isolates, a recombination analysis was performed and phylogeny was inferred based on multiple alignments of the complete genomes.

Budwood was collected in a commercial orchard in 2014, in São Joaquim, state of Santa Catarina, Brazil, from two apple plants cv. Fuji grafted on Maruba-kaido rootstocks of the same genetic origin. They were established by grafting on apple seedlings in a greenhouse. ASGV isolate M219–3 was collected from a plant displaying severe stem pitting and bark necrosis on the Maruba-kaido rootstock, weak foliage and plant decline. Isolate M220 was collected from a plant with normal vigor and no symptoms on the wood or the bark of the rootstock.

Samples of both isolates were analyzed for virus infection by ASGV, *Apple chlorotic leaf spot trichovirus* (ACLSV) and *Apple stem pitting virus* (ASPV). Biological indexing was carried out by bud chip grafting on woody indicator plants *M. domestica* cv. Radiant Crab (RC), cv. Lord Lambourne (LL-S5), *M. micromalus* GMAL 273 (MM), *M. platycarpa* (MP) and *P. veitchii* (PV). Symptoms were recorded over two years. Mechanical transmission of viruses contained in the two samples was attempted onto *Nicotiana occidentalis* 37B. Aqueous plant extracts were prepared by powdering leaves and bark pieces in liquid nitrogen and grinding in 0.05 M potassium phosphate, pH 7.2, containing 2% nicotine sulphate (*w*/*v*).

Total RNA, extracted from inoculated herbaceous and woody indicators by adsorption to silica (SiO_2) particles (Rott and Jelkmann 2001), was analyzed by RT-PCR for latent viruses ASGV, ACLSV and ASPV under conditions reported earlier (Nickel et al. 1999, 2001). For detection of ASGV in inoculated plants, oligonucleotide primers ASGV 5641F and ASGV 6396R were used under conditions reported earlier (Nickel et al. 1999). Total RNA was prepared from 100 mg of apple leaves powdered in liquid N_2 and extracted with the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. For cDNA synthesis, 5 μL (approximately 200 ng) of total RNA were mixed with 1 μL of 10 μM oligo-dT or a specific reverse primer and 4 μL of DEPC-treated water, incubated at 80 °C for 2 min and placed on ice for 3 min. The reverse transcription (RT) reaction consisted of 5 μL of 5× RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl), 1 μL of 10 mM dNTP mixture (dATP, dGTP, dCTP, dTTP each at 2.5 mM), 1 μL of 0.1 M DTT, 0.7 μL of RNase inhibitor (28 U, Promega), 1 μL of reverse transcriptase (200 U Superscript III, Invitrogen) adjusted to 25 μL with DEPC-treated water. The RT-mixture was incubated at 37 °C for 1 h. The reaction for PCR amplification contained 2.5 μL of 10× PCR buffer, 0.75 μL of 50 mM $MgCl_2$, 2 μL dNTPs (2.5 mM each), 0.5 μL of each primer (200 μM final concentration), 0.1 μL of Platinum *Taq* DNA polymerase (Invitrogen) and 2.5 μL of cDNA adjusted to 25 μL with water as above. Samples were submitted to the following amplification steps in a thermocycler: 94 °C initial denaturation for 2 min, 34 cycles of 50 s at 94 °C, 50 s at 55 to 58 °C, depending on specific primer temperatures (Table 1), and 60 s at 72 °C, for denaturing, annealing and chain extension, respectively, followed by a final extension step of 10 min at 72 °C. Oligonucleotide primers were designed based on ASGV RefSeq NC_001749, ASGV isolate UV1 (AF438409) and on sequences of own genome fragments (Table 1). PCR products were analyzed by electrophoresis in 1.2% agarose gels, stained with ethidium bromide, excised from gels over UV light and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The purification of amplicons, ligation and cloning were performed according to Sambrook and Russel (2001). Purified amplicons were cloned in pGEM-T-Easy (Promega) and the reaction was used to transform *Escherichia coli* strain DH5α. Transformant colonies were transferred to LB liquid culture medium and incubated at 37 °C for 12 h. Recombinant plasmids were purified using the Wizard Plus SV Minipreps DNA Purification System (Promega). Sequencing was carried out at Embrapa

