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## ABSTRACT

Sugarcane yellow leaf virus (SCYLV) is an aphid-transmitted virus for which *Melanaphis sacchari* is the main vector. Almost all sugarcane varieties grown in Florida are susceptible to SCYLV infection. In this study, we investigated the prevalence of SCYLV in accessions of *Sorghum bicolor* which is another natural host of this virus. Two field experiments, one in 2016 with 19 sorghum lines and the other in 2017 with 15 lines, were established at Belle Glade, FL. Stalks collected randomly in planted and ratoon crops were tested by tissue-blot immunoassay (TBIA) and reverse transcription-polymerase chain reaction (RT-PCR). Over the two-year period, 366 of 423 *S. bicolor* samples tested positive by TBIA but SCYLV was detected by RT-PCR in only 12 of 161 randomly selected subsamples. Full genome sequences of SCYLV, sugarcane mosaic virus (SCMV) and a new marafivirus were obtained by high-throughput sequencing (HTS) from three TBIA positive sorghum samples. HTS data for all three viruses were confirmed by RT-PCR. The

SCMV isolate from *S. bicolor* appeared to be a new strain of this virus species. Positive reaction of *S. bicolor* by TBIA using SCYLV antibodies could not be systematically associated with plant infection by SCYLV or another virus. This suggested the occurrence of a non-specific serological reaction with an unknown *S. bicolor* antigen. SCMV and the new marafivirus were also detected in *Sorghum almum*, suggesting that this weed is a reservoir for *S. bicolor*-infecting viruses in Florida.

## 1. Introduction

A metagenomic study recently reported the occurrence of six sugarcane-infecting viruses in Florida (Filloux et al., 2018): sugarcane bacilliform virus (SCBV), sugarcane mosaic virus (SCMV), sugarcane mild mosaic virus (SCMMV), sugarcane striate virus (SStrV), sugarcane yellow leaf virus (SCYLV), and an uncharacterized virus of the genus *Umbravirus*. Among these, SCYLV was the most widespread in both germplasm collections and commercial sugarcane (Filloux et al., 2018). During the last decade, this virus was also reported in three other hosts: barley in Tunisia in 2014 and more recently in both Columbus grass (*Sorghum almum*) and grain sorghum (*Sorghum bicolor*) in Florida (Bouallegue et al., 2014; ElSayed et al., 2018; Espinoza Delgado et al., 2015; Wei et al., 2016).

In sugarcane, SCYLV causes yellow leaf, a disease that was first described in Hawaii in 1989 (Schenck et al., 1997; Vega et al., 1997). Yellow leaf occurs in almost all areas worldwide where sugarcane is grown, including Florida which is the major cane producing area in the U.S.A. (Abu Ahmad et al., 2006; Rott et al., 2008). SCYLV infection can result in the intense yellowing of the midrib of the lower side of the leaf, and the subsequent necrosis from the tip

of the leaf toward its base. However, sugarcane infected by SCYLV in Florida is generally asymptomatic and no disease symptoms have been associated with this virus in *S. almum* and *S. bicolor*. Though the effect of SCYLV on the two sorghum species is unknown, the causal agent of yellow leaf has been associated with severe yield losses in cultivated sugarcane in India, Hawaii, Reunion Island, Louisiana and Brazil (Bagyalakshmi et al., 2019; Lehrer et al., 2009; Vega et al., 1997; Rott et al., 2008; Viswanathan et al., 2014). In Florida, cultivar-dependent yield losses between 11 and 27% were observed in infected sugarcane (Boukari et al., 2019; Comstock and Miller 2004). This is quite alarming since the highest potential yield losses reported so far were observed in cultivar CP96-1252 which presently covers about a third of Florida's commercial sugarcane production area (Boukari et al., 2019; VanWeelden et al., 2019).

Sugarcane is propagated by stalk cuttings and viruses can therefore be rapidly spread by infected planting material. The use of resistant cultivars is the method of choice to control yellow leaf but sources of resistance to virus infection were shown to be quite limited in both the ancestral sugarcane species (*Saccharum officinarum*, *Saccharum spontaneum*, etc.) and commercial sugarcane cultivars which are *Saccharum* interspecific hybrids (Comstock et al., 1998 and 2001; Filloux et al., 2018). Efforts to identify SCYLV resistance markers in sugarcane using quantitative trait loci (QTL) mapping have been limited and are very challenging due to the complex nature (polyploidy and aneuploidy) of this plant (Costet et al., 2012; Débibakas et al., 2014; Islam et al., 2018). Additionally, the large size of the sugarcane genome requires the identification of a large number of markers in order to cover each segregating locus and its high number of alleles (Garcia et al., 2006 and 2013; Wang et al., 2010). Consequently, identification of sources of resistance to SCYLV in sugarcane-related grasses with a less complex genome

87 (diploid rather than polyploid) may contribute to improve resistance of sugarcane to yellow leaf
88 in breeding programs.

89 The *Sorghum* genus was found to harbor not only SCYLV but also the sugarcane aphid
90 *Melanaphis sacchari*, which is considered the most efficient and widespread insect vector of
91 the virus (Rott et al., 2008; Wei et al., 2016). *Sorghum bicolor*, commonly known as sorghum or
92 great millet, durra, jowar, or milo around the world, is widely cultivated in tropical and
93 subtropical regions for its grain, which is used for human consumption, animal feed, fiber and
94 more recently ethanol production (Dillon et al., 2007). Sorghum, a cereal of African origin, is
95 also a grass species related to sugarcane and maize. The sorghum diploid genome has been
96 widely used to decipher the complex genome of sugarcane in the past, including identification
97 of markers for disease resistance (Le Cunff et al., 2008; McIntyre et al., 2005; Wang et al.,
98 2010). Thus, identification of resistance sources in sorghum could be useful in marker-assisted
99 breeding of SCYLV resistant sugarcane.

100 The first objective of this study was to determine the prevalence of SCYLV after natural
101 infection in a collection of *S. bicolor* lines whose genome sequences were already available
102 (Mace et al., 2013). To reach this goal, sorghum was planted in the field near sugarcane
103 infected by SCYLV and screened for virus-infection after colonization by *M. sacchari* using a
104 standard serological technique (tissue-blot immunoassay, TBIA). The second objective of our
105 study was to confirm serological data with additional molecular techniques such as the reverse
106 transcription-polymerase chain reaction (RT-PCR) and high-throughput sequencing (HTS). The
107 third objective was to obtain genomic sequences of sorghum-infecting viruses by HTS and to
108 determine their phylogenetic relationship with known virus species and strains.

## 2. Materials and Methods

### *2.1. Experimental fields*

Field trials were established at the Everglades Research and Education Center (EREC) in Belle Glade, FL over a two-year period. Seeds of sorghum lines (PIs, Table 1) were obtained from the USDA-Germplasm Resource Improvement Network (GRIN) (USDA 2020). The first trial was established on March 22, 2016 by planting seeds of 30 different sorghum lines (of which only 19 grew; Table 1) in 3.8 m lines separated by 0.8 m intervals on each side. The trial (26.7 x 15.2 m) was bordered on each side by the sorghum line Terral. This trial was in close proximity (within 5 meters) of a commercial sugarcane field of cultivar CP96-1252 where prevalence of SCYLV was greater than 90%. Four-month-old plants were harvested just above soil level on July 29, 2016. Ratoon plants were grown for four months until the end of flowering. The second trial was established on March 21, 2017 with seeds of 18 different sorghum lines (of which only 15 grew, Table 2) using the same design as for the first trial, but the border of the second trial was planted with sorghum line Dale. Three-month-old plants were harvested just above soil level on June 26, 2017. This second trial was also adjacent to the sugarcane commercial field of cultivar CP96-1252 with high prevalence of SCYLV (> 90%). A set of 10 plants of line Dale planted and grown in pots in an insect-free area (screened enclosure) served as negative control in 2017.

