INTRODUCTION

The recent epidemic of maize lethal necrosis (MLN) in sub-Saharan East Africa has severely constrained production of this staple crop, affecting food security in the region. Estimated losses in maize production attributed to MLN in East Africa range from 25% to 100% (reviewed in Redinbaugh and Stewart, 2018). The disease is caused by coinfection of maize with the emergent maize chlorotic mottle virus (MCMV) and any of several maize-infecting potyviruses endemic in East Africa and worldwide. Here, we examined the distribution of MCMV and sugarcane mosaic virus (SCMV), the major viruses contributing to MLN in Rwanda. These and other viruses in maize across Rwanda were further characterized by deep sequencing. When identified, MCMV had high titres and minimal sequence variability, whereas SCMV showed moderate titres and high sequence variability. Deep sequencing also identified maize streak virus and other maize-associated viruses, including a previously described polerovirus, maize yellow mosaic virus, and barley yellow dwarf virus, diverse maize-associated totoviruses, maize-associated pteridovirus, Zea mays chrysovirus 1, and a maize-associated betaflexivirus. Detection of each virus was confirmed in maize samples by reverse transcription polymerase chain reaction.

KEYWORDS
maize chlorotic mottle virus, maize lethal necrosis, potyvirus, Rwanda, synergy
transmit SCMV and other potyviruses (Teakle and Grylls, 1973; Louie, 1980; Cabanas et al., 2013; Mahuku et al., 2015a).

In Rwanda, MLN was first reported in the Northern Province in 2013 (Adams et al., 2014). Field observations based on typical symptoms later indicated that the disease was widespread across the country. Because highly diverse populations of SCMV are associated with emerging MLN (Mahuku et al., 2015a), traditional diagnostics like ELISA and PCR may not detect all viruses or variants associated with disease (see Redinbaugh and Stewart, 2018). In addition to diverse SCMV populations, other potyviruses contribute to MLN in East Africa, including a recently described East African strain of JGMV (JGMV-EA; Stewart et al., 2017). Sequences of other viruses, including a polerovirus and to人とviruses, have been frequently identified by deep sequencing in MLN-affected locations, often codetected with known MLN viruses (Chen et al., 2016a; Bernreiter et al., 2017; Goncalves et al., 2017; Yahaya et al., 2017; Guadie et al., 2018; Massawe et al., 2018). Whether these other viruses cause disease(s) is not known.

To date, most studies on MLN and RNA viruses associated with maize in East Africa have relied on few samples and/or very localized sampling. For example, Adams et al. (2014) used only four maize samples from the Northern Province to describe MLN in Rwanda. In this study, the sequences and distribution of viruses associated with MLN in Rwanda were examined using a total of 576 samples of maize and potential alternative hosts, collected in a comprehensive national survey. Samples were analysed for SCMV and MCMV by ELISA, and sequences from samples pooled by location were further analysed by RNA deep sequencing.

2 | MATERIALS AND METHODS

2.1 | Field sampling

To understand the distribution of MLN in Rwanda, as well as the variability and distribution of the viruses associated with it, a survey was conducted in 2015 in 19 of 30 districts from five provinces of Rwanda: the Eastern Province (Bugesera, Kihehe, Rwamagana, and Kayonza districts); Kigali (Kicukiro and Gasabo districts); the Northern Province (Burera, Gakenke, Musanze, and Rulindo districts); the Southern Province (Gisagara, Huye, Kamonyi, Nyamagabe, and Nyanza districts); and the Western Province (Karongi, Nyabihu, Rubavu, and Rutshuru districts). Using information from preliminary field reports, sampling sites were selected based on agroecological zone and purported MLN symptom prevalence. Fields where maize was grown for food, feed, or seed were sampled. Sampling was carried out in 2015 in January, during the wet growing season, and June, during the relatively dry growing season. Of districts, Burera, Gakenke, and Musanze in the Northern Province and Nyabihu and Rubavu in the Western Province were regarded as MLN hotspots, all of which border Uganda and/or the Democratic Republic of the Congo on the north or are contiguous with a border district (e.g., Gakenke; Figure 1). The other selected districts were considered low-incidence for MLN.

One to three sectors per district were sampled, with at least 5 km between maize fields. Eight samples were collected from each field, for a total of 576 samples collected from 72 fields across the country (Table 1). The sampling was done along two field diagonals, with three leaf samples collected in each. Two additional leaf samples were randomly collected from any location within the field, making a total of eight samples per field. Sample selection for symptoms was nonrandom, but symptom determination was not standardized, thus analyses based on symptoms could not be included. Most samples were collected from maize, but 15 samples were collected from other potential alternative hosts (four Napier grass [Pennisetum purpureum], two sugarcane [Saccharum officinarum] and nine sorghum [Sorghum bicolor]). A leaf section about 5 × 13 cm was cut from the second or third fully grown leaf from the top of each sampled plant, and placed in a paper bag. Leaf samples were kept on ice for transport to the laboratory.

Diverse agricultural practices across the country made collection of all maize at the same growth stage or field size impossible, but plants in the vegetative stages (V8–VT) were preferentially sampled, with older plants collected if no other stages were available for the location. Similarly, the sampled maize plants were of a mixture of hybrids and landraces; however, all maize grown and sampled were white endosperm types. A number of popular hybrids in Rwanda were represented, including ZM 607, PAN 691, PAN 4m-21, PAN 53, H629, and SIDCO 513, but information on which hybrids were sampled was inconsistently available; thus, these data were not included in the analyses. Global Positioning System coordinates for each sampled site were recorded, and sampled sites were mapped using Aeronautical Reconnaissance Coverage Geographic Information System (ArcGIS) v. 9.3 software and BatchGeo (BatchGeo LLC). Leaf samples were maintained at 4 °C for 2–3 days prior to being freeze-dried and shipped to the USA (USDA, ARS, Corn, Soybean and Wheat Quality Research Unit) for analyses. Aliquotted lyophilized samples were stored at −80 °C for ELISA and reverse transcription (RT)-PCR analyses.

