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Final Report

Identification and management of mosaic viruses and secondary pathogens in buffalo turf

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Identification and management of mosaic viruses and secondary pathogens in buffalo turf (TU19000)

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Content

Summary

Buffalo grass yellowing is an enigmatic disease that is causing great concern to turf farmers in Australia, particularly in the states of New South Wales and Queensland. Many control methods for this disease have been tested by farmers but with no industry-wide solutions found. In this project, we hypothesized that plant viruses, particularly panicum mosaic virus (PMV) and sugarcane mosaic virus (SCMV), were responsible for at least some of the yellowing symptoms but experiments were also done to search for any previously unrecognized viruses. The results of disease surveys on 27 farms in New South Wales, Queensland and Western Australia are described in this report.

The most damaging and widespread of the viral pathogens that was detected in buffalo grass was SCMV, which was present in all three states that were visited but was most prominent in New South Wales. SCMV was found on all seven farms that were surveyed in the Hawkesbury Valley and on two out of three farms in the Hunter Valley. In some paddocks, the incidence of SCMV infection approached 100% and all major buffalo grass cultivars were affected including 'Sir Walter', 'Sapphire', 'Palmetto' and 'Prestige'. Symptoms of infection were obvious, with strong leaf mosaic patterns, narrower leaf blades and shortened internodes observed. In the phylogenetic study, all SCMV isolates were related to an 'East African clade', containing maize virus isolates from Kenya and Tanzania, as well blue couch (*Digitaria didactyla*) and Sabi grass (*Urochloa mosambicensis*) virus isolates that were collected in Queensland. Furthermore, the phylogenetic analysis suggested multiple introductions of SCMV into buffalo grass from some of these alternative hosts rather than a single introduction and then dispersal through trade of infected turf. Diagnostic tests were also done for Bermuda grass latent virus (BGLV), which causes viral lethal necrosis of buffalo grass in Florida when present as a mixed infection with SCMV. While BGLV was not detected in buffalo grass, it was found in a green couch (*Cynodon dactylon*) plant collected from a turf farm in South East Queensland. This Is the first time BGLV has been recorded outside of the USA and demonstrates that the two agents responsible for lethal necrosis are present here in Australia and pose a threat to the buffalo grass turf industry.

PMV had a much more restricted distribution than SCMV on turf farms in Australia, as it was only found in cv. 'Palmetto' in the Hawkesbury Valley but in affected paddocks, was at a very high incidence. Panicum papanivirus 1, a satellite virus commonly associated with PMV in the USA, was not detected. Phylogenetic analyses suggested just the single introduction of PMV into Australia, and localized dispersal within the Hawkesbury Valley. Given the restricted distribution of this virus in Australia, eradication of PMV from Australian turf farms would be feasible and desirable in order to prevent St Augustinegrass Decline.

Virtually all farmers were unaware of the presence of plant viruses in their turf and had not implemented any management actions to control these pathogens. Although some farmers implemented some crop hygiene practices, mainly to prevent cross-contamination of turf varieties, none of these practices were adequate to prevent spread of plant viruses. As a consequence of a positive identification of SCMV on a turf farm in Western Australia, one farmer took immediate actions to eradicate the virus by treatment of the paddock with a herbicide.

Many cases of buffalo grass yellowing could not be attributed to viral infection and plants affected by this type of yellowing had poor root health. Fungi were isolated from the decaying roots and the most commonly recovered species were *Curvularia*. A causal relationship between the presence of this fungal pathogen and symptoms has not yet been demonstrated, although it should be considered a prime candidate for causing the poor root health. Interestingly, environmental factors that have previously been demonstrated to favour epidemics of *Curvularia* spp. are the overuse of nitrogen fertilizers and the build-up of thatch and grass clippings and many farmers commented that they thought that these factors were associated with the onset of buffalo grass yellows.

Yellowing is one of the commonest disease symptoms observed in grasses, and can be caused by a range of pathogens, as well as abiotic factors such as plant nutrition and water stress. It is therefore not surprising that the buffalo grass yellowing syndrome in Australia appears to have multiple independent causes, including plant viral and fungal pathogens and trace element deficiencies. Recommendations for future research to develop integrated disease management strategies are provided.

Keywords

Buffalo grass, turf grass, yellowing, sugarcane mosaic virus, panicum mosaic virus, nematodes, *Curvularia*, root rot, nutrition deficiency, disease management.

List of Abbreviations

BLAST – Basic local alignment search tool

BGLV – Bermuda grass latent virus

cv. – cultivar

DNA – Deoxyribonucleic acid

ELISA – Enzyme-linked immunosorbent assay

ITS – Internal transcribed spacer

JGMV – Johnsongrass mosaic virus

NSW – New South Wales

NGS – Next–generation sequencing

PCR – Polymerase chain reaction

PDA – Potato dextrose agar

PMV – Panicum mosaic virus

PRG – Project reference group

PPV1 – Panicum papanivirus 1QLD – Queensland

RT-PCR – Reverse transcription PCR

RdRp – RNA-dependent RNA polymerase

RNA – Ribonucleic acid

SCMV – Sugarcane mosaic virus

USA – United States of America

WA – Western Australia

Introduction

Stenotaphrum secundatum (buffalo grass or St. Augustine grass) is a hardwearing and vigorous warm season turfgrass species, thought to be endemic to the Atlantic coasts of the Americas and Africa (Busey, 2003; Sauer, 1972). It is either the most or second-most important turf species in Australia, depending on the ranking criterion used, value or volume produced (Hort Innovation Project TU17005, Turf Industry Statistics). The most significant diseases affecting buffalo grass in Australia are fungal diseases such as grey leaf spot (*Pyricularia* spp.), dollar spot (*Bipolaris* spp.), curvularia spot (*Curvularia* spp.), brown patch (*Rhizoctonia solani*) (Smiley et al., 1992) and 'wongoonoo patch' (*Gaeumannomyces wongoonoo*) (Wong, 2002). Management of fungal diseases can be obtained by, for example, cultural practices and application of fungicides and sometimes by just waiting for the weather to change to less favourable conditions for pathogen growth. In Australia, plant viruses have been reported to occur in various turf species but as they have not been regarded as major limiting factors to production, their presence has largely been ignored.

In Australia and the USA, panicum mosaic virus (PMV) and sugarcane mosaic virus (SCMV) are the two most common viruses that have been found in buffalo grass. In the USA, PMV is the most important of the two viruses and causes St. Augustine Decline (Cabrera & Scholthof, 1999). Plants can be infected by PMV alone or in combination with Panicum papanivirus 1 (PPV1), a virus known to increase the severity of symptoms (Cabrera & Scholthof, 1999). Plants infected with PMV at first have chlorotic mottling and stippling of the leaves but these symptoms progressively become worse, ending in generalised chlorosis that resembles iron or nitrogen deficiency. The plants also lose vigour and the turf becomes thinner, allowing invasion by weeds and other more vigorous turf species such as green couch. Infection of PMV can lead to plant death. There are no chemical cures for PMV infection and the best long-term control strategy is use of resistant varieties such as Floratam, Floralawn, Raleigh and Seville (Reinert et al., 1980).

Sugarcane mosaic virus is known as a pathogen of sugarcane and maize (Kuntze et al., 1997; Viswanathan et al., 2009), and for much of recorded history was only found in buffalo grass in the sugarcane-growing region of Florida (rural Palm Beach County) (Todd et al., 1964), where it caused mild mottling of the leaf and was not a great concern. However, in 2013, there was an outbreak of a much more virulent strain of the virus in neighborhoods of St. Petersburg, Florida, which led to leaf mosaic patterns, severe dieback and completely killed some infected lawns (Harmon, 2014; Harmon et al., 2015). More recent evidence suggests that this lethal necrosis disease is due to a mixed infection of SCMV and Bermudagrass latent virus (BGLV; P. Harmon pers. comm.). As the name suggests, BGLV was first found in an asymptomatic Bermudagrass (*Cynodon dactylon*) plant and thus far this virus has only been found in the USA (Tahir et al., 2017). Use of tolerant varieties such as 'Palmetto' and 'Bitterblue' and sanitation of equipment when mowing multiple lawns is recommended for disease control. Although SCMV is transmitted by aphids, the use of insecticides is not recommended as the aphids can transmit the virus in a matter of seconds, before the insecticide has a chance to kill the aphid, and the transmission is mostly by migratory, winged forms of the aphid, not those colonizing the plant. SCMV does have alternative grass hosts, but the host range varies according to the particular strain of the virus.

Johnsongrass mosaic virus (JGMV) has also been reported as a problem in Australia and not the USA, even though JGMV is found in the USA in other grass hosts. JGMV is genetically very closely related to SCMV, has an almost identical biology, and for years before the adoption of nucleic acid sequencing technologies, was considered a strain of the latter. The record of JGMV in buffalo grass was from a single diagnostic specimen submitted to NSW Agriculture and Fisheries in about 1990 from Windsor, NSW (Pares & Gillings, 1990). The diagnosis was based primarily on immunosorbent electron microscopy, with many more virus particles being trapped using anti-JGMV compared to anti-SCMV antibodies. However, nucleic sequencing does need to be done to confirm this identification, which would be possible as the disease specimen was archived in the NSW Plant Disease Herbarium.

During a preliminary survey of farms in the Hawkesbury Valley by the project team in April 2019, buffalo grass yellowing was found common and was widely reported by the farmers to have been very severe in the previous summer. We observed symptoms typical of virus infection and the infected 'Palmetto' tested positive for PMV, whereas 'Sir Walter', 'Matilda', 'Sapphire' and 'Shademaster' tested positive for SCMV. We also found a different type of symptom, a striate mosaic pattern, on 'King's Pride' and 'Sapphire' in the Hawkesbury Valley, suggestive of a virus infection but the causal agent remains unknown. In many cases, poor root health and leaf spot were found associated with the yellowing symptoms, and these may be caused by fungal pathogens that remain to be investigated.

At present, while there is a need for management options of buffalo grass yellowing, information on cause(s) of the problem, biology and epidemiology of the diseases is limited, hampering the development of effective disease

control strategies. This project was a scoping study primarily aimed at investigating the distribution and incidence of PMV and SCMV on turf farms around Australia but also to collect anecdotal information on factors affecting the severity of buffalo grass yellowing and strategies being used by the farmers to mitigate the disease. Additionally, plants with buffalo grass yellowing were tested for the presence of other potential pathogens including nematodes, soil-borne fungi and unspecified viruses.

Methodology

Formation of project reference group

In consultation with Hort Innovation, a project reference group (PRG) was formed, comprising Ms Jenny Zadro from Turf Australia, Mr John Keleher from Australian Lawn Concepts in Queensland and Mr Neale Tweedie from Atlas Turf in NSW. The PGR assisted the project team in identifying and contacting turf growers to arrange surveys, and provided advice and support to meet project objectives.