### *2.2. Determination of number of plants colonized by* M. sacchari *and aphid population sizes*

For the first trial, the number of plants colonized by *M. sacchari* and the number of aphids per colonized plant were determined by visual inspection on June 8, 2016 (planted

131 sorghum) and on October 24, 2016 (ratoon sorghum) when plants were three and two months
132 old, respectively. For the second trial, the same data were collected in planted sorghum on June
133 13, 2017 when plants were three months old. Data could not be collected in ratoon sorghum
134 for this trial because of delayed colonization of plants by *M. sacchari* and because plants were
135 severely damaged by hurricane Irma from September 10$^{th}$ to 13$^{th}$, 2017 (2.5 months after
136 harvest of planted sorghum). In each trial, 10 plants per sorghum line were randomly selected
137 and all leaves of each plant were evaluated for aphids. The average number of aphids per plant
138 was determined by dividing the total number of aphids per line by the number of colonized
139 plants.

140

141 *2.3. Detection of SCYLV in sorghum lines by tissue-blot immunoassay (TBIA)*

142 Ten stalks were randomly collected per line from each trial approximatively four months
143 after planting. Ten stalks per line were also sampled for the first trial four months after
144 harvesting (ratoon crop). For the second trial, only three samples (one stalk for Dale, PI 157033,
145 and PI 651496) were obtained and tested four and a half months after harvesting because the
146 remaining plants were damaged by hurricane Irma (as mentioned above). An imprint of the
147 cross section of the upper, middle, and lower part of each stalk was made on nitrocellulose
148 membranes. Each membrane also included two imprints of midribs from sugarcane leaves: one
149 SCYLV-infected leaf (positive TBIA control) and one SCYLV-free leaf (negative TBIA control).
150 Membranes were processed using SCYLV antibodies (1:7,000 dilution) as described by Schenck
151 et al. (1997) and modified by Girard et al. (2010) (Fig. 1). SCYLV antibodies were obtained from
152 Ben Lockhart at University of Minnesota (Scagliusi and Lockhart, 2000). Prevalence of SCYLV for

each sorghum line was expressed as the ratio of TBIA positive stalks per total number of stalks tested.

### *2.4. Detection of SCYLV in sorghum lines by one-step reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA was extracted from stalk cross sections of eight ratoon sorghum samples in 2016, 150 plant sorghum and three ratoon sorghum samples in 2017 with the Qiagen RNeasy Plant mini kit (Qiagen, USA), following the manufacturer's protocol. Each RNA sample was suspended in 30 µL of RNase-free water and stored at -20ºC until further use. The OneStep RT-PCR kit (Qiagen) and primers ScYLVf1 and ScYLVr1 were used as described by Girard et al. (2010) to test for SCYLV. Another round of one-step RT-PCR was performed with four randomly selected samples (three SCYLV negative and one weak positive) and their 1:10 and 1:100 dilutions to help support the accuracy of the RT-PCR assay. Amplification products (219 bp) were evaluated by electrophoresis on 1% agarose gels, stained with SYBR Green and observed under UV light using a Syngene G:Box F3 (Syngene, Pegasus Court, MD).

### *2.5. Detection of viruses in* S. bicolor *lines by high-throughput sequencing (HTS)*

Stalk pieces of three ratooned sorghum lines that tested SCYLV positive by TBIA (PI157033, PI651496, and Dale) were collected in 2017 at the end of the second trial. None of these plants showed disease symptoms that could be attributed to SCYLV or SCMV. Total RNA was extracted from these samples using the RNeasy Qiagen mini plant extraction kit. The RNA was sent to LC Sciences (Houston, TX) for cDNA library preparation and HTS as 150 nt pair-end

reads. CLC Genomic Workbench 11.0 (Qiagen) was used to assemble sequence reads into contigs. Contigs were subjected to BLASTx comparisons using a local NCBI database under the CLC platform. Geneious R11 was then used to finalize additional assemblies (Biomatters, New Zealand).

### *2.6. Detection of viruses in* S. almum *by HTS*

Two plants of *S. almum* were used. One plant without virus symptoms was collected at EREC in April 2016. The other plant with mosaic symptoms was sampled at Canal Point, FL in February 2015 and was known to be infected by SCMV (Mollov et al., 2016). Both plants also tested positive by TBIA with SCYLV antibodies as described above (Supplementary Fig. S1). Total RNA was extracted from these samples using the RNeasy Qiagen mini plant extraction kit. The RNA was sent to Seqmatic, California for cDNA library construction and RNASeq as 75 nt single end reads. CLC Genomic Workbench 11.0 (Qiagen) was used to assemble sequence reads into contigs. BLASTx analyses of contigs and additional assemblies were performed as described above for the HTS sequences obtained from *S. bicolor*.

### *2.7. Detection of SCYLV, SCMV, and sorghum marafivirus by two-step RT-PCR*

RNA extracted from sorghum samples as described above was used for the initial reverse transcription step with Promega's GoScript Reverse Transcription System following the manufacturer's protocol. The PCR reaction for each virus was performed in a final volume of 25 µL containing 2.5 µL of eluted cDNA, 12.5 µL of GoTaq Colorless Master Mix 2x (Promega), 1 µL of 10 µM of forward primer, 1 µL of 10 µM of reverse primer, and 8 µL of nuclease-free water.

Primer set (ScYLVf1 and ScYLVr1) and amplification conditions for SCYLV were as described by Girard et al. (2010). Primer set (Oligo 1n and Oligo 2n) and amplification conditions for SCMV were as described by Marie-Jeanne et al. (2000). The sorghum marafivirus sequences recovered by HTS were used to design diagnostic primers SBMV-F1 (GTTCCAATGGGTCGTCGGAT) and SBMV-R1 (TTTAGTCGCTCGTGAGGAGG) using Primer-blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The thermal cycling conditions were 95°C for 3 min, 30 cycles of 94°C (30 sec), 56°C (30 sec), 72°C (30 sec), and a final extension of 72°C for 7 min. Amplification products (219 bp for SCYLV, 327 bp for SCMV, and 476 bp for the marafivirus) were submitted to gel electrophoresis, stained and observed as described above.

### *2.8. Confirmation of sequence identity of SCMV amplicons obtained by two-step RT-PCR*

Two-step RT-PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen) and sent for sequencing at Eton Bioscience Inc. (San Diego,CA). The sequences were subjected to BLAST analysis at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

### *2.9. Phylogenetic analyses*

The entire genome coding region (ORFs 0-5) for SCYLV, the entire genome coding region of four isolates of SCMV, and the coat protein amino acid sequence of the two sorghum marafiviruses, all obtained by HTS, were each aligned to the corresponding sequences of isolates of the same virus species/genus retrieved from GenBank using Clustal W. Isolates of SCYLV and SCMV selected in the GenBank database represented the current known genetic diversity of these two viruses (Filloux et al., 2018; Li et al., 2019). Maximum likelihood

phylogenetic trees were inferred for each virus species/genus using MEGA X software with substitution model Tamura-Nei selected as best-fit model and 1,000 bootstraps (Kumar et al., 2018; Tamura and Nei 1993).

### *2.10. GenBank accession numbers*

The assembled genome sequences obtained by HTS are publicly available in GenBank with the following accession numbers: MN097766 for SCYLV from *S. bicolor*, MN097767, MN097768, and MN097769 for SCMV from *S. bicolor*, MN714644 for SCMV from *S. almum*, MN100128 for the marafivirus from *S. bicolor*, and MN714643 for the marafivirus from *S. almum.*

## 3. Results

### *3.1. Colonization of* S. bicolor *plants by the aphid* M. sacchari

The 19 sorghum lines grown in 2016 were all colonized by *M. sacchari* (Table 1). The average number of aphids per colonized plant ranged from four (sorghum line M81E) to 8,000 (line PI 571128). Only eight of the 19 lines also grew in the ratoon crop after harvest of the planted crop. All of these lines were again colonized by the sugarcane aphid, with an average of 30 (line PI 525695) to 5,300 (line PI 563295) aphids per plant among the eight ratooned sorghum lines. The 15 sorghum lines grown in 2017 were all colonized by *M. sacchari* (Table 2). The average number of aphids per colonized plant in planted sorghum ranged from eight (line PI 659692) to 9,200 (line PI 585749). Aphid data could not be obtained in ratoon sorghum because plants were damaged by hurricane Irma.