2.2 | Virus detection

2.2.1 | ELISA

Lyophilized leaf samples (200 mg) were homogenized in 1 ml of general extraction buffer (Agdia, Inc.), then screened using commercial double antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) kits for MCMV, SCMV, and MDMV detection (Agdia) according to the manufacturer’s recommendations, except that sample absorbances (405 nm) were determined 20 min after addition of the Agdia PNP buffer, as optimized to detect positive controls and avoid false positives from negative controls (healthy un inoculated plant samples). Samples were scored as positive if the mean absorbance of duplicate samples was greater than twice that of the healthy controls.
2.2.2 | RT-PCR

Primer sequences are summarized in Table S1. Total RNA was isolated from lyophilized leaf samples (50 mg) using Direct-zol (ZymoResearch) according to the manufacturer’s instructions. RT-PCR was used to confirm results in 60 samples that were positive for both MCMV and SCMV in ELISA, using primer pairs MCMV-895F/MCMV-1536R4 and SCMV-8679F/SCMV-9606R (Table S1). These primer pairs were designed to amplify sequences of East African isolates. Three other primer pairs were designed based on conserved sequences in eight MDMV genomes and used to assay for MDMV in the samples (MDMV-8950F/MDMV-9524R, MDMV-6127F/MDMV-6886R, MDMV-1938F/MDMV-2765R; Table S1). Although these primer pairs amplified MDMV sequences in positive controls using MDMV isolate OH, MDMV sequences were not amplified from any of the Rwandan samples tested under the same conditions. RT-PCR was also conducted to assess the presence of MCMV and SCMV in the 110 samples used to form the 20 RNA-Seq pools, using primers MCMV-RW22F/MCMV-RW1698R and SCMV-RW7874F/SCMV-RW9588R designed from RNA-Seq virus supercontig consensus sequences. One-step RT-PCR was carried out using SuperScript III polymerase for first-strand synthesis (ThermoFisher Scientific) and GoTaq DNA polymerase (Promega) for amplification (Stewart et al., 2017). Initial cDNA synthesis was carried out at 52 °C for 40 min then 94 °C for 2 min; followed by amplification with 32 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 20 s, extension at 72 °C for 1 min; and a final elongation at 72 °C for 7 min. PCR products were visualized on 1% agarose gels. RT-PCRs using primers designed against putative virus sequences identified in RNA-Seq were performed similarly using the same one-step RT-PCR protocol and adjusting amplification time based on expected amplicon size (1 min/kb; see primer list, Table S1).

2.3 | RNA sequencing and bioinformatics

RNA quality was assessed using a NanoDrop spectrophotometer, Qubit fluorometer, and TapeStation BioAnalyzer (Agilent...
Table 1: ELISA detection of maize chlorotic mottle virus (MCMV) and sugarcane mosaic virus (SCMV) in maize samples collected across Rwanda