Farm surveys and sample collection

Surveys were undertaken on ten turf farms in the Hawkesbury and Hunter Valleys of New South Wales, eight farms in the Sunshine and Gold Coast hinterlands in South East Queensland, and nine farms in the coastal district from Perth to Busselton, Western Australia. Surveys in Victoria were cancelled due to travel restrictions relating to the coronavirus pandemic. Each farm visit was 90-120 min in duration, and three to four farms were surveyed each day depending on travel times. Paddocks of buffalo grass were visually inspected for yellowing symptoms and symptoms of virus infections. The incidence of virus infection in each paddock was estimated based on an assessment of symptoms. Weedy grasses that were either growing as a contaminant of the turf or along the edges of the paddocks were also inspected for virus-like symptoms. A total of 138 samples were collected for analysis in the laboratory (40 samples from Queensland, 65 from NSW and 33 from WA). Living sods of grass were also collected and grown in the glasshouse to monitor symptom expression, as symptoms were not always unequivocal in the field.

At each farm, the turf growers were interviewed using a questionnaire (Appendix 2) to obtain their opinions on environmental and seasonal variation in the severity of buffalo grass yellowing, and management practices used to treat the disease.

ELISA and reverse transcription PCR

To detect PMV and SCMV, commercially available ELISA kits (Creative Diagnostics, Shirley, NY 11967, USA) were used following the manufacturer's instructions, except reaction volumes were reduced to 50 µl. To confirm the ELISA results, representative samples from each farm were also tested by reverse transcriptasePCR (RT-PCR) using primers PMV-F1/PMV-R1 specific to PMV and SCMV-F1/SCMV-R1 specific to SCMV developed by Geering et al. (2004).

To detect BGLV, a universal panicovirus RT-PCR assay was designed by aligning the RdRp gene sequences of all representatives of the virus genus, from which the following primers were chosen: PanicoRdRp-F2 (5' TGCGCWATWGGATTYGAYGC 3') and PanicoRdRp-R1 (5' CCTCATCATCCWCCGAGCA 3').

A RT-PCR diagnostic assay was also developed to detect the presence of a novel virus detected from next-generation sequencing, provisionally named grass nepovirus, in all samples collected. This assay uses the primer pair: Grassnepo-F1 (5' GAGACTATGCGTCATTTGATG 3') and Grassnepo-R1 (5 ' CAGCGATTAAATTATCATCTCC 3')/

All RT-PCR assays were done with a MyTaq One-Step RT-PCR kit (Bioline, Alexandria NSW, Australia) as per the manufacturer's instructions, except reaction volumes were reduced to 25 µl.

Next-generation sequencing (NGS)

To detect unrecognized viruses in buffalo grass plants with different symptom types and to obtain full genome sequences of the viruses, NGS was done. The total RNA population from a plant was extracted using a Trizol Plus RNA Purification kit (Catalog number: 12183555, Invitrogen, Carlsbad, CA 92088) as per the manufacturer's instructions. To remove DNA, the total RNA was treated with DNase I (New England Biolabs, MA) following the protocol of Asif et al. (2000) except that the polysaccharide removal step was omitted. The total RNA extracts were sent to the Australian Genome Research Facility (AGRF) for library preparation and 150-bp paired-end sequencing on an Illumina MiSeq sequencer with a 300 cycle kit. Prior to library construction, ribosomal RNA was removed using a Truseq Plant depletion protocol (Illumina) with 500 ng of input RNA. The sequence reads obtained were paired

and adapter and primer sequences removed from the obtained reads using the BBDuK Geneious plugin of Geneious Prime v. 2020.0.5 (Biomatters). After trimming for quality, *de novo* assembly was done using CLC Genomics Workbench 6.5 (CLC bio). The contigs produced after *de novo* assembly were sorted by length and matched against the sequences in the international nucleotide sequence databases using BLAST (Johnson et al., 2008). In Geneious, the whole-genome sequences of each virus were obtained by mapping the contigs that matched the virus from BLAST search to the reference genomes obtained from the NCBI Nucleotide database.

Phylogenetic analyses were done using the Maximum Likelihood method with 1000 bootstrap replicates implemented in MEGA X, and uncorrected pairwise nucleotide distances calculated with the same program (Kumar et al., 2018).

Fungal isolations

Fungal isolations were done by plating out diseased roots and leaves of buffalo grass plants showing yellowing on half-strength potato dextrose agar (½ PDA) following surface sterilisation of the samples by immersion in 70% ethanol for 30 s and blotting dry. Hyphal tips of fungi that grew were then sub-cultured on fresh ½ PDA plates to obtain pure cultures for morphological examination and molecular analysis.

Total genomic DNA (gDNA) was extracted using the Wizard Genomic DNA purification kit (Promega, Madison WI, USA) following manufacturer's instructions for DNA barcoding. DNA concentrations were determined using a DUO spectrophotometer (BioDrop, Cambridge, UK) and diluted using MilliQ water to a final concentration of 15 to 30 ng/µL prior to PCR amplifications. The internal transcribed spacer (ITS) region was amplified using the ITS1/ITS4 primers (White et al., 1990) and sequenced to identify the fungi to species level. PCR was performed in 20-µL reactions, each containing approximately 30 ng of gDNA (QIAGEN nuclease-free water served as a non-template control) and 4.0 µL of 5×MyTaq Red reaction buffer (Bioline, Alexandria NSW, Australia). The following cycling conditions were used: 2 min at 95 °C; 35 cycles of 95 °C for 20 s, 58 °C for 20 s and 72 °C for 30 s each; followed by a final extension at 72 °C for 5 min. PCR products were size-fractionated using 1.5% (wt/vol) agarose gels in 0.5× TBE buffer to examine DNA quality and size. PCR products were purified and directly sequenced, using the same primers used for PCR, at Macrogen Incorporated (South Korea) using an AB 3730xl DNA Analyzer (Applied Biosystems, Foster City CA, USA).

All the sequences were assembled and aligned with selected sequences obtained from GenBank (Appendix 1, Table 2) using Geneious Prime. Phylogenetic analyses were performed using Maximum Likelihood method with 1000 bootstrap replicates using MEGA X (Kumar et al., 2018).

Nematode isolations

Root and soil samples were collected from patches of turf with yellowing, and as a negative control, from apparently healthy turf growing within close vicinity of the diseased turf (< 5 m distance). When collecting the sample, an *c*. 5 cm diameter circle of turf was dug out to a depth of *c*. 10 cm and stolons, roots and soil placed in a ziplock bag and kept at ambient temperature. Nematode extractions and identifications were done by Ms Jenny Cobon and Mr Tim Shuey, plant nematologists at the Queensland Department of Agriculture and Fisheries. To extract nematodes from the soil, two hundred mL of soil from each sample was set up in a Whitehead tray over 3 days. The resulting solutions were sieved using a 38 µm sieve and then examined under the microscope for the presence of all plant-parasitic nematodes.

Outputs

Surveys for PMV and SCMV

New South Wales

SCMV was found on nine of the ten turf farms that were surveyed in NSW, both in the Hawkesbury and Hunter Valleys, and the incidence of infection approached 100% in some paddocks (Appendix 1, Figs 1 and 3). The effects of the virus were obvious, with infected plants having a yellower colour, narrower leaf blades and shorter internodes. Infected plants were most obvious at the earlier stages of re-growth of the paddock after it had been cut, when there was a strong contrast in the habit, colour and rate of growth of infected compared to healthy stolons. In some paddocks, infected plants occurred in strips, suggesting transmission by mowing (Appendix 1, Fig. 1).

All commonly grown buffalo grass cultivars in NSW were naturally infected by SCMV, including 'Sir Walter', 'Sapphire', 'Palmetto' and 'Prestige' (Appendix 1, Table 3). SCMV was also found in one crabgrass (*Digitaria*

sanguinalis) plant growing as a contaminant in a paddock of 'Sir Walter' in the Hawkesbury Valley.

PMV was only found in cv. 'Palmetto' on farms in the Hawkesbury Valley, although none of the farms surveyed in the Hunter Valley grew this cultivar. The symptoms of PMV were less severe than SCMV but nevertheless it caused some yellowing. A weedy crabgrass (*D. sanguinalis*) plant that was growing within a diseased sward of 'Palmetto' also tested positive for PMV.

Queensland

The majority of turf farms in south-east Queensland did not have any signs of viral disease, except for one farm in the Gold Coast hinterland. On this farm, a single paddock of cv. 'Prestige' was identified that had an estimated incidence of about 50% infection with SCMV (Appendix 1, Table 3). Infected plants had large, chlorotic, spindle-shaped lesions on the leaves, which was different to the mosaic symptom more commonly observed in NSW (Appendix 1, Fig. 3). Unusually, SCMV was not found in the paddocks of cv. 'Sir Walter', which was the main cultivar grown on the affected farm.

Despite the consistent association of PMV with cv. 'Palmetto' in New South Wales, all three farms growing this cultivar in south-east Queensland were free from this virus and represent a clean source of the turf for replanting in the Hawkesbury Valley (Appendix 1, Table 3).

Western Australia

The health status of buffalo grass in Western Australia was generally very high, the best that was observed throughout Australia. Root health was very good and virtually no leaf spots were observed. Most cases of yellowing appeared related to nutrient deficiency or the use of alkaline bore water for irrigation (Appendix 1, Fig. 4). The farmers reported that yellowing was induced when the grass was scalped. Most farmers stated that the yellowing could be reversed within 48 hours of appearance through use of soluble iron and manganese fertilizers. However, on one farm, strong virus-like symptoms were observed on the edge of a paddock of 'Palmetto' (>80% infection) and the plants tested positive for SCMV. The grower was aware and very concerned about the problem but had received an incorrect diagnosis from a local agronomist. This part of the paddock has now been sprayed-out with herbicide by the grower. Surprisingly, no evidence of infection was found in paddocks of 'Sir Walter' on the same farm.

A single, SCMV-infected plant was found on a second farm that was about 50 km straight-line distance from the first affected farm but no other evidence of infection was observed, despite extensive searching. Overall, the outbreak of SCMV on buffalo grass in Western Australia was very localized, suggesting a recent incursion of the virus.

SCMV was found in a paddock of Queensland blue couch (*D. didactyla*) on the only farm that was growing this turf species, which was an unsurprising result as the blue couch strain of SCMV is ubiquitous within this species. This turf farm was about 48 km straight-line distance from the farm with the infected 'Palmetto' and research is ongoing to test the hypothesis of cross-species transmission between blue couch and buffalo grass.

Surveys for Bermuda grass latent virus (BGLV)

Buffalo grass lethal necrosis in Florida is caused by a mixed infection of SCMV and BGLV, and therefore testing was done to determine whether a comparable situation existed in Australia. Like PMV, BGLV is a member of the genus *Panicovirus* and we therefore developed a universal panicovirus RT-PCR assay that was able to detect both viruses, as well as the two other representatives of this genus of viruses. RNA extracts that were previously prepared for screening for SCMV and PMV were retested using the universal panicovirus RT-PCR assay, and amplicons were sequenced to provide identifications. Of the 105 buffalo grass samples that were tested, four 'Palmetto' grass samples were positive for PMV, as already established using ELISA. A sample of common green couch (*Cynodon dactylon*), growing as a weed by the side of a track at a second turf farm in south-east Queensland, was also positive using the universal panicovirus PCR assay but negative for PMV by ELISA, suggesting the presence of a new panicovirus. The virus was identified to be BGLV by sequencing of the PCR amplicon. This detection of BGLV represents the first record of this virus in Australia and demonstrates that the two agents that cause viral lethal necrosis of buffalo grass in Florida are here too.