241

242 *3.2. Detection of SCYLV by TBIA and RT-PCR*

243 Clear stalk imprints of sorghum with no colored sections were considered negative for
244 SCYLV whereas imprints with bluish-purple dots (vascular bundles) or blue tissue coloring were
245 considered positive (Fig. 1). In 2016, between two and 10 of the 10 tested stalks per line tested
246 positive for SCYLV by TBIA in planted sorghum (Table 1). In ratoon sorghum of the same year,
247 between five and 10 of the 10 tested stalks per line were found TBIA positive. In 2016, a single
248 stalk of each of the eight lines in ratoon sorghum was tested by RT-PCR and four were found
249 positive for SCYLV (Dale, PI 157033, PI 300119, and PI 651496).

250 In 2017, between nine and 10 of the 10 tested stalks per line and 149 of the 150 stalks
251 tested in planted sorghum reacted positively by TBIA (Table 2). Only five of these 150 samples
252 tested positive for SCYLV by RT-PCR. Six of 10 stalks from the negative control sorghum (grown
253 in an insect free area) also tested positive for the virus by TBIA, but were RT-PCR negative
254 (Table 2). In a sensitivity assay, 1:10 and 1:100 dilutions of three negative samples and one
255 weak positive sample all tested negative for SCYLV by RT-PCR (Fig. 2). Due to hurricane Irma
256 damage, TBIA and RT-PCR data were obtained for only three ratoon sorghum samples: Dale, PI
257 157033 and PI 651496. SCYLV was detected in these three samples by both TBIA and RT-PCR
258 (Table 3).

259 In all TBIA assays, the sugarcane samples used as controls tested positive for SCYLV
260 when RT-PCR was positive and negative when RT-PCR was negative.

261

262 *3.3. Detection of viruses in S. bicolor by HTS*

263 The HTS resulted in 68,874,862 reads for sorghum line PI157033, 64,600,212 reads for
264 line PI651496, and 52,639,920 reads for line Dale. These reads assembled into 108,503; 204,169
265 and 164,552 contigs respectively for PI157033, PI651496, and Dale.
266 For PI157033, 10 contigs were initially identified to be associated with plant viruses:
267 seven to SCMV and three to SCYLV. Subsequent analysis resulted in single contigs for both
268 viruses. Using BLASTx comparisons, the 9,596 nt contig for SCMV had highest identity (97.4%)
269 with the polyprotein amino acid sequence of SCMV accession JX237862 from sugarcane in
270 Argentina (over a 92% query cover). The 5,891 nt contig for SCYLV was 99% identical to SCYLV
271 accession AF157029 from sugarcane in Florida.
272 For PI651496, 27 contigs were associated with plant viruses but none with SCYLV. All
273 contigs of PI651496 assembled into one 9,545 nt long contig with 97.6% similarity to SCMV
274 accession JX237862 (over a 93% query cover).
275 For Dale, 46 contigs were identified as plant virus-related. Of these, 32 were assembled
276 into a 9,536 nt contig with 97.6% similarity to SCMV accession JX237862 (over a 93% query
277 cover). Six contigs were related to SCYLV, but were only assembled by 545 (0.001%) reads. The
278 running length of these six contigs was about 3,746 nt, which is just above half of the SCYLV
279 genome length. The remaining eight contigs were assembled into a 6,240 nt consensus
280 sequence most similar to the amino acid sequence of maize rayado fino virus accession
281 AF265566 with 61.7% identity by BLASTx analysis.
282 The number of reads mapped back to the SCMV isolates were 262,457; 11,049,678 and
283 10,597,076 reads for sorghum lines PI157033, PI651496, and Dale, respectively. For PI157033,

306,624 reads mapped to the SCYLV consensus sequence. For Dale, only 1,440 reads mapped to the marafivirus.

### *3.4. Validation by RT-PCR of presence of viruses identified in* S. bicolor *by HTS*

SCMV infection in sorghum lines PI157033, PI651496, and Dale was confirmed by RT-PCR (Table 3). The marafivirus discovered by HTS was also detected using the RT-PCR assay developed in this study based on the coat protein sequence of this virus. All three sorghum lines PI157033, PI651496, and Dale were positive for SCYLV by RT-PCR (Table 3). An additional set of 15 sorghum samples that reacted positively by TBIA and negatively by SCYLV RT-PCR in 2017 were tested by RT-PCR for SCMV and the marafivirus. All samples tested negative for the marafivirus and SCMV was found in five of the 15 samples. RT-PCR amplicons (327 bp) of these positive samples had 94-99% sequence identity with SCMV isolates in GenBank.

### *3.5. Detection of viruses in* S. almum *by HTS*

The HTS resulted in 27,903,407 reads for the *S. almum* plant showing mosaic symptoms and 13,054,589 reads for the symptomless plant. The mosaic showing *S. almum* sample resulted in 109,646 contigs, one of which was the near complete genome of SCMV. The SCMV contig had 4.25 million reads which was more than 15% of total raw data reads. The 9,165 nt long SCMV contig was most similar to SCMV (KR108212 from China and JX237862 from Argentina) with 100% nt query coverage and 92.6% sequence identity.

The *S. almum* sample without mosaic symptoms initially produced 59,732 contigs and 16 of these contigs contained new virus sequences. After further analysis, these new virus

306 sequences were assembled into a single contig resembling the marafivirus genome obtained
307 from *S. bicolor*. The *S. almum* marafivirus contig was assembled from 46,417 reads which was
308 less than 0.4% of the total number of raw reads. The marafivirus consensus sequence from *S.*
309 *almum* was 6,138 nt long and BLASTn resulted in 76% identity to switchgrass mosaic virus
310 (JF727261) and maize rayado fino virus (HM133581) but query coverage was only 15% and 11%,
311 respectively. The switchgrass mosaic virus is considered as an isolate of maize rayado fino virus
312 and the International Committee on Taxonomy of Viruses (ICTV) only recognizes the latter as
313 the virus species.
314 None of the contigs of the two *S. almum* plants matched with SCYLV sequences although
315 these plants tested positive for SCYLV by TBIA (Supplementary Fig. S1).
316
317 *3.6. Validation by RT-PCR of presence of viruses identified in* S. almum *by HTS*
318 SCMV infection in the plant showing mosaic symptoms was confirmed by RT-PCR. The
319 marafivirus discovered by HTS was also detected in the asymptomatic plant of *S. almum* using
320 the RT-PCR assay developed in this study based on the coat protein sequence of the marafivirus
321 found in *S. bicolor*. An additional 36 plants of *S. almum* were collected in the Everglades
322 Agricultural Area (EAA) in Florida and tested for SCMV and the new marafivirus. Nine of these
323 36 plants showed mosaic symptoms and were positive for SCMV by RT-PCR. The sorghum
324 marafivirus was detected by RT-PCR in 15 of the 36 plants, including six that were infected by
325 both SCMV and the marafivirus.
326

### *3.7. Phylogenetic characterization of SCMV, SCYLV, and the marafiviruses identified in sorghum in Florida*

A phylogenetic tree was constructed with the entire genome coding sequences (ORF0-ORF5) of 14 SCYLV isolates (Fig. 3): isolate of sorghum PI157033 obtained in this study and 13 isolates retrieved from GenBank. These latter isolates represented the current known genetic diversity of SCYLV: genotypes BRA and REU (SCYLV clade I) and genotypes CUB, CHN1, FLA1, FLA2, and FLA3 (SCYLV clade II). Isolate of sorghum PI157033 from Florida clustered with isolate SCYLV-A [AF157029] from sugarcane in Florida, which was characterized as genotype BRA.