| District   | Sector   | MCMV  | SCMV  | MCMV + SCMV |
|------------|----------|-------|-------|-------------|
| Eastern    |          |       |       |             |
| Rwamagana  | Fumbwe   | 0/8   | 1/8   | 0/8         |
|            | Gahengeri| 1/16  | 3/16  | 0/16        |
| Bugesera   | Gashora  | 13/29 | 11/29 | 3/29        |
| Kirehe     | Gatore   | 22/41 | 5/41  | 5/41        |
| Bugesera   | Mayange  | 3/13  | 5/13  | 1/13        |
| Kayonza    | Nyamirama| 0/6   | 0/6   | 0/6         |
| Subtotal   |          | 39/113(35%) | 25/113(22%) | 9/113(8%) |
| Kigali     |          |       |       |             |
| Kicukiro   | Kanombe  | 5/8   | 0/8   | 0/8         |
| Gasabo     | Ndera    | 7/20  | 7/20  | 3/20        |
| Rubilizi   | Rubilizi | 2/2   | 0/2   | 0/2         |
| Subtotal   |          | 14/30(47%) | 7/30(23%)  | 3/30(10%) |
| Northern   |          |       |       |             |
| Rulindo    | Base     | 5/8   | 3/8   | 2/8         |
| Rulindo    | Bushoki  | 5/8   | 4/8   | 2/8         |
| Musanze    | Busogo   | 11/16 | 0/16  | 0/16        |
| Burera     | Cyanika  | 7/8   | 3/8   | 3/8         |
| Musanze    | Cuve     | 9/12  | 4/12  | 3/12        |
| Burera     | Gahunga  | 7/23  | 8/23  | 3/23        |
| Gakenke    | Gakenke  | 11/30 | 10/30 | 3/30        |
| Gakenke    | Gashenyi | 15/17 | 3/17  | 3/17        |
| Musanze    | Kinigi   | 7/10  | 3/10  | 2/10        |
| Burera     | Kinyababa| 11/11 | 7/11  | 7/11        |
| Musanze    | Nkotsi   | 6/18  | 3/18  | 0/18        |
| Burera     | Rugarama | 10/16 | 1/16  | 0/16        |
| Subtotal   |          | 104/177(59%) | 49/177(28%) | 28/177(16%) |
| Southern   |          |       |       |             |
| Nyanza     | Busasamana| 3/9 | 1/9  | 1/9         |
| Nyamagabe  | Gasaka   | 3/13  | 1/13  | 1/13        |
| Gisagara   | Kansi    | 12/19 | 5/19  | 4/19        |
| Huye       | Kigoma   | 6/8   | 3/8   | 3/8         |
| Gisagara   | Mamba    | 2/3   | 2/3   | 2/3         |
| Nyanza     | Mukingo  | 6/11  | 5/11  | 4/11        |
| Kamonyi    | Musambira| 4/4   | 0/4   | 0/4         |
| Huye       | Rusatira | 11/16 | 7/16  | 6/16        |
| Subtotal   |          | 47/83(57%) | 24/83(29%) | 21/83(25%) |
| Western    |          |       |       |             |
| Nyabihu    | Bigogwe  | 8/27  | 4/27  | 2/27        |
| Karongi    | Bwishyura| 12/27 | 6/27  | 3/27        |
| Rutsiro    | Gihango  | 9/18  | 5/18  | 4/18        |
| Nyabihu    | Mukamira | 11/27 | 3/27  | 1/27        |
| Rutsiro    | Mukura   | 3/3   | 0/3   | 0/3         |
| Rutsiro    | Mushabati| 9/15  | 8/15  | 6/15        |
| Rubavvu    | Nyakiriba| 12/14 | 4/14  | 2/14        |
| Rubavvu    | Rubavvu  | 20/31 | 18/31 | 12/31       |
| Karongi    | Rubengera| 2/8   | 2/8   | 2/8         |
| Subtotal   |          | 86/170(51%) | 50/170(29%) | 32/170(19%) |
| Total      |          | 290/573(51%) | 155/573(27%) | 93/573(16%) |
Technologies) according to the manufacturers’ recommendations. Twenty pools were prepared from 110 selected RNA samples representing each province, with three pools containing samples from a mixture of locations were also created (see Table S2 legend). Each pool contained 2.5 μg RNA in total, with equal amounts from each sample in the pool. Pooled RNA samples were submitted to USDA-ARS Genomics and Bioinformatics Research Unit (Stoneville, MS, USA). Libraries were prepared using Illumina TruSeq Total RNA stranded libraries with Ribo-Zero deple-
tion of rRNA (RS-122-2401 TruSeq Stranded Total RNA LT with Ribo-
Zero Plant). The sample pools were multiplexed (20 libraries, each containing 3–8 samples, for deep sequencing (Table 2; Table S2). Three additional pools (18–20) with samples from a mixture of locations were also created (see Table S2 legend). Each pool contained 2.5 μg RNA in total, with equal amounts from each sample in the pool. Pooled RNA samples were submitted to USDA-ARS Genomics and Bioinformatics Research Unit (Stoneville, MS, USA). Libraries were prepared using Illumina TruSeq Total RNA stranded libraries with Ribo-Zero depletion of rRNA (RS-122-2401 TruSeq Stranded Total RNA LT with Ribo-
Zero Plant). The sample pools were multiplexed (20 libraries, each consisting of 3–8 samples per pool with a minimum RQ value according to the Agilent 2200 TapeStation of 5.0–8.3) on a single lane on Illumina HiSeq 2500 in Rapid Mode, with 100 nt paired-end reads.

Sequences yielded a high-quality score of more than 96.3% of Q30 bases, at an average of 98.5% barcode perfection (a measure of barcode assignment and demultiplexing fidelity). The 20 sequenced samples were analysed using CLC Genomics v. 11.0. Adapters used for library preparation and polyA tails were trimmed and the sequences were mapped to the maize reference genome B73 RefGen_v4 (Jiao et al., 2017) using mapping parameters at default conditions (reading alignment with match score of 1 with a linear gap cost, 0.5 fraction length, 0.8 similarity fraction, and autodetection of paired distance). Reads that did not map to the maize genome were de
novo assembled using parameters including minimum contig length of 200 nt, autodetection of paired reads, and scaffolding performance with slow mapping option. The contigs were compared using BLAST to a custom plant virus database plus the maize genome to identify putative plant-associated virus sequences using an approach similar to one previously described (Stewart et al., 2014). The custom database was created by combining a filtered subset of NCBI virus sequences (downloaded 2018-01-24) and the maize genome B73 RefGen_v4. The 3.601,939 partial and complete virus sequences from NCBI were first filtered by size (>250 to <21,000 bp), then filtered based on descriptions to remove non-plant-associated
viruses to obtain a plant virus-enriched database of 58,111 sequences. De novo assembled contigs from reads not mapping to the maize genome were searched against the custom database by BLASTn (Altschul et al., 1990). Contigs with no BLASTn matches to that database were searched again using the tBLASTx algorithm (Gish and States, 1993) to identify matches with less conserved nucleotide sequences.

The contigs matching plant virus or plant virus-like sequences were assembled into supercontigs in Sequencher (Gene Codes v. 5.4.6), using program settings for dirty data with minimum match of 95% and 50 nucleotides minimum overlap, except for the pteridovirus, for which 85% and 25 nt overlap settings were used for the consensus assembly validated by RT-PCR. Assembly ends were trimmed against rapid amplification of cDNA ends (RACE)-verified reference sequences where possible, due to frequent sequence artefacts at the ends of de novo assembled contigs. To quantify reads per sample pool matching each virus, trimmed reads were mapped back to supercontig consensus sequences of putative maize-associated viruses, plus the reference sequences (see Table 2 legend) and the maize B73 genome. The default parameters were used for read alignment: match score of 1, 0.8 similarity fraction, 0.5 fragment length.