Table 1 Oligonucleotide primer pairs used to amplify the full genome sequence of Brazilian isolates M219–3 and M220 of *Apple stem grooving virus*

Primer F	Primer R	5′- 3′ Sequences	Annealing temperature	Nucleotide positions	Isolate	
					M219–3	M220
ASGV-1	ASGV-2	AAATTTAACAGGCTTAATTTCC GAACTTTTGGAAATTTGTTACG	55 °C	1–540		both
ASGV-5	ASGV-6	CAATGGGTCAAAGACAAATTTTG GATATGTCTTCATTTACCAAAAA	58 °C	1170–1935		X
SG-13	ASGV-18	GGACCCATGTTGAGAGCAATTGA TGGCAGCAAAGGTTTTCAATTC	55 °C	3981–6042	X	
SG5641-F	ASGV-20	ATGAGTTTGGAAGACGTGCTTC AGAGTGGACAAACTCTAGACTC	58 °C	5641–6495		both
ASGV-7	ASGV-10	TTTTTGGTAAATGAAGACATATC TTATAAAAGGCATTATGAACA	58 °C	1903–2934		X
ASGV-15	5659-R	CTTTACACAATTTTGAAAGAAGA AGCACGTCTTCCAAACTC	58 °C	4653–5659		X
ASGV-13	ASGV-16	GGACCCATGTTGAGAGCAATTGA AGAGCAAACAACTCAGTGTCCTG	58 °C	3978–5316		X
SG495-F	ASGV-6	TGGTCTTAGAGATCAATGCCTC GATATGTCTTCATTTACCAAAAA	55 °C	495–1930		both
SG1312-F	ASGV-6	TCATCCTGGACAAAGTCAACT GATATGTCTTCATTTACCAAAAA	55 °C	1312–1935	X	
ASSG-5	SG2807-R	CAATGGGTCAAAGACAAATTTTG ATCGTCTTGATCATGAGGTCA	55 °C	1171–2807	X	
SG2589-F	SG3978-R	GTACCCACCTGGATACCTTACA GCACTTTCAATTGCTCTCAACAT	55 °C	2589–3978		X
SG2589-F	ASGV-12	GTACCCACCTGGATACCTTACA TCAATTGCTCTCAACATGGGTCC	55 °C	2589–4003		both

(Brasília, DF, Brazil) by the Sanger method. Sequence assembly was performed using the Lasergene software (DNAStar Inc.).

Nucleotide sequences and deduced amino acid sequences of both isolates were aligned and compared with each other and with sequences available in GenBank using Clustal W (Thompson et al. 1994). A phylogenetic tree based on the complete nucleotide sequence alignment was built using the neighbor joining method with 1000 bootstrap replications for branch testing as implemented in Molecular Evolutionary Genetics Analysis (MEGA) software package v. 7.0 (www.megasoftware.net). These data sets were used for recombination and phylogenetic analyses. Confidence values for the recombination events were evaluated by RDP4 software package, which includes nine different modules for recombination detection: RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, 3Seq, Lard and PhylPro. Events detected by RDP with a *p*-value under 1×10^{-6} were considered significant. All complete genome sequences of ASGV isolates available in GenBank were included in the analysis.