Next-generation sequencing

A range of disease samples were selected for next generation sequencing with the dual purposes of discovering if any unrecognized viruses were infecting the plants, as well as assembling the genome sequences of Australian isolates of SCMV and PMV. Samples were chosen to represent a range of buffalo grass cultivars, farm locations and

different symptom types. For comparative purposes, symptomatic blue couch (*D. didactyla*) and Sabi grass (*U. mosambicensis*) plants, which are known hosts of SCMV along the east coast of Australia, were also sampled for sequencing.

Six near complete genome sequences of SCMV, each 9,520-9,803 nucleotides long, were obtained from three buffalo grass samples and one sample each of the other two grass species: one 'Sir Walter' sample contained two distinct virus sequences, which were 92.7 % identical to each other over the entire length of the genome sequence. A phylogenetic analysis using complete genome sequences (Appendix 1, Fig. 6) resolved all SCMV isolates obtained from the present study into one separate clade, except the 'Palmetto' isolate, which clustered with a maize isolate of SCMV from the USA (GenBank number: JX188385). A phylogenetic tree that was generated using the coat protein gene had a similar topology (Appendix 1, Fig. 7).

Interestingly, the three isolates of SCMV from 'Sir Walter' were not each other's closest relatives but one was more closely related to the isolate from blue couch, and the second, more closely related to the Sabi grass isolate (Appendix 1, Fig. 6). This result suggests separate introductions of SCMV into 'Sir Walter' from alternative hosts, rather than a single introduction and then dispersal of the virus by trade of turf.

Two near complete genome sequences of PMV of 3,999 and 4,018 nucleotide longs were obtained from two 'Palmetto' samples. We were able to construct a phylogenetic tree of PMV (Appendix 1, Fig. 8) using sequences of the partial coat protein gene obtained from Sanger sequencing. Comparisons with the buffalo grass strain of PMV from the USA were not possible, as there are no available sequences for this virus in the international nucleotide sequence databases. However, the virus isolates from Australia were relatively distantly related to the switchgrass isolates of PMV from the USA. No sequences corresponding to Panicum papanivirus 1 (PPV1), a satellite virus that is commonly associated with PMV infections of buffalo grass in the USA, were detected.

A putative new virus was identified in a sample of 'Sir Walter' from the Hawkesbury Valley (isolate 20200123nt-9), which was also infected with SCMV. The virus appears to have a bipartite genome, as two RNA segments were assembled (6,424 and 7,100 nucleotides long), one encoding a homologue of the RNA-dependent RNA polymerase (RdRp) protein, and the second, a homologue of the coat protein. When BLAST searches of the NCBI Nucleotide Database were done using the first of these RNA sequences, highly significant matches were obtained with members of the genus *Nepovirus*. The virus has been provisionally named Stenotaphrum spherical virus. Using the RT-PCR assay developed in the present study, Stenotaphrum spherical virus was detected in 13 turf farms in NSW, QLD and WA in several cultivars, including Sir Walter, Palmetto, Prestige, Kings Pride, Matilda, Neergabby, and Shademaster.

Fungi and nematodes associated with buffalo yellowing

In both NSW and QLD, many cases of buffalo grass yellowing were observed where the disease symptoms could not be attributed to infection by viruses. Plants showing these symptoms had very poor root health: the stolon and root mat was very weak and extensive root necrosis was observed (Appendix 1, Fig. 5). Experiments were done to investigate the cause of this root rotting, focusing on fungi and nematodes, which were considered the likely causes.

The most consistently isolated fungi from the rotting buffalo grass roots were *Curvularia* spp. (Appendix 1, Table 1). It is not possible to accurately identify this group of fungi using morphological features, and therefore DNA barcoding was done using the internally transcribed spacer of the ribosomal DNA, which is the universal DNA barcode for fungi. In a phylogenetic analysis, all *Curvularia* isolates grouped together in a well-supported clade representing a previously undescribed species, which is most closely related to *Curvularia ryleyi* (Appendix 1, Fig. 9). In the older scientific literature, the new *Curvularia* species would have been referred to as undescribed *Drechslera* species.

Curvularia/*Drechslera* spp. are known to be pathogens of turgrasses and cause various types of symptom, including root and crown rot, leaf spot and net blotch (Smiley et al., 1992). *Curvularia*/*Drechslera* spp. are active almost all year round, except when the weather is continuously cold or hot. Roots, rhizomes, lower stems and leaf can be infected. The disease is favored by prolonged periods of leaf wetness, mowing lawn lower than is recommended, excessive use of nitrogen fertilizers and thatch build up, which provides the food source for growth and reproduction of the pathogen (Smiley et al., 1992). Many of these risk factors matched conditions that were reported by farmers to coincide with buffalo grass yellowing.

Fusarium spp. were also isolated from the rotting buffalo grass roots but the correlation between the presence of this group of fungi and buffalo grass yellowing was weak, and unlikely to be significant (Appendix 1, Table 1). *Fusarium* spp. are common endophytes of grasses, and components of the normal microflora.

All fungi have been deposited in the Plant Pathology Herbarium, Brisbane (BRIP) as live cultures and are retrievable for future studies.

Nematodes were extracted from soil cores at sites of buffalo grass yellowing and reniform (*Rotylenchulus* sp.), stunt (*Tylenchorhynchus* sp.), stubby (*Paratrichodorus* sp.) and ring nematodes (*Criconemella* sp.) found (Appendix 3). These nematode species are low to moderately pathogenic, except stubby nematode, which is highly pathogenic but it was at a density well below the threshold to cause damage. Soil cores were taken from apparently healthy sites and often the same nematode species were found, sometimes in higher numbers, which can reflect the greater root mass of healthy plant and therefore the larger food source for the nematodes. Overall, there were no consistent correlations between the presence of a nematode species and buffalo grass yellowing.

Individualized reports of the diagnostic results have been sent to growers on whose farms the surveys were undertaken, or samples were submitted for testing. An example of diagnostic report is attached (Appendix 4).

Impact of buffalo grass yellowing

Turf growers reported great concerns that when buffalo grass was present, significant impact was observed. In severe cases, turf was not harvestable or saleable leading to complete loss (Appendix 1. Table 3).

Epidemiology of buffalo grass yellowing

During field surveys, anecdotal information on buffalo grass yellowing was collected from interviews with farmers (Appendix 1, Table 3), many of whom reported that yellowing has been around for a long time but has become more noticeable in the last few years, while others observed no change or decreasing incidence. Some farmers reported a positive correlation between humidity and incidence of yellowing but one farmer reported that rain reduced yellowing. The differences in observed epidemiology were likely because buffalo grass yellowing has several independent causes.

Existing management practices of buffalo yellowing

Most farmers we visited in New South Wales and Queensland had experienced buffalo grass yellowing at some time and experimented with methods to mitigate the symptoms with varying degrees of success (Appendix 1, Table 3). In New South Wales, one turf industry leader recognized two types of yellowing, one caused by viruses and the other from thatch build-up, and the viral problem was considered the more serious of the two. However, in general, the farmers did not differentiate different types of yellowing. A common theme among farmers in both states was that excessive nitrogen exacerbated the yellowing problem, and minimizing the use of urea or chicken manure helped control the yellowing problem. One farmer in Queensland who had success controlling buffalo grass yellowing had completely eliminated poultry manure top dressing as a management practice, and was only applying nitrogen as needed according to the results of a routine soil-testing program.

Some management practices applied by the growers in New South Wales were:

I. Metam sodium was used on two farms to fumigate the soil but yellowing returned after 12 months. The yellowing on this farm was associated with poor root health, not virus infection, and the rapid return of yellowing suggests that an aerially dispersed pathogen may be involved in the disease syndrome.
II. One farmer used a Charlie Carp-like soluble fertilizer with mancozeb and noticed mitigation of yellowing symptoms. Another farmer also reported Bumper (propiconazole) to reduce the problem. However, many others used fungicide (e.g. mancozeb) without effect.
III. One farmer applied Gypsum and found it reduced but did not completely get rid of the problem.
IV. Aeration was reported to be effective by one farmer.
V. One farmer incorporated Caliente mustard (a commercial blend of *Brassica juncea* and *Sinapsis alba*) as a green manure and replanted after each harvest. Using this practice, the farmer said that he had successfully controlled yellowing.
VI. One farmer reported benefits from dyeing the grass, long after coloration from the dye had faded.
VII. One farmer replanted with new turf after each harvest and noticed improvement in yellowing.

VIII. One farmer mentioned that yellowing was less of a problem in 'Palmetto'.

In Queensland, the following information was collected:

I. It was observed that yellowing started from the old turf ribbons left after harvest, forming parallel lines of symptoms. Several farmers had changed to replanting after harvest using shredded stolons, and cultivating the soil prior to replanting.
II. One farmer suggested that yellowing occurred from superheating of grass under grass clippings, and therefore, paddocks need to be swept or vacuumed.
III. Fungicides such as mancozeb were used but without effect in controlling yellowing.
IV. One farmer reported positive results from spraying for mites - leftover miticide used on a green couch paddock was sprayed onto buffalo grass as an 'experiment'.

In Western Australia, yellowing occurred but was considered a minor problem as the grass returned to a normal green colour within 48 hours of application of soluble iron and manganese fertilizers. Yellowing coincided with dry periods when alkaline bore water was used to irrigate turf, hence the yellowing was worst in summer and disappeared in winter when natural rainfall occurred. Yellowing occurred when the turf was inadvertently scalped but this yellowing could be treated with fertilizers. Overall, the farmers rated buffalo grass yellowing as a minor problem in Western Australia.

Seminars and papers

1. Tran NT, Teo AC, Thomas JE, Crew KS, Geering ADW (2020) Sugarcane mosaic virus infects *Stenotaphrum secundatum* in Australia. Australas Plant Dis Notes 15: 41.
2. A seminar describing the results of this project was presented to the Turf Industry SIAP on Friday 3 June 2020.
3. Geering ADW and Tran N (2020) Project update: understanding buffalo yellowing. Turf Australia Official Industry Magazine – Winter 2020, pp. 10-12.
4. Geering ADW and Tran N (2020) Preliminary results of buffalo yellowing scoping study. Turf New South Wales Newsletter June 2020, pp. 5-6.
5. Andrew Geering was invited to participate in a Turf New South Wales information day, but this event has now been postponed twice due to concerns over COVID-19.

Outcomes

For only a short scoping study, a lot of experimentation has been done and a large area of the country covered as part of the surveys. The project has demonstrated buffalo yellowing is a disease complex with many contributing factors, and three types of yellowing were observed, one associated with virus infections, the second associated with poor root health, and the third, a nutritional disorder. The first two types of yellowing were most prominent on the east coast of Australia, and this is where greatest concerns were held over the disease syndrome. There have been many outcomes from this project:

1) Baseline data on the types and distribution of plant viruses in buffalo grass in Australia has been provided, which will allow the development of strong and scientifically justifiable biosecurity policies at both national and state levels.
2) Two alternative diagnostic technologies, ELISA and RT-PCR have been either adopted or developed and importantly validated using local virus isolates for two of the most important viral pathogens of buffalo grass in the world. A rapid and accurate virus diagnostic service is available to the turf industry for the first time.
3) The genomes of several isolates of SCMV and PMV from turf grasses have been sequenced, which will allow the design of better diagnostic assays and provide information on the epidemiology of the viruses such as pathways of spread.
4) Bermuda grass latent virus has been detected in Australia for the first time, a pathogen that could pose a significant threat to the industry by acting synergistically with SCMV to cause lethal necrosis of buffalo grass.
5) A new nepovirus has been discovered, which may be an independent cause of disease in buffalo grass. Derivation of the genome sequence for this virus will allow diagnostic assays to be designed, which in turn will allow the distribution and epidemiology of this virus to be investigated.
6) Evidence has been provided as to the causes of buffalo grass yellowing, which will allow the development of a much more focused research program to develop an integrated disease management program. Likely

causes of buffalo grass yellowing have been identified, and conversely, organisms such as nematodes discounted.