2.4 | Phylogenetic analyses

Sequences were aligned using ClustalW (Thompson et al., 2002) in MacVector v. 15.5.4 with default parameters for alignment comparisons and matrices (multiple alignment open gap penalty 15; extend gap penalty 6.66; delay divergent 30%, and weighted transitions). SCMV tree was constructed using the maximum-likelihood statistical method with WAG substitution model, 1,000 bootstrap replicates and the neighbour-joining method for inference tree using MEGA X (Kumar et al., 2018) in CLC Genomics v. 11.0.

3 | RESULTS

3.1 | Identification of the viruses known to cause MLN

To understand the distribution of known MLN-causing viruses as well as the variability and distribution of other viruses in maize, 576 samples, 8 samples from each of 72 fields from five provinces in Rwanda, were collected. Burera, Gakenke, and Musanze in the Northern Province and Nyabihu and Rubavu in the Western Province were regarded as MLN hotspots. All of these considered hotspots border Uganda (Burera), Democratic Republic of the Congo (Nyabihu, Bubavu), both (Musanze), or are contiguous with a border district (Gakenke; Figure 1). The other selected districts were considered low-incidence for MLN. Using DAS-ELISA, 573 samples were tested for MCMV and SCMV (Table 1). MCMV was detected in 290 samples (50%), including five non-maize grass samples (three sorghum and two sugarcane). SCMV was detected less frequently than MCMV, with 155/573 (27%) samples testing positive, including one sugarcane sample in which MCMV was also detected. Of the maize samples, 93 (16%) were ELISA-positive for both MCMV and SCMV. MCMV and SCMV were ELISA-detected from samples widely distributed across the country (Figure 1; Table 1). For the selected samples, MCMV detection and MCMV/SCMV codetection rates were lowest in the Eastern Province, whereas SCMV rates of detection were similar across all locations (Table 1).

3.2 | RNA-Seq for virus discovery and prevalence

RNA-Seq from 20 pools of samples (Table S2) yielded a total of 487,604,962 reads with an average read length of 94 nt after quality trimming. After maize sequence subtraction, de novo assembly, and virus identification pipelines, 5,903 contigs matching putative plant virus sequences were identified. These were stringently assembled (at least 95% identity) into supercontigs to obtain a collection of putative plant virus consensus sequences (Table S3 and S4; File S1). The number of contigs per supercontig varied from 3 to 2,499, with assemblies from 1,929 to 12,980 nt (Table S3). Putative virus contigs were identified for each sample pool, and sequences with top hits matching sequences of MCMV and SCMV were found. A previously described polerovirus (maize yellow dwarf virus RMV2, MYDV-RMV2/MYDV-like/maize yellow mosaic virus, MaYMV), several distinct maize-associated tootivirus (MATV) sequences, maize pteridovirus 1 (MPTV-1), barley yellow dwarf virus (BYDV), Zea mays chrysovirus, and two betaflexivirus sequences with distant matches to cherry rusty mottle-associated virus (CRMaV) and apple chlorotic leaf spot virus (ACLSV), respectively, were also identified. The number of contigs assembled per supercontig varied, with 2,499 for one assembly of SCMV-like sequences, 820 for MYDV-RMV2, 444 for MCMV, 162 for another SCMV supercontig assembly, 90 for MATV, 76 for MPTV-1, and 44 for BYDV. For contigs with top BLASTn hits to maize streak virus (MSV), circular contigs could not be reliably assembled and only near-complete contigs >2 kb were further analysed (Table S5).

Reads from sample pools were mapped back to this sample set of assembled supercontig consensus sequences (File S1), reference sequences, and the B73 maize genome to assess abundance of reads per virus, with a ≥0.01% after rounding cut-off applied to score a sample pool as detection-positive. Extraordinary variability in reads mapping to maize versus virus sequences was observed (Table 2). The percentage of reads mapping to maize in each pool varied from 4.93% in pool 20 to 93.25% in pool 3. Of the total reads, 63.87% mapped to putative viral sequences in the compiled supercontig consensus and reference sequence set, with 53.64% mapping to MCMV and 8.26% to SCMV, whereas less than half of the total reads (34.54%) mapped to the B73 maize reference genome. The high proportion of viral RNA sequence reads compared to plant reads indicated extremely high virus titres, especially for MCMV. Total reads mapped to the virus sequence master list and maize genome per pool ranged from 94.43% to 99.27% (Table 2).
3.3 | Maize chlorotic mottle virus

Maize chlorotic mottle virus sequences were identified in each of the 20 sample pools. A total of 100 MCMV de novo assembled sequence contigs of at least 4 kb with ends trimmed according to reference sequences represented all sample pools except 3 (which had the lowest detection rate of MCMV reads), and were aligned at 99% sequence identity to obtain a single supercontig consensus sequence. Reads mapping to this MCMV consensus sequence ranged between 0.03% and 93.85% of total reads in each sample pool, showing a roughly inverse relationship with the number of reads mapping to the maize genome (Table 2), and indicating exceptionally high titres of MCMV in the sample set. Sample pools from the Eastern Province had the lowest rates (<2%) of MCMV-mapping reads, along with sample pools 6 (Gakenke district in the Northern Province), 11 (Kamonyi district in the Southern Province), and mixed samples 18, and 19 (pooled samples from a mix of locations selected primarily for ELISA-negative results for MCMV and SCMV; Table S3). From all other sample pools, including those from Kigali, Northern, Southern, and Western Provinces, >30% of reads mapped to MCMV.