The three SCMV genomic sequences from *S. bicolor* obtained in this study were 97% identical to one another at the nt level. These were also 97% identical to the SCMV genomic sequence obtained from *S. almum*. Based on a phylogenetic tree constructed with the complete genome coding sequence of 23 SCMV isolates (four obtained in this study and 19 retrieved from GenBank), the four isolates from sorghum in Florida formed a single phylogenetic group (Fig. 4).

The marafivirus sequences from *S. bicolor* and *S. almum* shared 82% nt identity over 96% of their genome. Based on the phylogenetic tree constructed with the amino acid coat protein sequence of ten marafiviruses (two isolates obtained in this study and eight retrieved from GenBank), the two marafivirus isolates from sorghum in Florida formed a unique lineage (Fig. 5).

## 4. Discussion

The screening of sorghum lines for SCYLV resulted in a high number of positive samples by TBIA but the virus was detected by RT-PCR only in few plants. This result was obtained despite the high counts of the SCYLV aphid vector in both 2016 and 2017 field experiments. The RT-PCR primers used for detection of SCYLV in this study may not attach to the target sequences of some virus isolates infecting sorghum, although these primers are efficiently used at Cirad's sugarcane quarantine to detect all known genotypes of SCYLV (Fernandez et al., 2019). However, SCYLV was not detected either by HTS in four *S. almum* plants that were TBIA positive (two plants in this study and data not shown for two additional plants).

TBIA is a serological technique widely used to detect accurately SCYLV in sugarcane (Komor 2011; Scagliusi and Lockhart 2000; Vega et al., 1997). Results obtained herein for sugarcane control samples were also consistent: TBIA positive samples were positive by RT-PCR and TBIA negative samples were negative by RT-PCR. Although some imprints of *S. bicolor* stalks had very defined bluish-purple dots associated with phloem cell (Figs. 1b and c), as it is the case with virus-infected sugarcane tissue, the majority of the imprints had partial or complete blue coloring (Figs. 1e and f). The latter were considered positive reactions because they were clearly different from the negative imprints (Figs. 1a and d). Quality of sorghum imprints also varied depending on sorghum line, stalk size and section used, and this may have contributed to our observations. Overall, TBIA was unreliable for detecting SCYLV in sorghum as it produced numerous false positive results in our study.

These false positive results may have been caused by a non-specific serological reaction between the SCYLV antibodies and a sorghum constituent or another virus (Chatenet et al., 2001; Lin et al., 2000; Whitfield et al., 2003). Antibodies used in this study were produced from

371 a mixture of purified SCYLV particles recovered from plants infected with a variety of SCYLV
372 genotypes (Scagliusi and Lockhart 2000). These plants could have been also infected by an
373 unknown virus. However, false positive results were also obtained when *S. bicolor* and *S.*
374 *almum* were tested with another source of SCYLV antibodies (prepared against a synthetic
375 peptide based on the SCYLV coat protein sequence) as compared to RT-PCR data (Rott et al.,
376 unpublished data). Two additional viruses, SCMV and a new marafivirus, were also detected in
377 plants from both species of sorghum by HTS, and plant infection confirmed by RT-PCR and
378 Sanger sequencing. However, a positive TBIA reaction was not correlated with detection of
379 these viruses by RT-PCR in *S. bicolor*, indicating that SCMV and the new marafivirus were not
380 involved in the false positive TBIA data.
381 Alternatively, PCR inhibitors in sorghum may have interfered with the RT-PCR assay,
382 yielding false negatives. Representative samples were also tested at 1:10 and 1:100 extract
383 dilutions but were still negative, suggesting that inhibitors in the sorghum RNA extracts were
384 not involved in the negative RT-PCR reactions. Furthermore, sorghum line Dale, which was
385 grown in a virus and vector-free environment, also tested positive by TBIA in 2017. All together,
386 these results are evidence that a reaction between sorghum plants and antibodies produced
387 against an epitope of the SCYLV coat protein produces false positives in serological assays. To
388 reach an unequivocal conclusion and to confirm these data, a large number of *S. bicolor*
389 samples needs to be tested for SCYLV using TBIA, RT-PCR, and a third detection method such as
390 HTS or loop-mediated isothermal amplification (Amata et al., 2016).
391 Although SCYLV was detected by RT-PCR in some sorghum plants, overall prevalence of
392 the virus in planted crop was not high enough to identify lines that were resistant or susceptible

393 to virus infection, despite colonization of all lines by *M. sacchari* in 2016 and 2017. This aphid
394 has been reported to be very efficient in spreading SCYLV from sugarcane to sugarcane and
395 prevalence of the virus is high (> 70%) in Florida sugarcane fields (Filloux et al., 2018). Low
396 prevalence in *S. bicolor* might be related to the shorter crop cycle of sorghum compared to
397 sugarcane (3-4 months for sorghum versus 12-18 months for sugarcane). Sugarcane is also
398 grown for several years (2-3 ratoon crops in Florida) thus allowing build-up of plant infections
399 by the virus. Prevalence of the virus appeared to be higher in sorghum ratoon crop (as
400 confirmed by RT-PCR in 4 of 8 samples in 2016 and 3 of 3 samples in 2017) and merits further
401 investigation. However, only a few sorghum lines showed good ratooning capacity, which limits
402 the possibility of a large-scale screening for disease resistance in this plant species.
403 SCYLV is not known to be seed-transmitted, and it was assumed that the sorghum seeds
404 obtained from GRIN were virus-free. Consequently, the source of infection of SCYLV in *S. bicolor*
405 could be either infected sugarcane plants or infected *S. almum* plants that grew near the
406 experimental fields. *S. almum* has been reported as a secondary host of SCYLV in Florida and
407 this plant species is also colonized by *M. sacchari* (Espinoza Delgado et al., 2015). The SCYLV
408 isolate from *S. bicolor* characterized in this study was 99% identical to a sugarcane isolate from
409 Florida that was sequenced 20 years ago (Moonan et al., 2000), suggesting that the same virus
410 strain is infecting sorghum and sugarcane and that spread from one plant species to another is
411 naturally possible. Recombinant isolates of SCYLV have also been recently reported in *S. bicolor,*
412 and this plant may be another host for development of new genotypes of the virus (ElSayed et
413 al., 2018; Filloux et al., 2018).

Only a limited number of *S. bicolor* plants were tested for SCMV but prevalence of this virus appeared to be high (33% for 15 randomly selected plants tested by RT-PCR and 100% for three plants tested by HTS). None of these plants showed mosaic symptoms, either because the virus only recently infected plants or because infected lines were tolerant to mosaic. Nevertheless, this result suggested that SCMV was introduced into the research plots from plants surrounding the experimental location at Belle Glade, FL, most likely from one or more of the five hosts that have been reported for SCMV in Florida (Hincapie et al., 2018). Sugarcane cultivars currently grown in Florida are resistant to mosaic and the virus was not found in commercial sugarcane in a recent virus metagenomics study (Filloux et al., 2018). Several plants of *S. almum* collected in this study in the EAA showed mosaic symptoms and were found infected by a strain of SCMV that was 97% identical to the three virus isolates obtained from *S. bicolor*. This suggested that *S. bicolor* became infected by SCMV from *S. almum*. Furthermore, the four sorghum isolates of SCMV from Florida sequenced in this study formed a unique lineage that did not match with other sequenced isolates retrieved from GenBank and representing the diversity of this virus species. This suggested that the sorghum isolates from Florida belong to a new strain of SCMV whose virulence and host range needs to be investigated.