7) Epidemics of SCMV have been identified at an early stage in Queensland and Western Australia, which will substantially improve prospects that the virus can be either eradicated from these farms or its spread minimized in these regions.

Monitoring and evaluation

1. The project has made significant progress in understanding the buffalo grass yellowing syndrome on Australian turf farms, and provided directions for future research to more accurately identify the causes of the disease and to develop an integrated disease management plan.
2. Diagnostic services have been provided free-of-charge on samples collected during field surveys or sent in by the growers who missed out on the surveys. Individualized diagnostic reports have sent to farmers, allowing immediate responses in some cases. For example, a farmer in Western Australia has already acted to eradicate SCMV on his farm to prevent the virus becoming more widespread.
3. The project has over-delivered on the number of farms that were surveyed in three states, New South Wales, Queensland and Western Australia, before the COVID-19 pandemic. Field surveys to Victoria were curtailed because of COVID-19 travel restrictions.
4. The project has also over-delivered on investigating alternative causes of buffalo grass yellowing, such exploring the role of fungi and nematodes.

Recommendations

Management practices

Buffalo grass yellowing has several independent causes and it is essential that farmers obtain a proper diagnosis.

Viruses

There is no cure for plant viruses and containment and eradication must be the focus of management.

To eradicate the viruses, it is necessary to spray out infected plants with herbicides such as glyphosate. It is important to completely kill the plant as the virus is present in all plant parts, including underground rhizomes, and if regrowth occurs, then the virus will reappear.

PMV can survive for limited times in decaying plant material. After a paddock is sprayed out, it should be cultivated to speed up the decay of the dead plants and the paddock rested for a few months.

A paddock should be replanted with turf that is certified to be free of plant viruses.

To prevent reintroduction of plant viruses, it is important that mower blades are decontaminated using at least high-pressure water hoses to dilute inoculum to below levels that cause infection. However, the virus is only completely inactivated through use of 1% commercial bleach.

To minimize spread of the viruses, the turf should be mowed only when dry to minimize sap-contamination of the mower blades.

Fungi

No specific fungicides can be recommended at this stage without further research.

Several management practices were observed to improve root health and therefore yellowing symptoms, including cultivating the paddock after harvest, green manuring with Caliente mustard mixes, and replanting with shredded stolons rather than allowing regrowth from old turf ribbons. Increasing carbon:nitrogen ratios in the soil will likely reduce buffalo grass yellowing caused by fungal pathogens and have other benefits for soil health. Avoidance of poultry manures and reducing nitrogen applications will also likely prevent the build-up of fungal pathogens to damaging levels.

Abiotic factors

For yellowing caused by abiotic factors, it is suggested that soil, plant and/or irrigation water tests be done to

identify deficiencies and act accordingly. For example, buffalo grass yellowing in WA could be rapidly resolved by applying soluble iron and manganese fertilisers. Yellowing associated with grass clippings can be prevented by sweeping or vacuuming of the paddock after mowing.

Biosecurity risk

The research undertaken in this project has allowed the distributions of SCMV and PMV in three states of Australia (New South Wales, Queensland, and Western Australia) to be determined. COVID-19 travel restrictions prevented surveys in Victoria; however, there would be benefit in undertaking Victorian surveys to gain a broader understanding of SCMV and PMV distributions within Australia.

PMV has a more restricted distribution than SCMV and was only found on two of seven farms in the Hawkesbury Valley in cv. 'Palmetto'. There are good prospects that PMV could be eradicated from the surveyed farms, thus preventing the virus from becoming a more serious problem for the industry. Eradication of the virus from Australia is not feasible, however, as it has been present since at least 2008 and would have been spread to residential properties through the sale of turf.

Eradication of PMV from farms would need to be a voluntary activity of the grower, but there may be a role for the companies that own the licensing rights of susceptible varieties to contribute to the eradication program to protect their commercial interests. Furthermore, there are broader benefits to the whole turf industry from eradication of PMV from turf farms, as other varieties other than cv. 'Palmetto' may be susceptible to infection.

Despite the fact that PMV is now present in parts of New South Wales, it should still be regarded as a biosecurity risk to other states in Australia as the satellite virus, Panicum papanivirus 1, which is commonly associated with PMV in the USA has not been found in Australia. Every effort should be made to prevent introduction of the satellite virus into Australia, as its presence exacerbates virus symptoms in some host species such as *Panicum miliaceum*.

In this study, PMV was only found in NSW but further surveys across multiple seasons and locations may extend the current known range of the virus within Australia. Notwithstanding this outcome, the turf industry should consider imposing pre-entry conditions and restrictions on the import of plants and plant products from NSW to prevent the potential spread of this virus to other States in Australia. SCMV has been found in NSW, Queensland and Western Australia and therefore there is not the same justification to introduce pre-entry import conditions and restrictions for this virus. However, given the rarity of SCMV on buffalo grass turf farms in Queensland and Western Australia, there should be a code adopted by the turf industry for buffalo grass to be inspected for this virus before being moved out of NSW.

SCMV was only found on two farms in Western Australia, and only at a high incidence on one farm. Given the dispersed nature of the industry in Western Australia and the strong Mediterranean-type climate that prevents growth of voluntary grasses during summer, the eradication of SCMV from turf farms in Western Australia may be feasible. An eradication program has begun on the farm with the worst disease problem and while the majority of infected plants have likely been removed, regular inspections and spot spraying with herbicide will be needed over several years to remove the final traces of infection. Ongoing surveillance is also required on neighboring farms, especially on the farm where the single infected stolon was found.

A long-term objective for the turf industry should be to include surveys for viruses as part of the turf certification schemes.

Development of an integrated disease management plan for buffalo grass yellowing

Buffalo grass yellowing is a single encompassing name for at least two types of disease, one caused by viruses and the second by fungi. Each type of disease needs a different research approach to develop an integrated disease management plan.

Viruses

SCMV is the most serious viral pathogen of buffalo grass in Australia and will be the most difficult to control because as it is transmitted by aphids, and vector control using insecticides is ineffective for non-persistently transmitted viruses such as this. While SCMV should be the highest priority for research, there are also knowledge gaps for PMV, particularly the question of whether any Australia buffalo grass varieties are either resistant or tolerant to infection by the virus. Given that PMV has been present in the Hawkesbury Valley since at least 2008 (Thomas & Steele, 2011), it is a significant observation that PMV is still restricted in distribution to cv. 'Palmetto' and that natural spread to other cultivars has not occurred, particularly as hygiene practices such as disinfestation of mower blades has not been routinely done. This result suggests that most Australian cultivars of buffalo grass may either be immune or less susceptible to infection by PMV, but this hypothesis needs to be experimentally validated.

There are a number of avenues of research that need to be pursued for SCMV.

i) Virus strain variation needs to be investigated, particularly differences in the virulence and host range of the virus isolates. In the USA, cv. 'Palmetto' has useful resistance against SCMV and this cultivar was mainly free of this virus in Australia. However, there were two notable exceptions, one farm near Perth and a second in the Hawkesbury Valley.
ii) The yield impact of SCMV on buffalo grass needs to be quantified.
iii) Properly controlled glasshouse and field experiments need to be done to investigate whether any Australian cultivars of buffalo grass have resistance or tolerance to SCMV infection.
iv) The relative importance of mowing versus aphid vectors in the spread of the virus needs to be determined through field experimentation.
v) The rate of spread of SCMV into freshly planted, healthy turf needs to be investigated.
vi) There need to be more thorough surveys for alternative hosts of SCMV in the field. The experimental host range of the virus among commonly occurring grass weeds and some crop species such as sweet corn also needs to be determined. The buffalo grass virus isolates from Australia fall within a group of maize-infecting strains from East Africa, and therefore infection of buffalo grass may pose a threat to the corn crops in the Hawkesbury Valley.
vii) The two disease-causing agents responsible for buffalo grass lethal necrosis in Florida have now been found in Australia, namely SCMV and BGLV, although only in Queensland. Further research needs to be done to understand the status of BGLV in Australia, such as its geographic distribution and natural host range. There is a risk that BGLV could become even more common and cause the emergence of a more damaging disease than is being observed at the moment.
viii) A new nepovirus has been discovered in buffalo grass and virtually nothing is known about this virus, including its ability to cause disease, modes of spread, and geographic distribution. This virus was not tested for in the general surveys and therefore its possible role in buffalo grass yellowing needs to be investigated.

Fungi

It is hypothesized that the second type of buffalo grass yellowing that is typified by poor root health is caused by an undescribed species of *Curvularia*. Much work also needs to be done to investigate this disease problem.

i) The new *Curvularia* species needs to be properly described and formally named.
ii) Proof that the new *Curvularia* species causes the disease needs to be provided through satisfaction of Koch's Postulates, which stipulates that pure cultures of the fungus need to be inoculated onto buffalo grass and the disease symptoms reproduced.
iii) More surveys for *Curvularia* need to be done, especially in Queensland, and a stronger correlation established between infection by this fungus and buffalo grass yellowing. Other questions remain to be investigated including whether there are other causes of yellowing such as infection by *Pythium* or *Rhizoctonia*.
iv) The variability of *Curvularia* needs to be established – how many species of *Curvularia* are associated with buffalo grass yellowing.
v) Properly controlled field trials need to be done to examine the efficacy of a range of fungicides at controlling buffalo grass yellowing, including some specific for oomycetes such as *Pythium*. The results of these trials will provide indirect evidence for the involvement of a pathogen in the yellowing syndrome, as well contribute to development of a control method. Grasses are also responsive to applications of soluble silicon fertilizers, which improve the plant's defense system against a broad range of pathogens and are effective in crops such as rice.
vi) The resistance or tolerance of Australian buffalo grass cultivars to *Curvularia* needs to be investigated.
vii) Environmental factors that affect disease progress need to be investigated, such as the impact of different rates of nitrogen application and organic (e.g. poultry manure) versus inorganic fertilizers. Observations during this project also suggest that viral infections may predispose the plant to infection by *Curvularia*.

Strategic

There would be strategic benefits for the turf industry to provide continuing support for a plant protection research officer. While private consultants partially fulfill the plant protection requirements of the industry, particularly for commonly encountered pest and diseases, they do not have the specialist research facilities and sometimes the training required to make precise identifications for the majority of pathogens. It is easiest to demonstrate this point with plant virology, as identifications cannot be done without access to a molecular diagnostic laboratory and other equipment such as an electron microscope. The detection of the new virus in cv. 'Sir Walter' in the Hawkesbury

Valley was only achievable in such a short timeframe through having a diagnostician that was familiar with next generation sequencing technologies. At the farm in Western Australia where SCMV was detected, the disease was incorrectly diagnosed as a nutrient deficiency by an agronomist and remained untreated over a couple of years, allowing the problem to become more severe and provide the time for the virus to spread to neighboring farms. Even though fungi are relatively easier to identify than viruses, fungi still need to be cultured and DNA barcoding done for accurate identification, especially for ascomycetes such as *Curvularia* species.