To assess sequence diversity, MCMV coat protein (CP) sequences were RT-PCR amplified and Sanger sequenced from seven individual plant samples (1, 18, 43, 145, 207, 208, and 424) that were included in RNA-Seq pools. MCMV CP sequences (corresponding to nt 3,384–4,094 based on RefSeq NC_003627.1) were deposited as GenBank accession nos. MK684197–MK684203. A nonredundant collection of 13 distinct complete CP sequences identified from among the 87 de novo assembled contigs spanning the CP sequence were also analysed. These sequences were deposited as GenBank accession nos. MK684204–MK684216. These MCMV CP sequences shared 98.9%–100% nucleotide sequence identity and 99.2%–100% amino acid sequence identity. The Rwandan MCMV CP nucleotide sequences shared between 94.9%–100% identity to the entire GenBank collection of 109 additional MCMV CP nucleotide sequences accessed 2019-08-27. Of the database MCMV CP sequences, only 12 shared <98% sequence identity with any of the Rwandan sequences based on a ClustalW alignment: GenBank accession nos. JF422772 from China; AY587605 from Thailand; EU35865 from Nebraska USA; NC_003627/X14736, KS1 isolate from Kansas USA and the reference sequence; MH645620, also named MCMV.KS1 but from Kenya; MF510219–MF510222 from Ecuador; KJ782300 from Taiwan; and KFO10583 from Yunnan, China. Together, the results demonstrated very low diversity of the MCMV sequences.

3.4 | Potyviruses

A total of 4,054 de novo assembled contigs representing each of the 20 sample pools (although below the 0.01% read-mapping threshold in pool 3; Table 2) had top sequence hits to SCMV. Unlike MCMV sequences, these contigs were diverse and most were not near full-length sequences. The subset of 32 contigs that were at least 9 kb were assembled into five supercontigs and one single contig sequence at 99% sequence identity: one supercontig of 9 contigs from pools 1 and 11; another supercontig of 9 contigs from pool 2; a supercontig of 5 contigs from pool 18; a supercontig of 4 contigs from pool 13; a supercontig of 3 contigs from pool 20; and a single near-full-length contig from pool 10 (File S1). These, along with NCBI full-length SCMV sequences (SCMVH72, JX047424.1; BBJ JNO21933.1; Rwanda isolates R1, KF744392.1, R2, KF744391.1, and R3, KF744390.1; and OH isolate, JX188385.1) were used to map reads from each pool. The percentage of total reads mapping to the collection of SCMV consensus and reference sequences ranged from 0.00% (pool 3) to 36.73% (pool 6). Some reads mapped to SCMV sequences in several sample pools with no ELISA-positive samples (pools 7, 11, 12, and 19), suggesting that RNA-Seq is more sensitive and/or detected more sequence variants than ELISA.

Sugarcane mosaic virus sequence diversity was assessed by comparing seven partial sequences encoding nuclear inclusion b and coat protein (NIb/CP; nt 7,950–9,338 based on reference sequence NC_003398.1, where NiB is encoded from nt 6,837–8,399 and CP from nt 8,400–9,338). Sequences from the subset of de novo assembled contigs spanning the comparison sequence (deposited as GenBank accessions MK684190–MK684196), and Sanger sequences of RT-PCR amplicons from eight individual sample pools (18, 121, 145, 208, 338, 424, 538, and 550; deposited as GenBank accessions MK684217–MK684224; Figure 2) were compared to database sequences. The 15 Rwandan NiB/CP nucleotide sequences were 90.8%–99.4% identical to each other and as low as 65.5% identical to other SCMV nucleotide sequences (see Figure 2 for sequence comparison set). Amino acid sequences of NiB/CP were 92.1%–100% identical among the Rwandan sequence set, and shared sequence identities as low as 88.2% with other SCMV amino acid sequences compared. As observed previously for SCMV and potyviruses more generally, hypervariability was observed in the CP N-terminal sequences (Figure 3). SCMV sequences identified in this study were similar to other Rwandan and Asian isolates (Table S3). Phylogenetic analyses suggested that most of the Rwanda SCMV sequences are most closely related to, and monophyletic with, previously reported sequences from Rwanda and Ethiopia. The only outlier in our sample set (sample RWS20) grouped in another branch with sequences from China and Ecuador (Figure 2).

No potyvirus contigs in the dataset had top BLASTn hits to JGMV or MDMV. Reference sequences for each were included in read mapping samples, but no sample pool had reads mapping to either of these reference sequences above the 0.01% set detection threshold (Table 2). MDMV was not detected by RT-PCR using any of several primer sets (Table S1). However, JGMV-specific sequences were amplified from a small subset of samples including samples 338 (pool 1, Bugesera), 222 (pool 4, Kigali), 456 (pool 9, Gisagara), 158 and 466 (pool 16, Western Rubavu). Sanger-sequenced amplicons from sample 158 had 100% identity to JGMV-EA (GenBank accession no. KX897165) nt 236–648, with no BLASTn hits to other
FIGURE 2  Phylogenetic analyses of 15 sugarcane mosaic virus (SCMV) partial nuclear inclusion b and coat protein amino acid sequences from Rwanda and 39 selected references from NCBI, based on translated nt coordinates 7,950−9,338 of SCMV RefSeq NC_003398.1 and corresponding sequences from other isolates. Tree was constructed using the maximum-likelihood statistical method with WAG substitution model, 1,000 bootstrap replicates, and the neighbour-joining method for inference tree using MEGA X; bootstrap values <50 are not shown. Labels in bold indicate Rwandan sequences from this study, with contigs indicated with underscore and code number (SCMV_RwS), and others showing Sanger-sequenced amplicons (SCMV_Rw-CP).
potyvirus sequences, indicating that virus sequences below the RNA-Seq detection threshold in our pooled samples or with our 0.01% read mapping threshold could still be detected by RT-PCR.