Both ASGV isolates were complexed with ACLSV and ASPV, detected by RT-PCR and/or biological indexing. Mixed-infected budwood of both ASGV isolates induced significant mortality of inoculated woody indicator plants. Surviving plants showed differential reactions that indicate biological differences between the two isolates in terms of virulence towards sensitive hosts. On PV the inoculum with isolate M219–3 killed the outgrowth of the second season, while inoculum with isolate M220 allowed a weak bud outgrowth and twig development, displaying yellow leaf flecking and vein chlorosis typical of ASGV infection (Siebert and Engelbrecht 1981). Since apple plants in commercial orchards are usually infected by several latent viruses, this reaction of the indicator may have been influenced by a mixed virus infection. All plants of indicator MM, which usually reacts with strong leaf symptoms to ASGV infection, did not survive infection by both inocula. Inoculum containing ASGV isolate M219–3 induced: on ASPV indicator RC, severe epinasty, necrosis of posterior midveins, red flecking of leaves and stunted growth; on ACLSV indicator LL-S5, small crinkled leaves; and on ACLSV indicator MP, stunted growth, strong line patterns and chlorotic rings and small leaves. Inoculum containing ASGV isolate M220 induced: on RC, red flecking but allowed growth of large leaves; on LL-S5, allowed large leaves and strong twig growth; on MP, marked twig elongation, weaker line pattern and larger leaves. Mechanical inoculation of *N. occidentalis* 37B with M220 inoculum induced, beginning at 7 days post-inoculation (dpi), leaf chlorosis and

Table 2 Percentage of nucleotide and deduced amino acid sequence identities of ASGV isolates M219–3 and M220 to other ASGV isolates available in GenBank

ASGV isolates	% genome identity (nt)		% deduced amino acid identity					
			ORF 1		ORF 2		CP	
	M219–3	M220	M219–3	M220	M219–3	M220	M219–3	M220
KX668488 M219–3_apple_ Brazil	*	92.2	*	92.3	*	95.3	*	93.3
KX686111 M220_apple_ Brazil	92.2	*	92.3	*	95.3	*	93.3	*
NC0011749_apple_ Japan	92.2	97.1	92.3	98.2	95.0	98.4	94.5	98.3
AB004063_lily_Japan	82.5	83.0	87.7	87.8	96.3	95.0	96.2	97.1
AF438409 UV01_apple_ Brazil	***	***	***	***	***	***	95.8	96.6
AY596172_pear_Korea	80.2	79.4	85.8	85.6	94.7	93.1	97.9	93.7
AY646511_citrus_Taiwan	81.5	81.8	87.1	87.5	96.3	95.6	93.7	94.5
D16681_lily_ Japan	82.4	83.0	88.1	88.2	96.3	95.0	96.2	97.1
EU553489_citrus_USA	82.0	81.5	87.8	86.6	96.6	96.0	95.8	93.3
FJ355920_citrus_Taiwan	81.4	81.8	87.6	87.4	95.3	96.6	94.5	95.4
HE978837_apple_ India	81.1	81.5	84.9	85.1	94.7	94.7	95.4	96.2
JN701424_pear_China	86.2	86.6	89.4	90.2	93.8	94.4	95.8	95.8
JQ308181_apple_China	86.2	85.9	89.2	90.2	93.8	94.1	94.5	95.4
JQ765412_citrus_China	81.4	81.5	87.0	87.3	96.6	95.0	92.9	94.1
JX080201_apple_Germany	82.0	82.4	87.7	87.9	96.3	97.2	95.8	96.6
JX416228_citrus_Taiwan	81.6	81.9	87.2	87.2	96.6	96.0	91.6	92.9
KC588947_citrus_China	81.2	81.3	86.7	86.9	95.3	94.7	92.4	93.7
KC588948_citrus_China	86.1	87.0	90.6	93.6	97.2	95.6	94.1	95.0
KF434636_apple_China	92.8	96.6	92.6	98.1	94.4	96.6	94.5	98.7
KJ579253_apple_China	85.5	86.7	88.2	88.9	97.2	97.2	97.1	96.2
KR106996_pear_China	79.5	79.2	85.7	84.7	96.6	95.0	96.6	93.2
KR185346_pear_China	82.5	83.0	88.7	88.2	97.2	96.3	96.6	96.2
KU198289_pear_China	82.5	82.8	88.6	88.0	97.2	96.3	96.6	95.8
KU605672_pear_China	86.1	87.0	89.6	90.2	96.6	97.5	94.1	97.0
LC084659_citrus_Japan	81.5	81.7	87.3	87.2	96.9	95.6	93.3	94.1
LC143387_citrus_Japan	82.4	83.4	88.7	89.8	97.2	96.3	95.4	97.0
LT160740_apple_India	82.4	82.0	87.4	87.4	95.6	96.6	94.9	94.9

necrotic spots that developed rapidly into severe vein necrosis leading to collapsing leaves. Symptoms induced by M219–3 inoculum appeared on this indicator at 15 dpi. They consisted of light chlorotic mottling, tiny necrotic spots along the veins, developing slowly into brown spots, coalescing to black vein necrosis, not leading leaves to collapse.