A benefit for the turf industry investing in the training of a specialist turf pathologist is that the person would acquire industry experience and be able to quickly recognize new pests and diseases in the event of an incursion. Given the small size of the turf industry research budget in Australia, it would be important that the turf pathologist had a generalist knowledge of insect pests.

Extension

The short duration and small budget for this project did not allow for a wide-reaching extension program. There would be benefit in presenting the results of this project in a series of regional grower meetings, perhaps part of a larger roadshow highlighting the outputs of several research projects.

Refereed scientific publications

Tran, N. T., Teo, A. C., Thomas, J. E., Crew, K. S., Geering, A. D. W. 2020. Sugarcane mosaic virus infects *Stenotaphrum secundatum* in Australia. Australasian Plant Disease Notes **15**, 41. https://link.springer.com/article/10.1007/s13314-020-00410-y

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Intellectual property, commercialisation and confidentiality

No project IP, commercialisation or confidentiality issues to report

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Appendices

Appendix 1. Tables and Figures.

Appendix 2. Survey questionnaire.

Appendix 3. Nematode testing diagnostic report.

Appendix 4. Example of an individualized diagnostic report sent to growers.

Appendix 5. Refereed scientific publications

Appendix 6. Honours thesis

Appendix 1. Table and Figures

Table 1. Fungi isolated from roots and leaves of buffalo grass that showed yellowing

Accession number	Organisms [a]	Host	Location	Plant part isolated
BRIP 71296 a	*Curvularia* sp.	Sir Walter	Queensland	Root
BRIP 71297 a	*Fusarium* sp.	Sir Walter	Queensland	Root
BRIP 71299 a	*Curvularia* sp.	Palmetto	Queensland	Root
BRIP 71300 a	*Fusarium* sp.	Palmetto	Queensland	Root
BRIP 71301 a	*Fusarium* sp.	Palmetto	Queensland	Root
BRIP 71303 a	*Curvularia* sp.	Palmetto	Queensland	Root
BRIP 71305 a	*Fusarium* sp.	Sir Walter	New South Wales	Root
BRIP 71401 a	*Curvularia* sp.	Sir Walter	New South Wales	Root
BRIP 71307 a	*Curvularia* sp.	Sir Walter	New South Wales	Root
BRIP 71309 a	*Curvularia* sp.	Sapphire	New South Wales	Leaf
BRIP 71310 a	*Curvularia* sp.	Sapphire	New South Wales	Root
BRIP 71311 a	*Curvularia* sp.	Sir Walter	New South Wales	Root
BRIP 71402 a	*Curvularia* sp.	Sir Walter	New South Wales	Root
BRIP 71312 a	*Fusarium* sp.	Sir Walter	New South Wales	Root
BRIP 71314 a	*Curvularia* sp.	Sir Walter	New South Wales	Root
BRIP 71315 a	*Curvularia* sp.	Sir Walter	New South Wales	Leaf
BRIP 71316 a	*Curvularia* sp.	Matilda	New South Wales	Root
BRIP 71317 a	*Mortierella* sp.	Matilda	New South Wales	Root
BRIP 71318 a	*Curvularia* sp.	Matilda	New South Wales	Root
BRIP 71319 a	*Curvularia* sp.	Kings Pride	New South Wales	Root
BRIP 71320 a	*Curvularia* sp.	Kings Pride	New South Wales	Root
BRIP 71321 a	*Curvularia* sp.	Kings Pride	New South Wales	Root
BRIP 71322 a	*Curvularia* sp.	Kings Pride	New South Wales	Root
BRIP 71323 a	*Fusarium* sp.	Kings Pride	New South Wales	Root
BRIP 71324 a	*Curvularia* sp.	Kings Pride	New South Wales	Root

[a] Species identification was achieved through light microscopy and sequencing of the internal transcribed spacer (ITS) using the ITS1/ITS4 primers.

Table 2. GenBank accessions used in the phylogenetic analysis of *Curvularia*

Species	Accession number [a]	Host	Location	GenBank accessions
Bipolaris maydis	CBS 136.29	*Zea mays*	USA	AF071325
Curvularia alcornii	MFLUCC 10-0703	*Z. mays*	Thailand	JX256420
C. americana	UTHSC 08-3414	*Homo sapiens*	USA	HE861833
C. asianensis	MFLUCC 10-0711	*Panicum* sp.	Thailand	JX256424
C. australiensis	BRIP 12044	*Oryza sativa*	Australia	KJ415540
C. australis	BRIP 12521	*Sporobolus caroli*	Australia	KJ415541
C. bannonii	BRIP 16732	*Jacquemontia tamnifolia*	USA	KJ415542
C. beasleyi sp. nov.	BRIP 10972	*Chloris gayana*	Australia	MH414892
C. beasleyi sp. nov.	BRIP 15854	*Leersia hexandra*	Australia	MH414893
C. beerburrumensis sp. nov.	BRIP 12942	*Eragrostis bahiensis*	Australia	MH414894
C. boeremae comb. nov.	IMI 164633	*Portulaca oleracea*	India	MH414911
C. bothriochloae	BRIP 12522	*Bothriochloa bladhii*	Australia	KJ415543
C. buchloes	CBS 246.49	*Buchloë dactyloides*	USA	KJ909765
C. caricae-papayae	CBS 135941	*Carica papaya*	India	HG778984
C. chiangmaiensis	CPC 28829	*Z. mays*	Thailand	MF490814
C. coatesiae sp. nov.	BRIP 24261	*Litchi chinensis*	Australia	MH414897
C. coicis	CBS 192.29	*Coix lacryma-jobi*	Japan	AF081447
C. crustacea	BRIP 13524	*Sporobolus* sp.	Indonesia	KJ415544
C. cymbopogonis	CBS 419.78 *	*Yucca* sp.	Netherlands	HG778985
C. dactyloctenicola	CPC 28810	*Dactyloctenium aegyptium*	Thailand	MF490815
C. dactyloctenii	BRIP 12846	*Dactyloctenium radulans*	Australia	KJ415545
C. ellisii	CBS 193.62	air	Pakistan	JN192375
C. eragrosticola sp. nov.	BRIP 12538	*Eragrostis pilosa*	Australia	MH414899
C. eragrostidis	CBS 189.48 *	*Sorghum* sp.	Indonesia	HG778986
C. graminicola	BRIP 23186	*Aristida ingrata*	Australia	JN192376
C. harveyi	BRIP 57412	*Triticum aestivum*	Australia	KJ415546
C. hawaiiensis	BRIP 11987	*O. sativa*	USA	KJ415547
C. heteropogonis	CBS 284.91	*Heteropogon contortus*	Australia	KJ415549
C. hominis	CBS 136985	*Homo sapiens*	USA	HG779011
C. kenpeggii sp. nov.	BRIP 14530	*Triticum aestivum*	Australia	MH414900
C. lamingtonensis sp. nov.	BRIP 12259	*Microlaena stipoides*	Australia	MH414901
C. lunata	CBS 730.96	Human lung biopsy	USA	JX256429
C. mebaldsii sp. nov.	BRIP 12900	*Cynodon transvaalensis*	Australia	MH414902
C. mebaldsii sp. nov.	BRIP 13983 *	*C. dactylon* × *transvaalensis*	Australia	MH414903
C. oryzae	CBS 169.53	*O. sativa*	Vietnam	KP400650
C. perotidis	CBS 350.90	*Perotis rara*	Australia	JN192385
C. petersonii sp. nov.	BRIP 14642	*Dactyloctenium aegyptium*	Australia	MH414905
C. platzii sp. nov.	BRIP 27703 b	*Cenchrus clandestinum*	Australia	MH414906
C. ravenelii	BRIP 13165	*Sporobolus fertilis*	Australia	JN192386
C. reesii sp. nov.	BRIP 4358	air	Australia	MH414907
C. ryleyi	BRIP 12554	*Sporobolus creber*	Australia	KJ415556
C. sporobolicola sp. nov.	BRIP 23040 b	*Sporobolus australasicus*	Australia	MH414908
C. trifolii	ICMP 6149 *	*Setaria glauca*	New Zealand	JX256434
C. uncinata	CBS 221.52	*O. sativa*	Vietnam	HG779024
C. warraberensis sp. nov.	BRIP 14817	*Dactyloctenium aegyptium*	Australia	MH414909

[a] All accessions are ex-type isolates, except one marked with an asterisk.

Table 3. Summary of buffalo grass yellowing farm survey results in Feb – March 2020 [a]

Survey aspects	Hawkesbury Valley NSW	Hunter Valley NSW	Southeast QLD	Periurban Perth WA
General information				
Number of farms surveyed	7	3	8	8
Cultivars surveyed	Palmetto, Sir Walter, Sapphire, Prestige	Sir Walter, Matilda, soft leaf buffalo	Palmetto, Sir Walter, Sapphire, Prestige	Palmetto, Sir Walter, Sapphire, Prestige, Neergabby (ST15), New Frontier
Types of buffalo yellowing and incidence				
Viruses [b]				
Symptoms	Mosaic yellowing [e]	Mosaic yellowing	Mosaic yellowing	Mosaic yellowing
Causes	SCMV and PMV	SCMV	SCMV	SCMV
Number of farms infected by SCMV	7/7	2/3	1/8	2/8
Cultivar infected by SCMV	Palmetto, Sir Walter, Sapphire, Prestige	Sir Walter	Prestige	Palmetto
Estimated incidence SCMV (%)	5-100%	40-50%	50%	1% on one farm, and 80% on the other
Number of farms infected by PMV	2/7	0	0	0
Cultivar infected by PMV	Palmetto	0	0	0
Estimated incidence PMV (%)	50-100%	0	0	0
Fungi [c]				
Symptoms	Root rot and leaf spot leading to yellowing [e]	Root rot and leaf spot leading to yellowing	Root rot and leaf spot leading to yellowing	n/a
Causes	*Curvularia* sp.	*Curvularia* sp./*Fusarium* sp.	*Curvularia* sp./*Fusarium* sp.	n/a
Number of farms tested [c]	4/7	3/3	1/8	n/a
Incidence (%)	n/a	n/a	n/a	n/a
Abiotic stressors [d]	n/a			
Symptoms	n/a	General yellowing [e]	General yellowing	General yellowing
Causes	n/a n/a	Heating by grass clipping worsened yellowing, salted water	Nutrient deficiencies, thatch buildup, grass clippings	Nutrient deficiencies, high pH in irrigation water, grass clippings
Number of farms infected	n/a	1/3	5/8	5/8
Incidence (% area)	n/a	n/a	n/a	n/a
Unknown causes				
Symptoms	Interveinal yellowing/Striation [e]	Striation	Interveinal yellowing/Striation [d]	Striation
Causes	Unknown	Unknown	Unknown	Unknown
Number of farms infected	4/7	3/3	5/8	5/8
Epidemiology				
First notice of yellowing	1-4 years ago	1-5 years ago	1-4 years ago	n/a
Changes on incidence over time	Various patterns reported: Increase/no change/decrease	Various patterns reported: Increase/no change	Various patterns reported: Increase/no change/decrease	No change
Seasonal effect on yellowing	Various patterns reported: No/Yes when high humidity and disappeared when cold	Various patterns reported: No/Yes more severe in growing season in Oct - Dec; rain reduced yellowing	Various patterns reported: No/Yes when soil stress such as high humidity or drought	Various patterns reported: No/Yes more severe in winter and autumn
Yellowing symptoms returns after harvest	n/a	n/a	1 response: As soon as new growth appears	n/a
Spread of yellowing to new area	Various patterns reported: No/Yes by machinery	n/a	Various patterns reported: No/Yes by machinery	n/a