3.5 | Maize streak virus

In contrast to all the other viruses identified, whose genomic material is RNA, MSV is a DNA virus that produces RNA transcripts during its infection cycle. MSV sequence contigs were identified by RNA sequencing in 12/20 sample pools, and were not found in pools 1–4, 7, 14–15, and 17. Reads mapped to the contig collection at or over the 0.01% threshold in 9/12 sample pools (Table 2). Reads mapping to MSV were 0.16% of all reads across the sample pools. Of the MSV contigs identified, 31 were >2 kb complete or near-complete genome sequences, and represented sample pools 5, 10, 11, 12, 13, 16, 18, and 19 and unknown (missing location barcodes post-sequencing). These 31 complete and near-complete MSV sequences from single contigs were deposited in GenBank (accession nos. MN428839–MN428869). These contigs had top hit matches with at least 97% identity to one of nine different MSV GenBank sequences, eight of which were identified in Kenya (Pande et al., 2017, and MF415878.1 direct submission) and one of which was found in Nigeria (KX787931.1). Top hits of RNA-Seq contigs >2 kb are shown in Table S5 (contigs File S2). The MSV sequences identified in Rwanda were MSV-A sequences (Martin et al., 2001) that shared high identity with Kenyan isolate sequences belonging to recombination groups XVII and V (Pande et al., 2017).

3.6 | Luteovirids

Assembled sequence contigs matching a previously described polerovirus, variously described as MYDV-RMV2 (Wang et al., 2016) maize yellow mosaic virus (MaYMV; Chen et al., 2016b) or MYDV-like (Massawe et al., 2018) were ubiquitous, identified in all RNA-Seq pools. The 659 contigs all assembled at 96% sequence identity, with the consensus sequence and individual contigs with closest matches to published complete sequences of MYDV-RMV isolate KALRO, MYDV-like EA isolate 97, and MaYMV isolate MV115 (GenBank accession nos. MH205607, MF974579, and MF684369, respectively). Only 0.01%–0.63% of reads per pool mapped back to the consensus supercontig and MYDV-like EA isolate 97 sequence, much lower than the rates for MCMV or SCMV reads (Table 2), supporting the expectation of lower titres for this frequently detected but probably phloem-limited virus. MaYMV presence was confirmed by RT-PCR in a subset of Rwandan samples, including at least one sample in each of the sample pools 6, 7, 12–14, 16–18, and 20, using primers DM03/DM04, and ORF0 was RT-PCR amplified using primers DM43/DM79 and Sanger sequenced in five samples, as described previously (Massawe et al., 2018).

In addition to polerovirus sequences, contigs most closely matching sequences of the luteovirus BYDV were identified in RNA-Seq sample pools 5 and 7. Pool 5 sequences assembled into three distinct supercontigs and two single contigs at 98%–99% identity and 100 nt overlap: the 5,660 nt supercontig (5-1; of 25 contigs) was near full-length virus sequence most closely matching BYDV-PAS isolate KS PAS-1 (GenBank accession no. KYS93456; Table S3). A 3,266 nt supercontig (5-2, assembly of 11 contigs) shared 99% nucleotide sequence identity with BYDV-PAV isolate Chk04 (GenBank accession no. KT252975) from Pakistan. A 2,458 nt supercontig (5-3, assembly of 11 contigs) shared 99% nucleotide sequence identity with BYDV-PAV isolate Chk04 (GenBank accession no. KT252975) from Pakistan. A 2,458 nt supercontig (5-3, assembly of 10 contigs) most closely matched BYDV-PAV isolate KS PAV (91% identity; GenBank accession no. KYS93458). A single contig of 814 nt from RNA-Seq pool 5 was distinct, with a top match of 92% identity to BYDV-PAV isolate 064 (GenBank accession no. EF521850); and a second single contig of 478 nt (contig 20) shared 96% identity with BYDV-PAV isolate 064 sequence. From pool 7, a 5,417 nt supercontig of 29 contigs assembled at 99% identity and most closely matched BYDV-PAV isolate 064. BYDV presence was confirmed by RT-PCR in 2/11 samples from pools 5 and 11 (one sample from each pool) using primers Lu1/Lu4 (Robertson et al., 1991).

3.7 | Maize-associated totiviruses

Maize-associated totivirus sequence contigs were found in each of the 20 RNA-Seq sample pools, with 628 de novo assembled contigs in total. Sample pool 19 was selected for totivirus Sanger sequence
validation of an alignment of 100 >2 kb contigs (MATV Rwanda 1; Figure S1) by primer walking and Sanger sequencing (sequence deposited as GenBank accession nos. MN248735; Figure S1). MATV Rwanda 1 sequence was verified in two of five samples comprising pool 19 by RT-PCR with primers designed across the genome using the contig consensus and GenBank accession KT272800 sequences as references. From all of the RNA-Seq pools, the 240 contigs >2 kb assembled at 99% sequence identity into 10 near-complete virus sequences of 4.5–5.6 kb and were named MATV Rwanda 2–11 (deposited as GenBank accession nos. MN428829–MN428838; Table S4). All had top BLASTn hits to NCBI database MATV sequences with identities of 92%–99% (Table S4). However, near full-length Rwandan MATV sequence assemblies were divergent from each other, representing at least two distinct totivirus species (Table S4). The Sanger-confirmed MATV Rwanda 1 sequence shared 98.3% nucleotide sequence identity with the reference sequence, MATV 2 isolate EC_Portoviejo (NC_030398). Similarly, MATV Rwanda 6–11 sequences shared high sequence identity (80%–99%) with the reference sequence and were represented across locations in Rwanda (Table S4). However, MATV Rwanda 2–5 were highly divergent from the reference sequence, with only 16%–19% query coverage to the reference sequence by BLASTn (Table S4). MATV MATV Rwanda 2–5 sequences were also found across Rwanda (Table S4) and shared greatest sequence identity to each other and MATV isolate 16-0130b from Tanzania (GenBank accession no. MK066243.1).