The complete genome sequences of the two ASGV Brazilian isolates were obtained and deposited in GenBank under the accession numbers KX668488 (M219–3) and KX686111 (M220). Sequences of the isolates M219–3 and M220 comprise 6495 nucleotides (nt), including both non-translated regions (5′-NTR with 35 nt, 3′-NTR with 140 nt) and excluding the poly-A tail. Both sequences were similar to those previously described (Yoshikawa et al. 1992; Ohira et al. 1995; Terauchi et al. 1997; Tatineni et al. 2009; Liebenberg et al. 2012; Zhao et al. 2012; Chen et al. 2014a, 2014b; Dhir et al. 2015; Jo et al. 2016) and included a large open reading frame (ORF1) starting at nt position 36 and ending at nt 6354, coding for a polyprotein of 241 kDa, and a smaller ORF (ORF2) overlapping ORF1, starting at nt position 4787 and ending at nt 5749, coding for a movement protein (MP) of 36 kDa. When compared to other ASGV isolates, M219–3 showed between 92.8 and 79.5% nucleotide identity and M220, 97.1–79.2% (Table 2). The two isolates, M219–3 and M220, shared 92.2% nucleotide sequence identity, 92.3% sequence identity at the deduced amino acid (daa) level for ORF1, 95.3% for ORF2 and 93.3% for the CP gene. A high degree of daa identity, 95.8% and 96.6%, was observed between the CPs of M219–3 and M220, respectively, with the Brazilian isolate UV01 (AF438409).

Phylogenetic analysis based on the complete genome nucleotide sequences supported the results from sequence