Continued on next page

Survey aspects	Hawkesburry Valley NSW	Hunter Valley NSW	Southeast QLD	Periurban Perth WA
Existing management practices				
Positive results reported	Minimising fertilisers and urea	Decreasing chicken manures	Miticides (1 grower experimented)	Application of soluble iron and manganese fertilisers
	Bumper (propiconazole)	Aeration before planting	Replanting after harvest using shredded stolons	Sweep/vacuum grass clippings
	Caliente mustard used as green manure Dyeing grass Replanted new turf after harvesting One report of less yellowing on Palmetto Fungicides, e.g. mancozeb + Charlie Carp-like soluble fertiliser	Gypsum application		
No effect reported	Metam sodium to fumigate soil		Fungicides, e.g. mancozeb	
Hygiene practices applied	Clean mower blades between paddocks 1 grower reported watering reducing yellowing	Clean mower blades between paddocks	Clean mower blades between paddocks	Clean mower blades between paddocks
Effect of high rate of nitrogen	Different responses: Increased yellowing/ no effect/ lessened the problem	Different responses: Increased yellowing/reduced yellowing	Different responses: Worsen problem - no use of chicken manures recommended/no effect	Lessened yellowing
Seeking technical advice	A consultant in Melbourne for disease diagnostic: *Fusarium* spp., *Rhizoctonia* spp.reported	A plant pathologist for pathogen ID and soil scientist for soil testing	A consultant/another turf grower	
Impact (rating from 0-10 (0 = no effect, 5 = medium, 10 = very serious)				
Quantity	5-9	5-9	1 grower rated 8, i.e. the infected grass was not harvestable	0
Quality	9-10	9-10	2 grower rated 8, i.e. the infected grass was not saleable	0
Receiving negative feedback from customers	Frequently	Frequently	Sometimes	Never

[a] Diagnostic tests were done for nematodes on soil samples collected from NSW and Qld. Nematodes species *Rotylenchulus* sp., *Tylenchorhynchus* sp., *Paratrichodorus* sp., *Criconemella* sp., and *Pratylenchus* sp. were identified. While these nematode species have low to moderate pathogenicity on turf grasses, their observed density was well below the threshold to cause damage, thus the test results are not shown.

[b] Panicum mosaic virus (PMV) and sugarcane mosaic virus (SCMV) were tested by ELISA using kit from Creative Diagnostics and reverse transcription PCR (RT-PCR). The results show incidence of infection estimated from field surveys following by the varieties that virus infection was observed and confirmed by ELISA and RT-PCR.

[c] Fungi were isolated from roots and leaves of the plants showing yellowing. The fungal isolates obtained were identified by light microscopy and sequencing of the ITS regions. The data indicate number of farms that was tested for fungi, does not necessary reflect true distribution and incidence of fungal-associated yellowing on buffalo grass.

[d] Identified through field surveys and interviewing with growers.

[e] photographs of different types of symptoms are in Figures 2 – 5.

n/a = not available

Figure 1. A buffalo paddock showing yellowing (A) and a stolon that was infected with sugarcane mosaic virus showing yellowing and stunting compared to healthy green grass in the background (B).

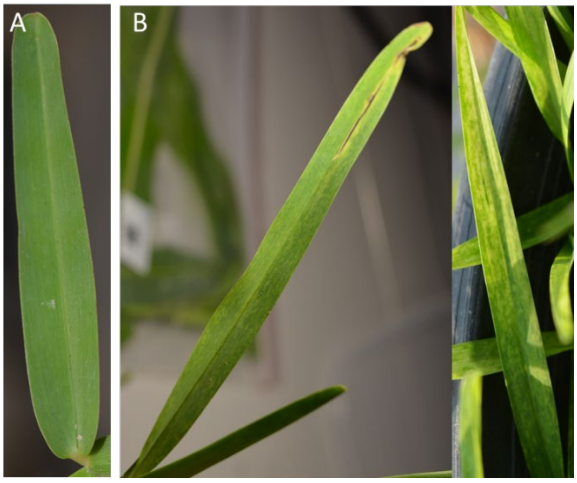

Figure 2. Healthy Palmetto (A) and panicum mosaic virus infected Palmetto showing mosaic on leaves (B).

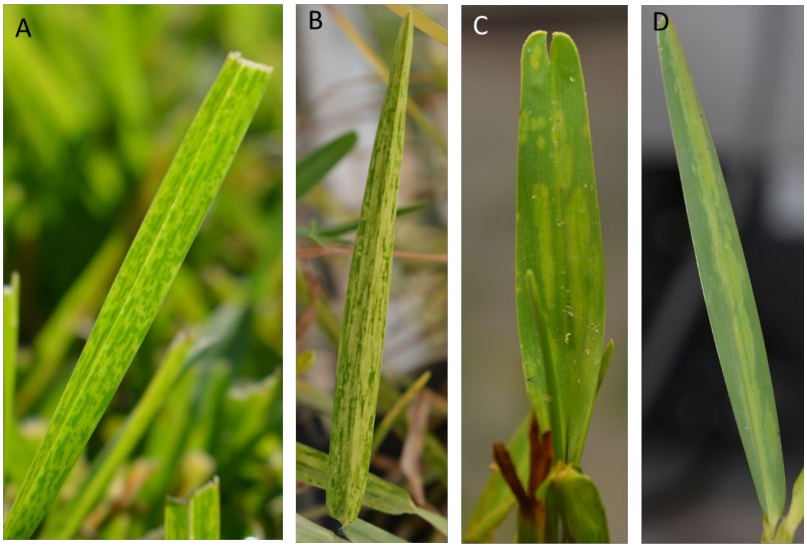

Figure 3. Sugarcane mosaic virus infected (A) Sir Walter, (B) Sapphire, (C) Prestige, and (D) Palmetto showing different types of mosaic patterns.

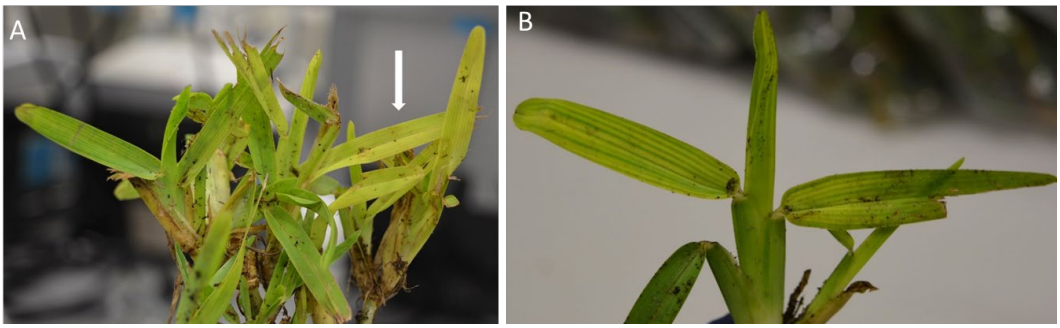

Figure 4. (A) General yellowing (even yellowing, arrow) observed on Sir Walter, and (B) interveinal striation observed on Neergabby (ST15).

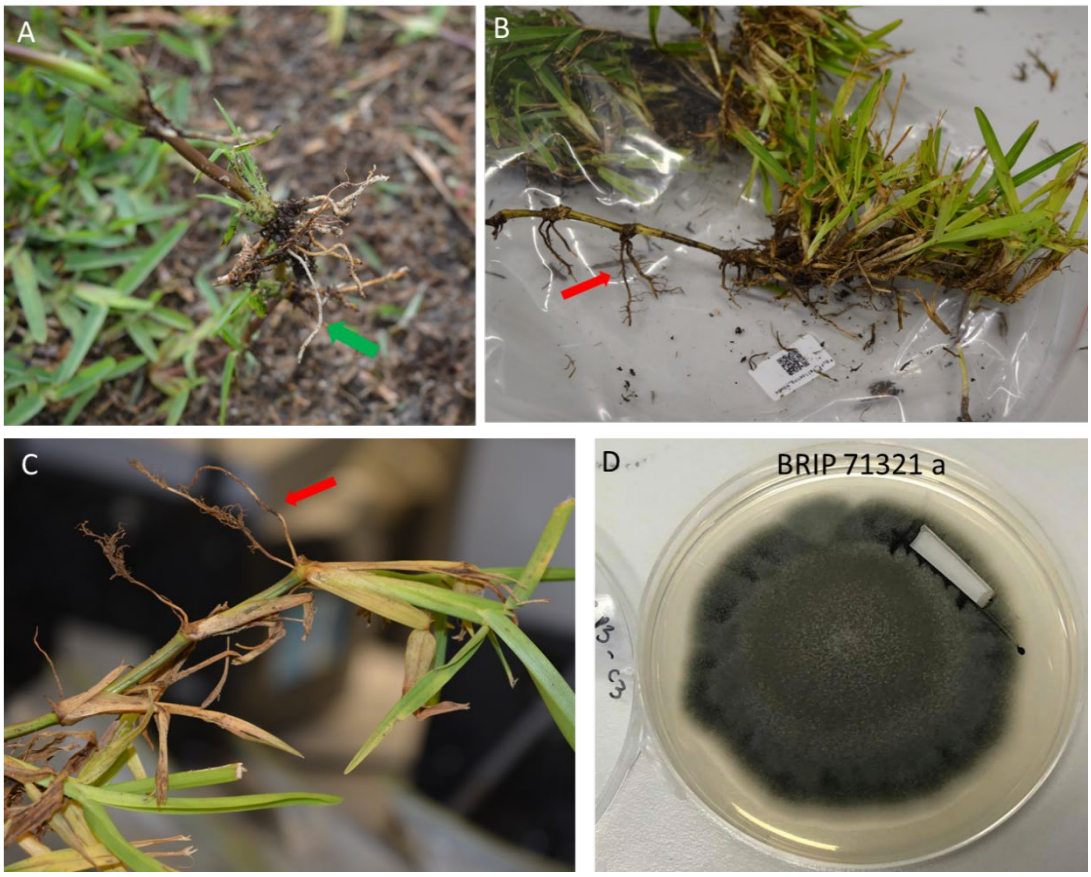

Figure 5. Healthy buffalo grass showing healthy roots (A, green arrow), buffalo grass with yellowing symptoms and root rots (B and C, red arrows), and a *Curvularia* sp. isolate recovered from the rotten roots (D).