HTS confirmed infection by SCYLV for two of three samples of *S. bicolor* that tested positive by TBIA, but SCYLV was not found by HTS in one sample (PI 651496) that tested positive for this virus by RT-PCR using two independent RNA extractions and RT-PCR assays. High titers of SCMV in sorghum line PI651496 may have prevented random amplification of SCYLV sequences in this sample. Inconsistency between HTS and RT-PCR detection of SCYLV has

436 previously been reported (Filloux et al., 2018). Similarly, the limited number of SCYLV reads
437 obtained for line Dale might be related to the presence of other viruses. Cultivar Dale, which
438 was infected by SCYLV and SCMV was also found infected by a third virus belonging to the
439 genus *Marafivirus,* family *Tymoviridae*. The marafivirus identified in this study shared less than
440 80% genome-wide sequence identity with other known marafiviruses, which is below the
441 species demarcation criterion within the genus as specified by the ICTV (King et al., 2011). The
442 second criterion for identification of a new marafivirus is less than 90% identity of the amino
443 acid sequence of the coat protein. The highest coat protein sequence identity between the *S.*
444 *almum* marafivirus and the *S. bicolor* marafivirus isolates and virus sequences in the GenBank
445 database was 57% and 58% with the oat blue dwarf virus (NP044448), respectively.
446 Furthermore, the two marafivirus isolates from sorghum formed a separate lineage in the
447 phylogenetic tree inferred from the coat protein amino acid sequences of marafiviruses
448 retrieved from GenBank. Therefore, the marafivirus isolates from *S. almum* and *S. bicolor* can
449 be considered a new species belonging to the genus *Marafivirus.* We propose to name this
450 newly discovered virus sorghum marafivirus.
451

452 ## 5. Conclusion

453 *S. bicolor* lines were colonized by the aphid vector of SCYLV in south Florida but the
454 prevalence of the virus in these plants was low based on RT-PCR screening. Detection of SCYLV
455 in sorghum by TBIA appeared not reliable because of non-specific reactions of sorghum using
456 this serological assay. A new strain of SCMV and a new marafivirus were discovered in mixed
457 infections with SCYLV in non-symptomatic plants of *S. bicolor*. Presence of these viruses in

propagating material needs to be determined to evaluate their risk to germplasm exchange. The weed *S. almum* appears to be an inoculum source for SCMV and the sorghum marafivirus. The insect vectors of these viruses as well as their effect on sorghum growth and yield also needs to be investigated.

## CRediT authorship contribution statement

**Wardatou Boukari:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing - Original draft. **Dimitre Mollov:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – Original Draft. **Chunyan Wei:** Investigation. **Lihua Tang:** Investigation. **Samuel Grinstead:** Investigation. **Muhammad Nouman Tahir:** Investigation. **Eva Mulandesa:** Investigation. **Martha Hincapie:** Investigation. **Robert Beiriger:** Methodology, Investigation. **Philippe Rott:** Conceptualization, Methodology, Investigation, Visualization, Writing – Original Draft, Supervision, Project administration, Funding acquisition.

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613

## Tables

**Table 1**

Detection of sugarcane yellow leaf virus by tissue-blot immunoassay (TBIA) in sorghum lines colonized by the aphid *Melanaphis sacchari* in planted and ratoon crops in 2016.

**Table 2**

Detection of sugarcane yellow leaf virus by tissue-blot immunoassay (TBIA) and reverse-transcription polymerase chain reaction (RT-PCR) in sorghum lines colonized by the aphid *Melanaphis sacchari* in planted crop in 2017.

**Table 3**

Detection of three viruses infecting three lines of *Sorghum bicolor* in ratoon crop at Belle Glade, FL in 2017.

## Figure legends

**Fig. 1.** Stalk imprints of *Sorghum bicolor* after processing by tissue-blot immunoassay with antibodies for sugarcane yellow leaf virus. Imprints in (a-c) were made with the cross section of the middle part of a stalk whereas imprints in (d-f) are from the upper part of a stalk. The diameter of imprints is 30-35 mm. (a and d) Colorless negative imprint revealing absence of the virus. (b and c) Positive imprint with bluish-purple dots (highlighted by red arrows) revealing the virus in the phloem. (e and f) Positive imprint with bluish-purple coloration of vascular and parenchymatic tissues.

**Fig. 2.** Detection of sugarcane yellow leaf virus (SCYLV) in undiluted and diluted samples of *Sorghum bicolor* by RT-PCR using primer pair ScYLVf1/ScYLVr1. 1 = 1 KB DNA ladder, 2 = Water control, 3 = Negative *S. bicolor* (Dale) control, 4 = Negative *Saccharum* control, 5 = Positive control (*Saccharum* plant infected by SCYLV), 6 = Dale #5 (no dilution), 7 = Dale #5 (1:10 dilution), 8 = Dale #5 (1:100 dilution), 9 = PI 585749 #7 (no dilution), 10 = PI 585749 #7 (1:10 dilution), 11 = PI 585749 #7 (1:100 dilution), 12 = PI 571128 #6 (no dilution), 13 = PI 571128 #6 (1:10 dilution), 14 = PI 571128 #6 (1:100 dilution), 15 = PI 571128 #10 (no dilution), 16 = PI 571128 #10 (1:10 dilution), 17 = PI 571128 #10 (1:100 dilution), 18 = 1 KB DNA ladder. The amplicon (shown by white arrow) has a size of 219 bp.

**Fig. 3.** Phylogenetic tree of sugarcane yellow leaf virus (SCYLV) constructed with the complete genome coding sequence (ORF0 to ORF5) of 13 virus isolates retrieved from GenBank and one isolate from sorghum obtained in this study. The 13 GenBank isolates represent the most recent genetic diversity reported for SCYLV (Filloux et al., 2018). The phylogenetic tree was inferred using the maximum-likelihood method in MEGA X with substitution model Tamura-Nei

selected as the best-fit model. The SCYLV isolates were listed as accession number/isolate name/host plant/country of origin, and the isolate obtained in this study has been marked with a star. Bootstrap values based on 1000 replications and higher than 50 are indicated at branches. Scale bar units are in number of substitutions per nucleotide. The bold brackets represent SCYLV genotypes and the vertical lines correspond to the two clades of SCYLV.

**Fig. 4.** Phylogenetic tree of sugarcane mosaic virus (SCMV) constructed with the complete genome coding region of 19 virus isolates retrieved from GenBank and four isolates from sorghum obtained in this study. The 19 GenBank isolates represent the most recent genetic diversity reported for SCMV (Li et al., 2019). The phylogenetic tree was inferred using the maximum-likelihood method in MEGA X with substitution model Tamura-Nei selected as the best-fit model. The SCMV isolates were listed as accession number/isolate name/host plant/country of origin, and the isolates obtained in this study have been marked with a star. Bootstrap values based on 1000 replications and higher than 50 are indicated at branches. Branches with bootstrap values <50 were collapsed. Scale bar units are in number of substitutions per nucleotide.

**Fig. 5.** Phylogenetic tree constructed with the amino acid sequences of the coat protein of the eight virus members of the genus *Marafivirus* retrieved from GenBank and the marafiviruses from sorghum obtained in this study. The phylogenetic tree was inferred using the maximum-likelihood method in MEGA X with substitution model Tamura-Nei selected as best-fit model. The sequences were listed as accession number/isolate name/country of origin, and the two isolates obtained in this study have been marked with a star. Bootstrap values based on 1000 replications and higher than 50 are indicated at branches. The grapevine fleck virus

(NC_003347) was used as an out-group. Scale bar units are in number of substitutions per amino acid.