3.8 | Maize pteridovirus 1

Contigs of a putative maize pteridovirus (MPtV-1) were found in 13/20 RNA-Seq sample pools (1–4, 7–11, 14–15, and 19–20). A single near-complete 5,854 nt supercontig consensus sequence for MPtV-1 RNA 1 was assembled from 148 contigs (Figure S2). This sequence had 98% nucleotide sequence identity to the MPtV-1 isolate SSF4 partial RNA 1 sequence reported from South Sudan (GenBank accession no. MF372912) and 64% identity to a portion of RNA 1 of Japanese holly fern mottle virus (JHFMoV; GenBank accession no. FJ907327). Both JHFMoV and MPtV-1 have two reported RNAs. Although contigs for RNA 2 were not found among the RNA-Seq contigs, sequenced reads mapped to both reported MPtV-1 RNA sequences (GenBank accession nos. MF372912 and MF372913) in each of the sample pools in which contigs were identified (Table 2). The presence of MPtV-1 was confirmed by RT-PCR in each of the five RNA samples comprising pool 19 (see Figure S2). Near-complete RNA 1 and partial RNA 2 sequences confirmed by Sanger sequencing of RT-PCR products were deposited in GenBank (accession nos. MN248736 and MN428828).

These were confirmed by RT-PCR and Sanger sequencing of samples 325 and 330 (primers Table S1; Figure S3). Zea mays chrysosivirus 1 sequences were only recently reported, identified from ancient and modern maize samples (Peyambari et al., 2018). The Rwanda sequence (deposited in GenBank as accession nos. MN248737) was named Zea mays chrysosivirus 1 isolate Rwanda.

3.10 | Maize-associated betaflexivirus

Two contigs, 3,270 and 1,381 nt, were both identified from sample pool 14 (Western Province, Karongi district; Table S3; Figure S4), with significant sequence identities to betaflexiviruses. These were validated by RT-PCR and Sanger sequencing. Although the top BLASTn and tBLASTn hits of the contig sequence and translated predicted ORFs were to different virus sequences in GenBank, the Rwandan betaflexivirus sequences were found in the same pool and correspond to portions of genomic sequence and replicase that appeared to be non-overlapping. RT-PCR amplification across the contigs (primers Table S1) and Sanger sequencing identified 676 nt intervening sequence, joining the two contigs into one 5,649 nt supercontig (Figure S4). The supercontig sequence was deposited as GenBank accession no. MN714158. The nucleotide sequence was highly dissimilar from other GenBank sequences but most closely matched betaflexiviruses including CRMaV, a positive-sense RNA unclassified virus in the family Betaflexaviridae (Villamor et al., 2013), with 13% of the supercontig sequence sharing 66% nucleotide sequence identity with CRMaV (Table S3). Translations of the predicted ORFs of the maize-associated betaflexivirus (MaBV) partial sequence matched a complete 1569 amino acid betaflexavirid replicase including start codon, N-terminal methyltransferase motifs, C-terminal helix and RNA-dependent RNA polymerase motifs and stop codon, as well as 322 amino acid putative movement protein (MP) downstream (Figure S4a). The predicted translation of MaBV replicase sequence had a top tBLASTn hit to Scaevola virus A isolate WA-3 (JN127346.1) and also shared sequence identity with Actinidia seed-borne latent virus (LC438404.1). The predicted translation of the second ORF encoding a putative MP that was identified within the MaBV sequence had a top hit to carrot betaflexivirus 1 isolate CBV-1 S20 (KF533711.1; 67% query coverage with 33.6% amino acid sequence identity of the predicted translated proteins), corresponding to the N-terminal portion of its putative MP. This is the first discovery of MaBV.

4 | DISCUSSION

Maize was surveyed across Rwanda, using ELISA to detect MLN viruses MCMV and SCMV, and RNA sequencing to identify the presence and sequences of known and novel viruses. MCMV, SCMV, and coinfected plants were detected across the country. RNA sequencing identified MCMV, SCMV, MYDV-like/MaYMV,
BYDV, diverse MATV sequences, MPTV, Zea mays chrysovirus 1 and MaBV sequences, all of which were validated by RT-PCR assays. MCMV, SCMV, MaYMV, and MATVs were distributed across Rwanda and detected in all areas sampled. As previously found, MCMV sequences in Rwanda were highly conserved (Braidwood et al., 2018), while SCMV sequences were highly diverse. MSV was detected at low rates, and was not detected in samples from the Eastern Province, Kigali, or three of four districts surveyed in the Western Province. MPIV was found in less than half of the sample pools, BYDV was detected only in samples collected in the Northern Province, and betaflexivirus sequences were only detected in Karongi.

Maize lethal necrosis in East Africa is mainly attributed to coinfections of MCMV and SCMV. The rate of codetection of both MCMV and SCMV in tested Rwanda samples was low (16.1%; note that this is not necessarily reflective of overall incidence of coinfection because samples were nonrandomly selected). The lower than expected codetection rate could be due, in part, to both the lower sensitivity of ELISA compared to RT-PCR and false negatives for the antisera. A minimum rate of false negatives for SCMV of 25% was estimated by Mahuku et al. (2015a). Similar observations were made by Adams et al. (2014) when they could not detect SCMV using ELISA and RT-PCR although high-throughput sequencing indicated its presence. The specificity of ELISA, source of antisera and differences in CP sequences among SCMV isolates may be some of the reasons behind this observation (see also Mahuku et al., 2015a).