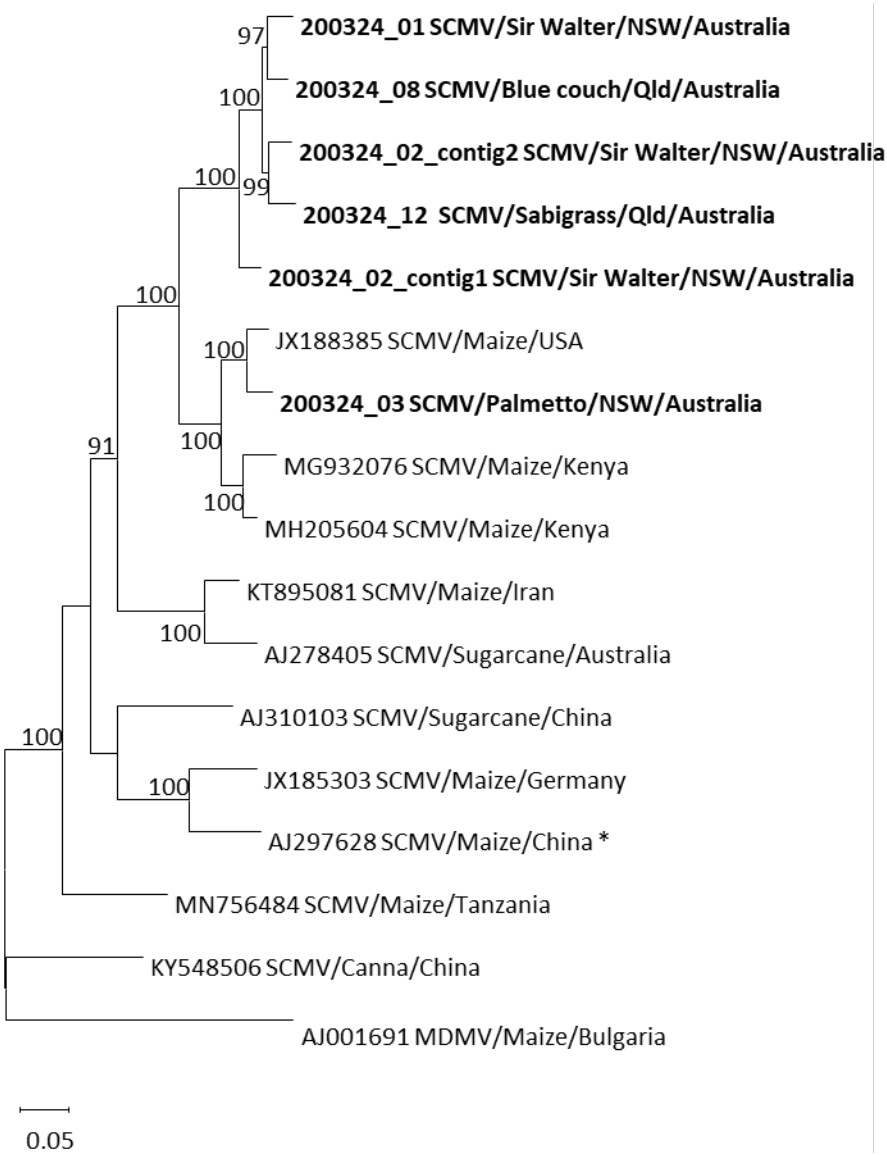

Figure 6. Phylogenetic tree of sugarcane mosaic virus (SCMV) constructed with the complete genome sequences using the Maximum Likelihood method in MEGA X. Bootstrap values from 1000 replicates greater than 70% are indicated at the nodes. The virus isolates were listed as accession number followed by virus name/host plant/country of origin. Isolates obtained from the present project are in bold font and exemplar isolate is marked with an asterisk (*). Maize dwarf mosaic virus (MDMV) (AJ001691) was used as an out-group. The scale bars showed a genetic distance of 0.05.

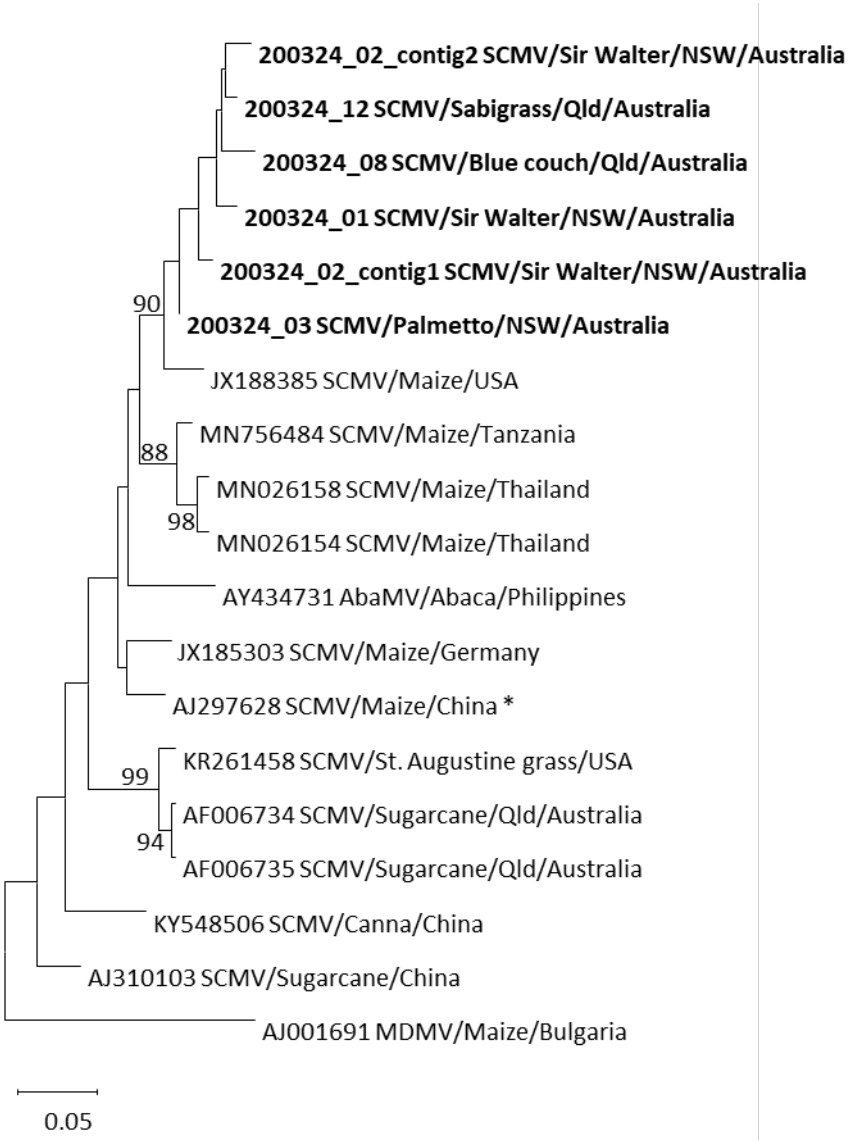

Figure 7. Phylogenetic tree of sugarcane mosaic virus (SCMV) constructed with the coat protein genes using the Maximum Likelihood method in MEGA X. Bootstrap values from 1000 replicates greater than 70% are indicated at the nodes. The virus isolates were listed as accession number followed by virus name/host plant/country of origin. Isolates obtained from the present project are in bold font, and exemplar isolate is marked with an asterisk. Maize dwarf mosaic virus (MDMV) (AJ001691) was used as an out-group and Abaca mosaic virus (AbaMV), a strain of SCMV was included for comparision. The scale bars showed a genetic distance of 0.05.

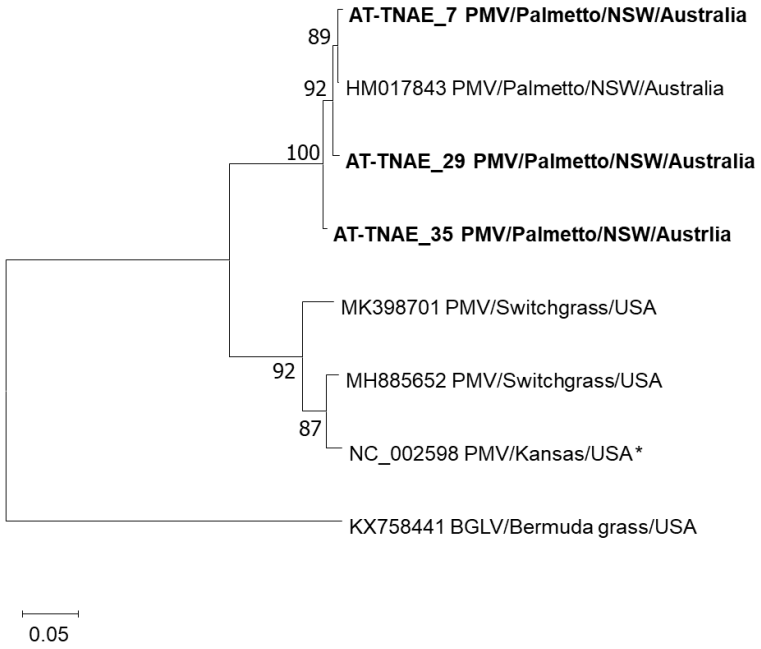

Figure 8. Phylogenetic tree of panicum mosaic virus (PMV) constructed with the partial coat protein genes using the Maximum Likelihood method in MEGA X. Bootstrap values from 1000 replicates greater than 70% are indicated at the nodes. The virus isolates were listed as accession number followed by virus name/host plant/country of origin. Isolates obtained from the present project are in bold font, and exemplar isolate is marked with an asterisk. The tree was rooted to bermuda grass latent virus (BGLV). The scale bars showed a genetic distance of 0.05.