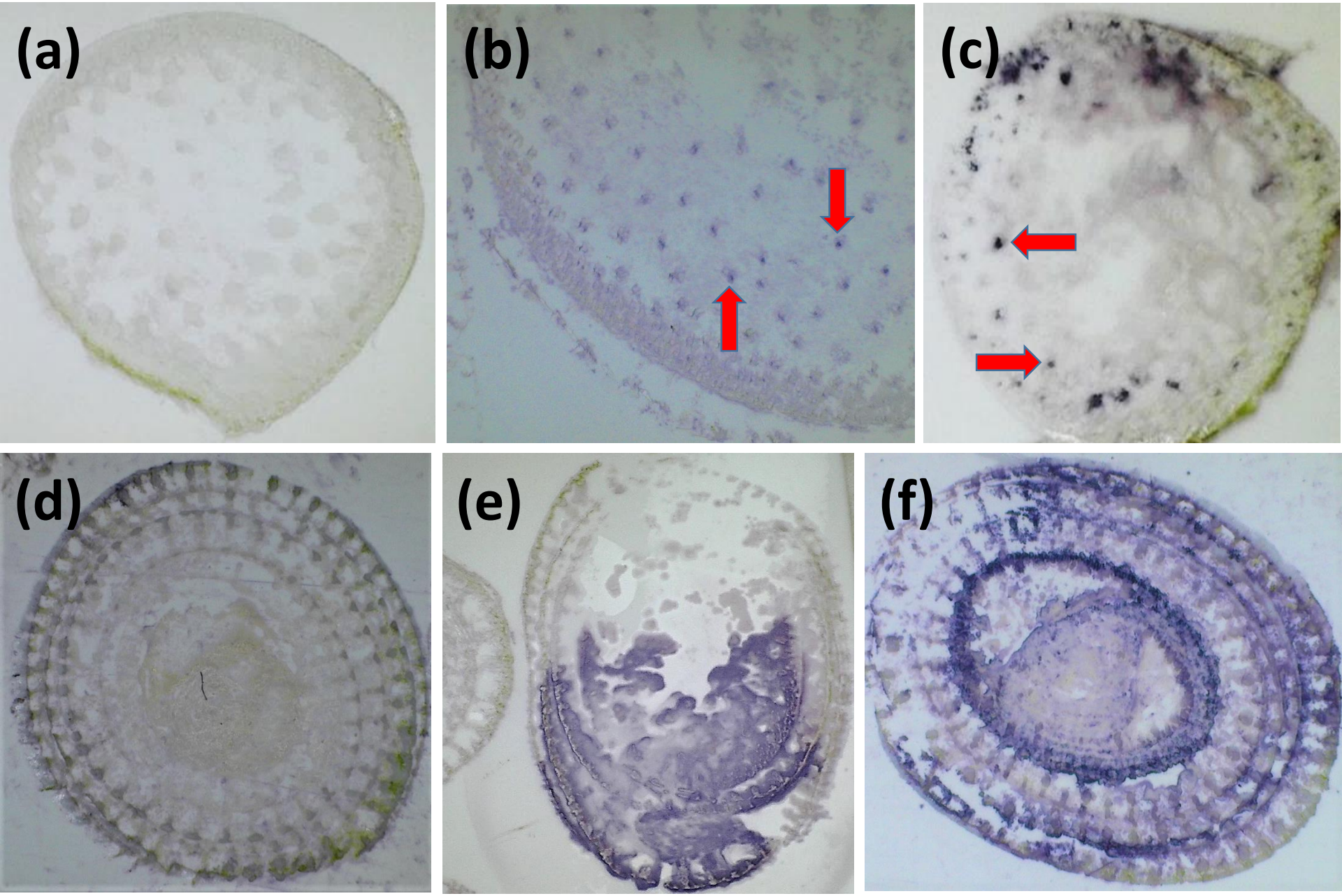



Figure 1

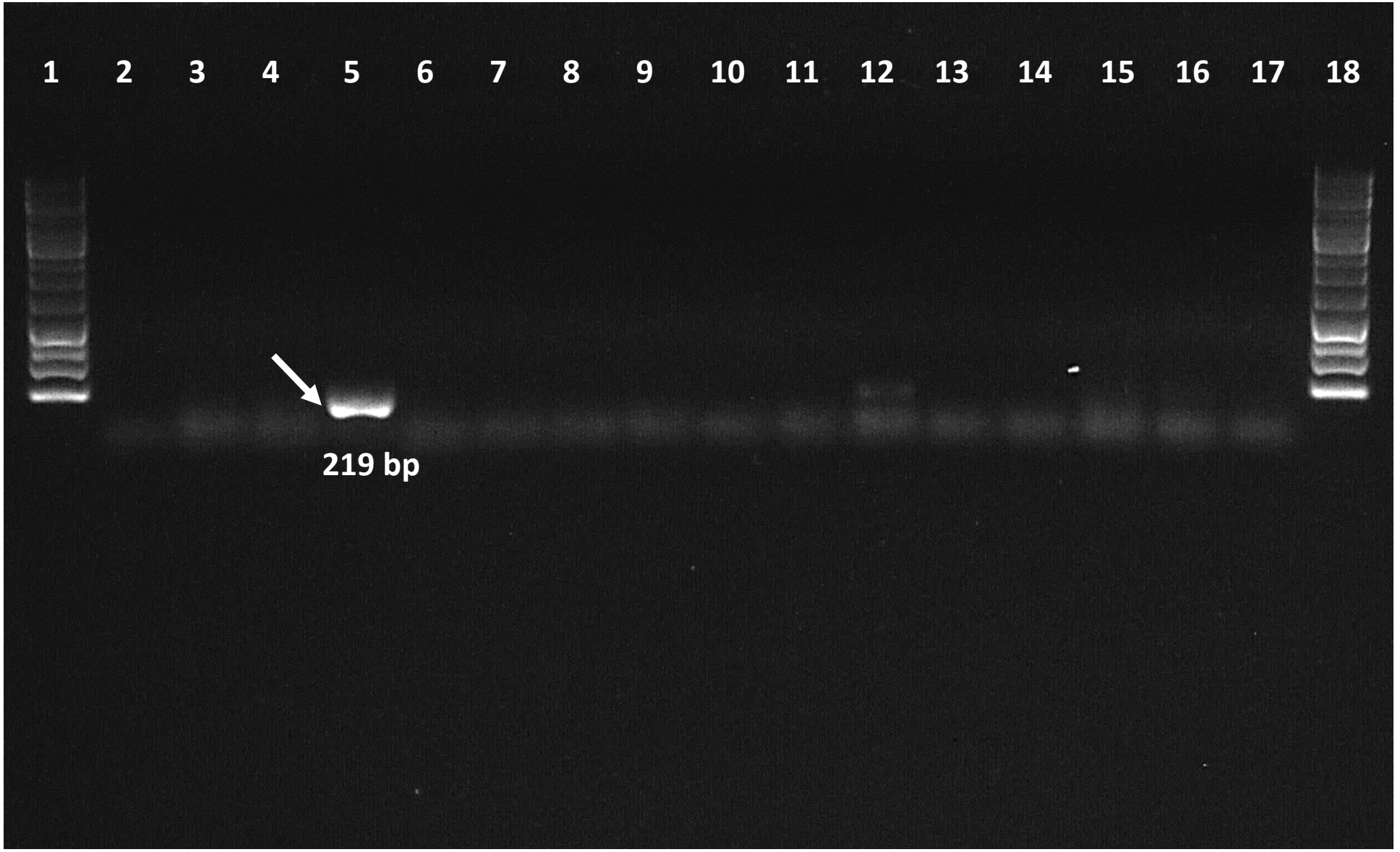
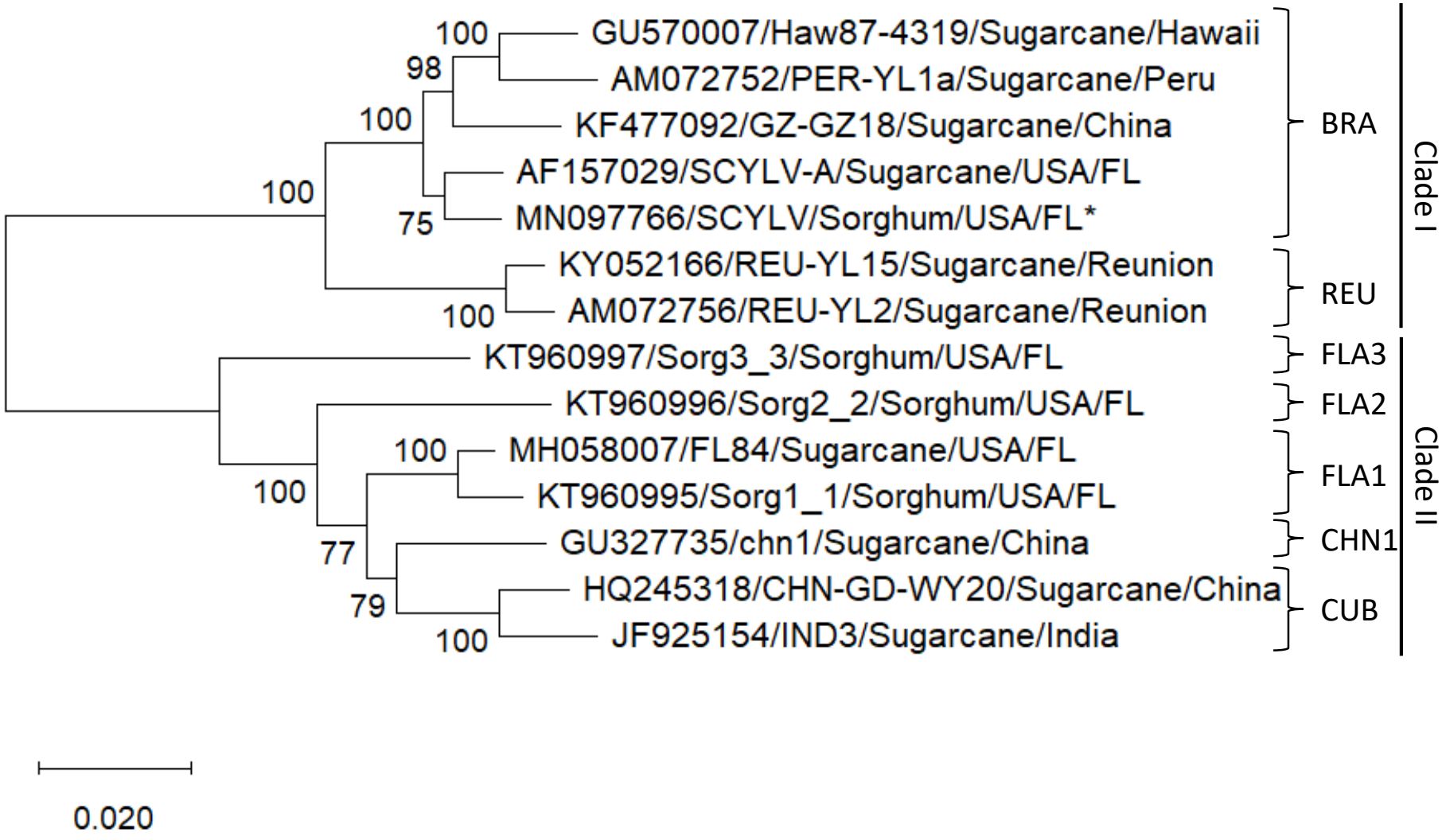