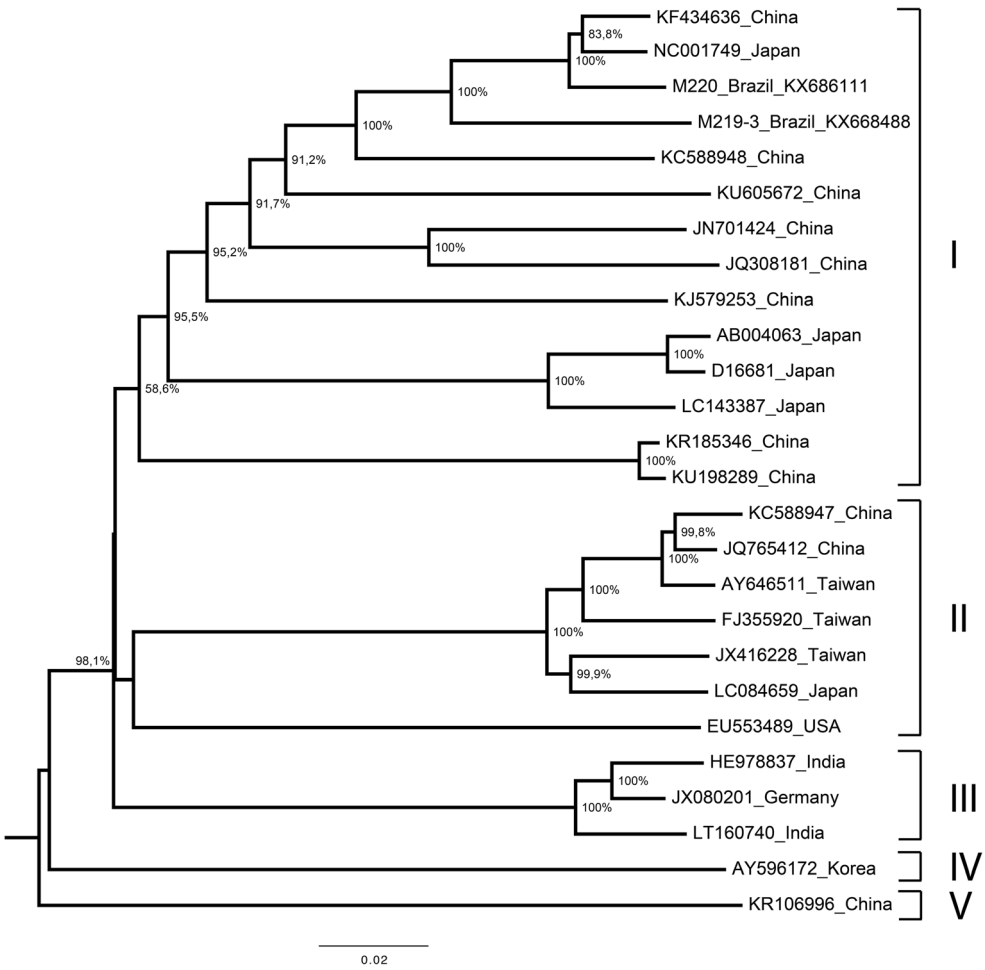

Fig. 1 Neighbor joining phylogenetic tree based on the complete nucleotide sequences of ASGV isolates M219–3 and M220. Numbers at the branches indicate bootstrap percentages (1000 replications). The scale bar indicates nucleotide substitutions per site

comparisons and showed that ASGV isolates are divided into five groups. The Brazilian isolates formed a monophyletic cluster (Fig. 1) with Asian isolates from different hosts (apple, citrus, lily and pear). The North American citrus isolate clustered in group II with six Asian citrus isolates, while two pear isolates formed the single-isolate groups IV and V. Group III includes apple isolates from India and Germany. Thus, no clear grouping according to geographic origin of the isolates was observed. Phylogenetic and recombination analyses revealed that both isolates are potential recombinants. It was found that a larger portion of isolate M219–3 is contributed by the major parent LC143387, a citrus isolate from Japan, and to a smaller extent by M220, whereas isolate M220 is potentially a recombinant of two other ASGV isolates, ASGV type member NC_001749 from Japan and JN701424, a pear isolate from China (Table 3), respectively,

Table 3 Recombination events detected in Brazilian ASGV isolates M219–3 and M220

Potential Recombinant (host/geographic origin)	Major Parent (host/geographic origin)	Minor Parent (host/geographic origin)	Beginning breakpoint	Ending breakpoint	*p*-value (RDP)	Detection methods[#]
KF434636 (apple/China)	LC143387 (citrus/Japan)	M220 (apple/Brazil)	489	4010	$3{,}74 \times 10^{-27}$	A,B,C,D,E,F,G,H
KJ579253 (apple/China)	M220 (apple/Brazil)	*JN701424 (pear/China)	466	2005	$1{,}84 \times 10^{-24}$	A,B,C,D,E,F,G,H,I
LT160740 (apple/India)	JX080201 (apple/Germany)	M219–3 (apple/Brazil)	4011	4344	$1{,}17 \times 10^{-17}$	A,B,C,D,E,F,G
M219–3 (apple/Brazil)	*LC143387 (citrus/Japan)	M220 (apple/Brazil)	489	4010	$3{,}74 \times 10^{-27}$	A,B,C,D,E,F,G
M220 (apple/Brazil)	NC001749 (apple/Japan)	*JN701424 (pear/China)	6467	536	$9{,}67 \times 10^{-23}$	A,C,D,E,F,G

*Only one parent and a recombinant needed to be in the alignment for a recombination event to be detectable. The sequence listed was used to infer the existence of a missing parental sequence

[#] A, RDP; B, GENECONV; C, BootScan; D, MaxChi; E, Chimaera; F, SiScan; G, 3Seq; H, Lard; I, PhylPro

major and minor parents (Table 3). The existence of these phylogenetic relationships establishes a consistent evolutionary link of the characterized Brazilian ASGV isolates to their parental ascendants.

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