Coinfection of maize plants with MCMV and SCMV (MLN) was most common in samples selected in Northern and Western Provinces of Rwanda that were already considered MLN hotspots, and in the Southern Province. A survey by Adams et al. (2014) with limited geographic coverage had also indicated the presence of MLN in the Northern Province. Not surprisingly, RNA sequencing was more sensitive than ELISA for detection, and results indicated that ELISA-positive plants were likely to have higher virus titres measured by sequenced read mapping than ELISA-negative plants. RT-PCR assays also detected more positives than ELISA on the same samples. However, ELISA assays were given greater weight in this study because they are generally more resistant to false negatives due to small variations in sequence, and false positives due to low levels of cross-amplification or contamination to which amplification-based assays are prone. Nevertheless, surveillance programmes in the region have consistently and reliably used RT-PCR to detect the virus. We detected MCMV in other grasses tested (Napier grass, sugarcane and sorghum) while SCMV was only detected in Napier grass and sugarcane in single and coinfections with MCMV. Mahuku et al. (2015a) also detected these viruses in several non-maize grasses, including sugarcane, Johnsongrass, and Setaria spp. Although detailed studies on alternative hosts for MLN viruses are needed, the potential ability of these hosts to act as virus reservoirs further complicates MLN management in mixed farming systems characteristic of sub-Saharan Africa.

Ubiquitous in Rwandan samples was the previously reported polerovirus (Massawe et al., 2018), which has been described across the globe in recent years in surveys for MLN viruses in China (Wang et al., 2016; Chen et al., 2016b), East Africa (Mahuku et al., 2015a; Massawe et al., 2018; Wamaitha et al., 2018), Burkina Faso (Palanga et al., 2017), Nigeria (Guadie et al., 2018), Brazil (Goncalves et al., 2017), and Ecuador (Bernreiter et al., 2017). Although frequently found where MLN has created an interest in maize viruses, whether this virus in any way contributes to, moves with, or shares any but incidental colocation with MLN is not clear. However, it is frequently found in mixed infections with known MLN viruses. Most recently, it was described in South Korea in maize, without any recognized association with MLN (Lim et al., 2018). However, any symptoms and disease impact this polerovirus may have, and which vectors transmit it, remain to be reported. BYDV sequences were also identified in two Northern Province sample pools. BYDV has previously been reported in East Africa and to infect maize (Stoner, 1977).

Putative MATV sequences have been found by several groups previously, also identified during surveys for MLN viruses. Contigs identified in maize from Rwanda were diverse, with highest similarities to sequences reported in Ecuador (Alvarez-Quinto et al., 2017) and Tanzania (Read et al., 2019), and sequences deposited in GenBank from Kenya, Rwanda, Ethiopia, and South Sudan in sub-Saharan Africa, including isolates designated in MATV 1 and MATV 2 groups, as well as a third MATV 3 group. Chen et al. (2016a) first reported a 3,965 nt totivirus genome sequence from maize (MATV 1, GenBank accession no. KP984504) in Guizhou Province, China, from samples showing “leaf shrinkage”, and with phylogenetic analyses showing that it clustered in the family Totiviridae, containing mostly but not exclusively fungal-associated viruses. Because totiviruses are historically primarily associated with fungi that may be associated with plants, experimental determination of their host is challenging. To address this lack of clarity as to the host of these viruses, Chen et al. (2016a) tried to amplify fungal internal transcribed spacer (ITS) sequences without success from MATV 1-positive plants, and Alvarez-Quinto et al. (2017) cultured endophytic fungi from maize but were not able to detect totivirus in culturable fungi or to amplify fungal ITS sequences from totivirus-positive samples lacking culturable fungi. However, the biology of the host association and pathogenicity, if any, of the totiviruses for which sequences have been identified remains to be elucidated. Zea mays chrysovirus 1 is a recent discovery from ancient and modern maize samples (Peyambari et al., 2018) for which Koch’s postulates and host demonstration have likewise not yet been reported. Like totiviruses, characterized chrysosviruses are primarily mycoviruses, so definitively determining the host and potential disease biology may be challenging.

We also identified and validated the sequence of MPTV, which was broadly distributed among Rwandan samples from across the country that had very little diversity among samples and was essentially identical to a sequence in GenBank from another sub-Saharan African country (MPTV-1 isolate SSF4; GenBank accession no. MF372912 and RNA 2, designated isolate SSF5, MF372913). The next most similar virus sequence reported is JHFMoV, a bipartite virus identified by Valverde and Sabanadzovic (2009), which
causes disease in its Japanese holly fern host. As is the case for the totiviruses, whether this pteridovirus causes disease in maize remains to be determined. Finally, we reported a betaflexivirus in one sample pool in Rwanda for the first time, which we named MaBV.

Viruses that cause MLN, MCMV and SCMV, are found throughout Rwanda in samples tested, and show sequence diversity within populations that are specific to each virus, including high sequence identity for MCMV and high diversity for SCMV as found previously for neighbouring countries. In addition to these viruses, many other virus sequences are found in maize in Rwanda including MaYMV and several other viruses with unknown biology and impact. These viruses represent broad diversity among and within virus species. Together, these findings provide comprehensive data on distribution of known MLN-associated viruses in Rwanda, as well as comprehensive sequence data on other maize-associated viruses and virus populations across all provinces of Rwanda, which will be valuable in managing disease and formulating future experiments to understand and manage viral diseases including MLN.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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