Figure 2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GU570007/Haw87-4319/Sugarcane/Hawaii
AM072752/PER-YL1a/Sugarcane/Peru
KF477092/GZ-GZ18/Sugarcane/China
AF157029/SCYLV-A/Sugarcane/USA/FL
MN097766/SCYLV/Sorghum/USA/FL*
KY052166/REU-YL15/Sugarcane/Reunion
AM072756/REU-YL2/Sugarcane/Reunion
KT960997/Sorg3_3/Sorghum/USA/FL
KT960996/Sorg2_2/Sorghum/USA/FL
MH058007/FL84/Sugarcane/USA/FL
KT960995/Sorg1_1/Sorghum/USA/FL
GU327735/chn1/Sugarcane/China
HQ245318/CHN-GD-WY20/Sugarcane/China
JF925154/IND3/Sugarcane/India
BRA
REU
FLA3
FLA2
FLA1
CHN1
CUB
Clade I
Clade II
0.020

Figure 3

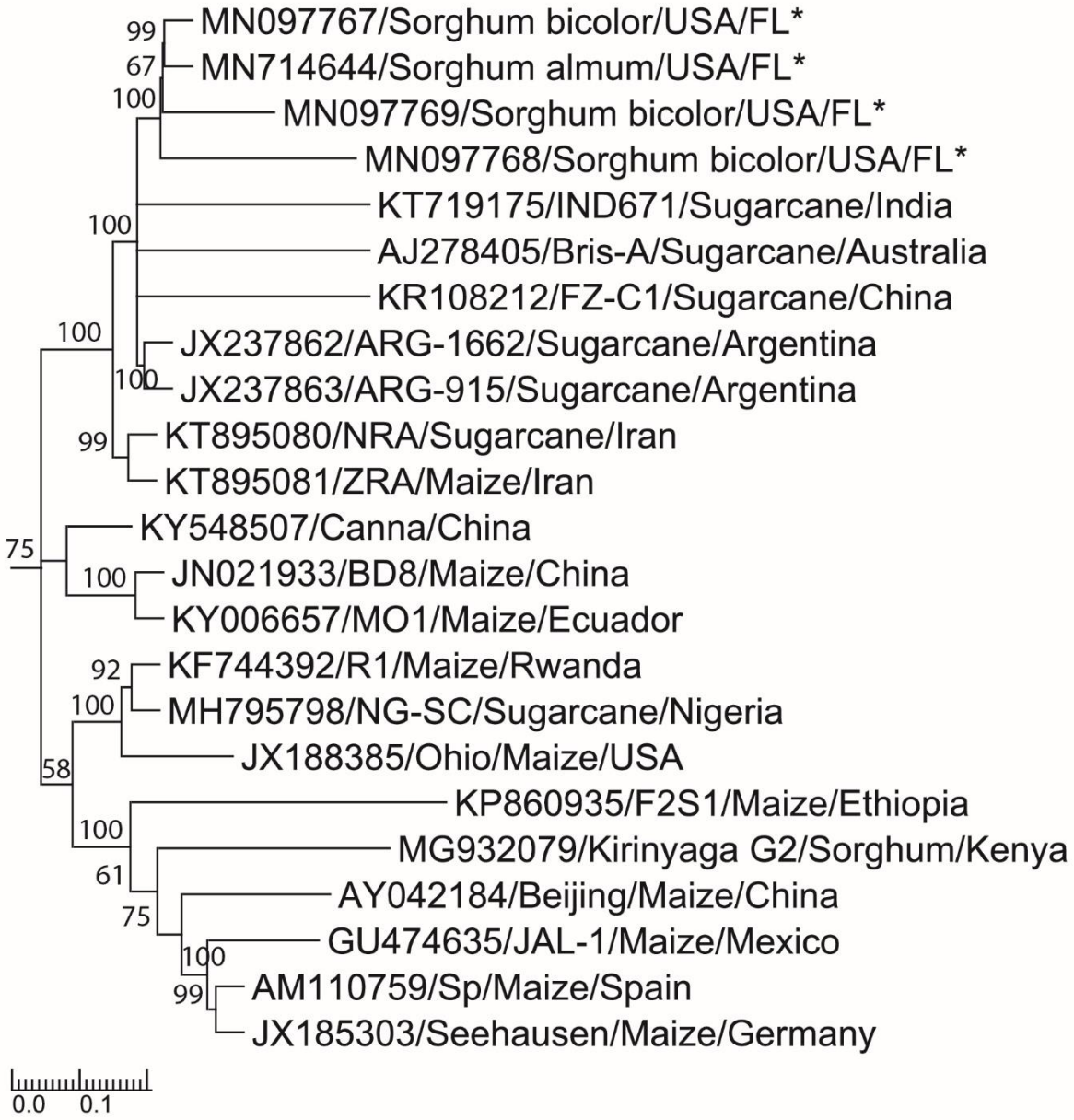
MN097767/Sorghum bicolor/USA/FL*
MN714644/Sorghum almum/USA/FL*
MN097769/Sorghum bicolor/USA/FL*
MN097768/Sorghum bicolor/USA/FL*
KT719175/IND671/Sugarcane/India
AJ278405/Bris-A/Sugarcane/Australia
KR108212/FZ-C1/Sugarcane/China
JX237862/ARG-1662/Sugarcane/Argentina
JX237863/ARG-915/Sugarcane/Argentina
KT895080/NRA/Sugarcane/Iran
KT895081/ZRA/Maize/Iran
KY548507/Canna/China
JN021933/BD8/Maize/China
KY006657/MO1/Maize/Ecuador
KF744392/R1/Maize/Rwanda
MH795798/NG-SC/Sugarcane/Nigeria
JX188385/Ohio/Maize/USA
KP860935/F2S1/Maize/Ethiopia
MG932079/Kirinyaga G2/Sorghum/Kenya
AY042184/Beijing/Maize/China
GU474635/JAL-1/Maize/Mexico
AM110759/Sp/Maize/Spain
JX185303/Seehausen/Maize/Germany
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Figure 4


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KY123917/Grapevine asteroid mosaic-associated virus isolate CS/France
69
AY884005/Citrus sudden death-associated virus/Brazil
64
NC001793/Oat blue dwarf virus/USA
67
FJ915122/Blackberry virus S/USA
MN100128/Sorghum bicolor marafivirus/USA/FL*
95
100
MN714643/Sorghum almum marafivirus/USA/FL*
AF265566/Maize rayado fino virus/Costa Rica
90
AY040531/Bermuda grass etched-line virus/USA/CA
FJ436028/Grapevine Syrah virus 1/USA
99
AY706994/Grapevine rupestris vein feathering virus/France
NC 003347/Grapevine fleck virus/Italy
0.20

Figure 5

**Table 1** Detection of sugarcane yellow leaf virus by tissue-blot immunoassay (TBIA) in sorghum lines colonized by the aphid *Melanaphis sacchari* in planted and ratoon crops in 2016

| Crop season | Sorghum line | Number of plants colonized by aphids[a] | Average number of aphids/ colonized plant[a] | TBIA positive stalks/ Total number of stalks tested |
|---|---|---|---|---|
| Planted sorghum | Dale | 8/10 | 5 | 8/10 |
| | Keller | 7/10 | 7 | 2/10 |
| | M81E | 5/10 | 4 | 7/10 |
| | PI 157033 | 10/10 | 64 | 9/10 |
| | PI 300119 | 6/10 | 5 | 6/10 |
| | PI 525695 | 10/10 | 300 | 8/10 |
| | PI 533834 | 10/10 | 306 | 10/10 |
| | PI 533910 | 10/10 | 475 | 10/10 |
| | PI 534133 | 10/10 | 652 | 7/10 |
| | PI 563295 | 5/10 | 6 | 9/10 |
| | PI 565121 | 10/10 | 622 | 10/10 |
| | PI 571128 | 3/10 | 8000 | 8/10 |
| | PI 576130 | 3/10 | 5 | 10/10 |
| | PI 585749 | 8/10 | 9 | 9/10 |
| | PI 613536 | 3/10 | 1200 | 7/10 |
| | PI 651496 | 4/10 | 5 | 9/10 |
| | PI 653617 | 6/10 | 85 | 6/10 |
| | PI 659692 | 7/10 | 8 | 4/10 |
| | Terral | 10/10 | 610 | 6/10 |
| | All 19 lines | 125/190 | 651 | 145/190 |
| Ratoon sorghum[b] | Dale | 10/10 | 2700 | 10/10 |
| | PI 157033 | 10/10 | 2200 | 9/10 |
| | PI 300119 | 10/10 | 415 | 10/10 |
| | PI 525695 | 10/10 | 30 | 10/10 |
| | PI 563295 | 10/10 | 5300 | 9/10 |
| | PI 613536 | 10/10 | 360 | 5/10 |
| | PI 651496 | 10/10 | 4000 | 9/10 |
| | Terral | 10/10 | 1470 | 7/10 |
| | All 8 lines | 80/80 | 2059 | 69/80 |

[a] Number of plants colonized by aphids and average number of aphids per plant was determined on June 8, 2016 for planted sorghum and October 24, 2016 in ratoon sorghum when plants were 3 and 2 months old, respectively.

[b] Eight samples of ratoon sorghum (one stalk per line) were tested for SCYLV by RT-PCR and four of them were positive: Dale, PI 157033, PI 300119, and PI 651496.

**Table 2** Detection of sugarcane yellow leaf virus by tissue-blot immunoassay (TBIA) and reverse-transcription polymerase chain reaction (RT-PCR) in sorghum lines colonized by the aphid *Melanaphis sacchari* in planted crop in 2017

| Sorghum line | Number of plants colonized by aphids[a] | Average number of aphids/ colonized plant[a] | TBIA positive stalks/ Total number of stalks tested | Number of RT-PCR positive stalks/Total number of stalks tested |
|---|---|---|---|---|
| Dale | 10/10 | 44 | 10/10 | 1/10 |
| Dale (Neg. Control)[b] | 0/10 | 0 | 6/10 | 0/10 |
| Keller | 10/10 | 1775 | 10/10 | 0/10 |
| PI 157033 | 10/10 | 2400 | 10/10 | 0/10 |
| PI 330272 | 10/10 | 277 | 10/10 | 0/10 |
| PI 533834 | 7/10 | 749 | 10/10 | 0/10 |
| PI 533910 | 10/10 | 914 | 9/10 | 0/10 |
| PI 534133 | 10/10 | 2819 | 10/10 | 0/10 |
| PI 534138 | 10/10 | 344 | 10/10 | 0/10 |
| PI 563295 | 9/10 | 40 | 10/10 | 0/10 |
| PI 571128 | 10/10 | 7450 | 10/10 | 3/10 |
| PI 585749 | 10/10 | 9200 | 10/10 | 0/10 |
| PI 651496 | 10/10 | 160 | 10/10 | 0/10 |
| PI 653617 | 10/10 | 963 | 10/10 | 1/10 |
| PI 659692 | 8/10 | 8 | 10/10 | 0/10 |
| Terral | 10/10 | 1502 | 10/10 | 0/10 |
| All 15 lines (except Neg. control) | 144/150 | 1910 | 149/150 | 5/150 |

[a] Data were collected on June 13, 2017 when sorghum plants were three months old in planted crop. Aphid numbers could not be determined in ratoon sorghum because plants were severely damaged by hurricane Irma (September 10-13, 2017). Three remaining

stalks in ratoon sorghum were collected on 16th November 2017 and all three tested positive for SCYLV by TBIA and RT-PCR (Table 3).

[b] Negative control plants were grown in pots in an insect-proof screened enclosure about 40 km from the field trial.

**Table 3** Detection of three viruses infecting three lines of *Sorghum bicolor* in ratoon crop at Belle Glade, FL in 2017

| | | Sorghum line[a] | | |
|---|---|---|---|---|
| Virus[b] | Diagnostic technique[c] | Dale | PI 157033 | PI 651496 |
| SCYLV | TBIA | + | + | + |
| | One-step RT-PCR | + | + | + |
| | HTS | + | + | - |
| | Two-step RT-PCR | + | + | + |
| SCMV | HTS | + | + | + |
| | Two-step RT-PCR | + | + | + |
| marafivirus | HTS | + | - | - |
| | Two-step RT-PCR | + | - | - |

[a] All samples collected on 16 November 2017.

[b] SCYLV = sugarcane yellow leaf virus, SCMV = sugarcane mosaic virus.

[c] TBIA = tissue-blot immunoassay; RT-PCR = reverse-transcription polymerase chain reaction, HTS = high-throughput sequencing. RT-PCR assays for SCYLV were performed with two different RNA extracts of each sample.