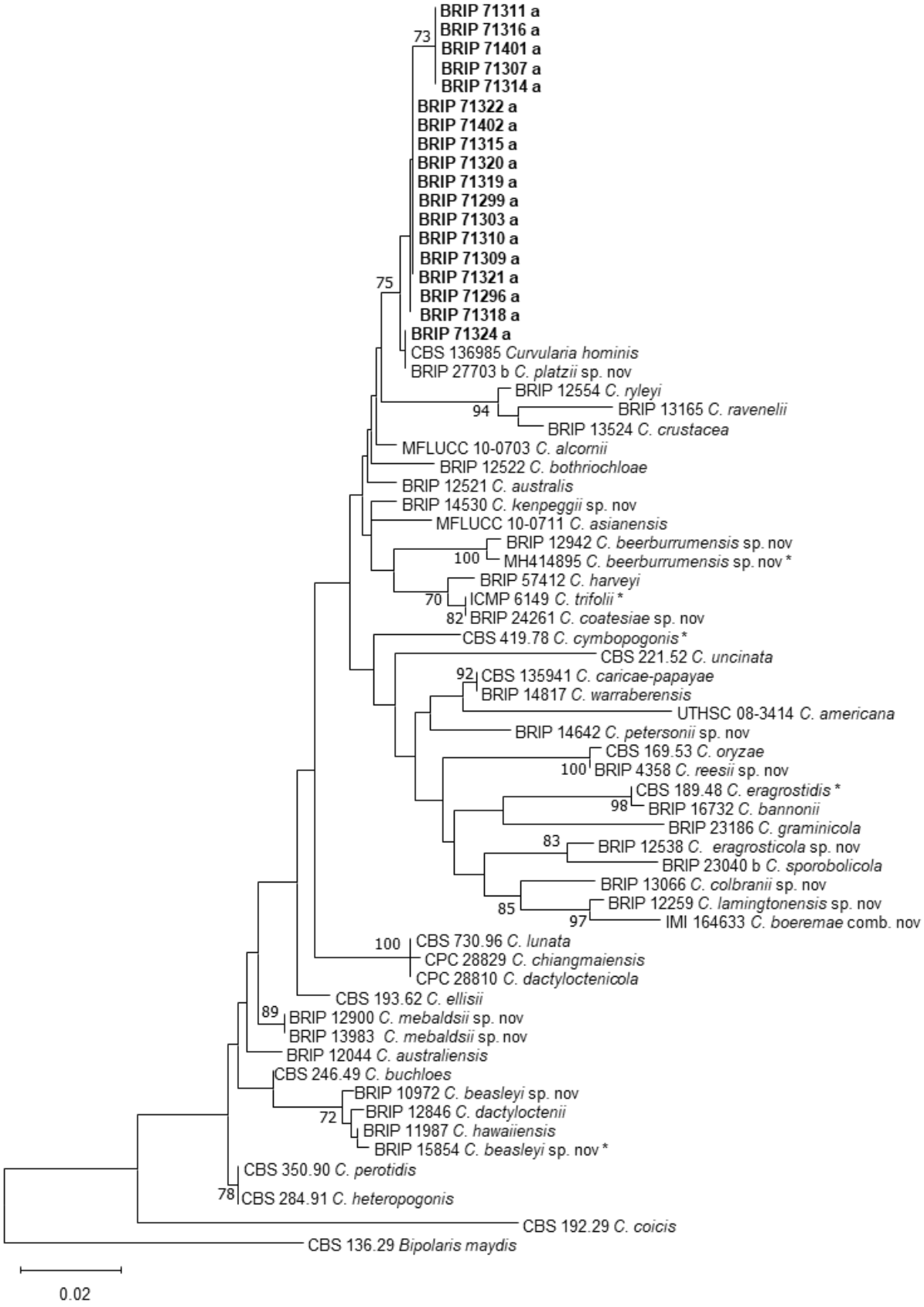

Figure 9. Phylogenetic tree of the *Curvularia* spp. isolated from buffalo grass samples showing yellowing symptoms based on Maximum Likelihood algorithm of the ITS sequence alignment. Bootstrap values from 1000 replicates greater than 70% are indicated at the nodes. The scale bars showed a genetic distance of 0.02. Isolates obtained from the current project is in bold font, all reference isolates are ex-type except one marked with and asterisk. The tree was rooted to *Bipolaris maydis*.

FIELD SURVEY QUESTIONNAIRE – MOSAIC VIRUSES IN BUFFALO GRASS

1. General information:

Company:……

Address:……

GPS:……

Respondent :……

Date:……

How many years have you been growing turf for sale? …… years

2. Yellowing of buffalo grasses: Incidence and distribution

2.1. List by variety the areas of buffalo grass (*Stenotaphrum secundatum*) that you grow.

Cultivars	Yellowing symptoms (Y/N)	Area (ha)		Symptomatic sample collected		
		Total	Infected	Y/N	Sample ID	Preliminary diagnosis? PMV/SCMV/JGMV?
Palmetto						
Sir Walter						
Shademaster						
Sapphire						
Prestige						
Matilda						
King's Pride						
.....						
.....						
.....						
Weedy grasses						

2.2. What type of symptoms have you seen?

……

Any of the below?

Panicum mosaic virus

Sugarcane mosaic virus

Unkown causes/ Striate symptoms

3. Epidemiology

3.1. When did you first become aware of buffalo yellowing?

3.2 Have you seen an increase or decrease in yellowing incidence on your farm over time? Increase/No change/Decrease

3.3. Is the yellowing problem seasonal? Yes/No
If yes, when ……? or is it present throughout the year? Yes/No

3.4. Is the problem associated with any specific weather conditions? Yes/No
If yes, under what conditions it becomes more severe?.....

3.5. How soon do you see the yellowing symptoms return after harvest?…….. weeks

3.6. Has the yellowing problem spread into new areas on your farm? Yes/No
If yes, by what means? Machinery/movement of turf/insects
If "yes", how quickly do you act on the new outbreak?

- ☐ Immediate response
- ☐ Short-term response (0-3 months)
- ☐ Long-term response (>3 months)
- ☐ Never

4. Management

4.1. Have you tried to manage the problem? Yes/No
If yes, by what means?

- ☐ Fungicides (specify ……)
- ☐ Insecticides (specify ……)
- ☐ Mechanical means (chipping out infected clumps, etc)
- ☐ Cultural practices (irrigation, fertilizers: nitrogen, potassium etc.)
- ☐ Spot-spraying around infected clumps with herbicide …….
- ☐ Rotation of paddocks

4.2. Have you succeeded in managing the yellowing problem and has the control been short or long term? Yes/No
If yes, by what means?

- ☐ Fungicides (specify ……)
- ☐ Insecticides (specify ……)
- ☐ Mechanical means (chipping out infected clumps, etc)
- ☐ Cultural practices (irrigation, fertilizers: nitrogen, potassium etc.)
- ☐ Spot-spraying with herbicide …….
- ☐ Rotation of paddocks

Comments: ……

4.3. Do high rates of fertilizer nitrogen ☐ lessen the problem, ☐ make no difference, or ☐ make the problem worse

4.4. What farm hygiene practices do you exercise

- ☐ Clean mower blades between paddocks – how?
- ☐ Control weedy grasses on paddock verges.
- ☐ Other……

4.5. Have you ever sought technical advice on the yellowing problem? Yes/No

If yes, did you contact:

- ☐ another turf grower
- ☐ a product sales representative (……)
- ☐ a chemical company representative (……)
- ☐ a consultant (……)
- ☐ a qualified agronomist (……)
- ☐ a plant pathologist (……)

4.6. Any further comments on your experience with managing or controlling the yellowing problem?

……
……
……

5. Economic impact

5.1. In your opinion and based on your experience, rate the effect of yellowing problems from 0 to 10 (0 = no effect, 5 = medium, 9 = very serious) on:

Quantity produced and sold…….

Quality of product sold ……..

Comments:……

5.2. Have you received negative feedback from customers about the yellowing problem?

Frequently /Sometimes/Rarely

Comments: ……

Queensland Government

Department of
Agriculture and Fisheries

10 March 2020

Dr Nga Tran
EcoSciences Precinct
Boggo Rd
DUTTON PARK QLD 4102

Dear Nga,

Nematode results – Queensland Laboratory numbers NO7531 - 7542

Twelve soil samples were received at the Nematology Diagnostic Laboratory of Agri-Science Queensland, DAF, Dutton Park, on 06/03/2020.

To extract nematodes from the soil, two hundred mLs of soil from each sample was set up in a Whitehead tray over 3 days. The resulting solutions were sieved using a 38 um sieve and then examined under the microscope for the presence of all plant-parasitic nematodes.

Table 1. Plant-parasitic nematodes extracted from soil using the Whitehead tray method over 3 days.

		Plant-parasitic nematodes/200 mL soil (corrected for extraction efficiency)			
Lab number	**Sample markings**	**Reniform *Rotylenchulus sp.***	**Stunt *Tylenchorhynchus* sp.**	**Stubby *Paratrichodorus* sp.**	**Ring *Criconemella* sp.**
NO7531	4 Sir Walter healthy	0	0	0	0
NO7532	5 Sir Walter yellowing	0	0	0	0
NO7533	20 Sir Walter healthy	180	20	0	0
NO7534	21 Sir Walter yellowing	765	7	7	0
NO7535	36 Saphire healthy	0	11	0	0
NO7536	36 Saphire yellowing	2	23	0	0
NO7537	42 Saphire healthy	2	0	0	0
NO7538	42 Saphire yellowing	2	2	0	0
NO7539	50 Sir Walter healthy	2	0	5	0
NO7540	51 Sir Walter yellowing	0	0	5	0
NO7541	54 Sir Walter healthy	0	0	0	99
NO7542	55 Sir Walter yellowing	0	0	0	90

Ecosciences Precinct
GPO Box 267
Brisbane
Queensland 4001 Australia
Business Centre 13 25 23
Website www.daf.qld.gov.au
ABN 66 934 348 189

Queensland
Government

Department of
Agriculture and Fisheries

If you require any further information regarding this matter, please do not hesitate to contact me on telephone 07 3708 8442 or email jennifer.cobon@daf.qld.gov.au

Yours sincerely,

Jennifer Cobon
Principal Experimentalist (Nematology)

Ecosciences Precinct
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Queensland 4001 Australia
Business Centre 13 25 23
Website www.daf.qld.gov.au
ABN 66 934 348 189

24 April 2020

Queensland Alliance for Agriculture and Food Innovation (QAAFI)

Addressed to farm owner/manager

Dear farm owner/manager

Buffalo grass yellowing survey results

Background

As part of Hort Innovation project TU19000, field surveys have been done to investigate the involvement of plant viruses in the buffalo grass yellowing disease syndrome on Australian turf farms. Yellowing is a very common symptom in grasses and can be caused by a wide variety of factors in addition to viruses, such as root-infecting fungi, nematodes, phytoplasmas, water stress and nutrient deficiencies. Hence, there will not be a single explanation for the buffalo grass yellowing seen on every farm and it should not be assumed that every instance of yellowing is caused by a virus.

In Australia and the USA, the two most common viruses that infect buffalo grass are panicum mosaic virus (PMV) and sugarcane mosaic virus (SCMV). The symptoms of infection of these two viruses overlap, and they can only be accurately identified using molecular tests in the laboratory. Both viruses are transmitted on the blades of mowers and edging equipment. Aphids also transmit SCMV in the non-persistent manner, which means that they can acquire and transmit the virus after very short feeding probes (5 second probes) and there may not be any evidence of aphid colonisation of the plant. Insecticides are ineffective at preventing spread of SCMV, as they don't kill the aphid fast enough to prevent transmission. Counter-intuitively, insecticides may speed up spread of the virus, as after exposure, the aphid may go into a frenzied feeding state before death. A critical difference in biology of the two viruses is that PMV does not have any insect vectors, and transmission is solely by mechanical methods. There are no chemical cures for either virus, and the only control method is to remove infected grass and replant with healthy grass. Neither virus will survive in the soil, but it is important to ensure there is no regrowth of old turf from deep stolons. At the moment we cannot recommend a disease-resistant variety.

Buffalo grass yellowing at turf farm

Paddocks of Sir Walter were inspected for yellowing symptoms (Fig. 1), including symptoms of PMV and SCMV. Evidence of SCMV infection (Fig. 2) was found in all paddocks with incidence estimated at 50%. Results of the virus tests are shown in Table 1.

Plants with interveinal and striate yellowing were observed and this yellowing was not always associated with virus infection. These plants had poor root health, which is the likely cause of the symptoms (Fig. 2D). Nematode tests on two samples identified ring nematode (*Criconemella* sp.) at a density of <100 nematodes/200 mL of soil (Table 1). While this nematode species has low to moderate pathogenicity on turf grasses, the observed density was well below the threshold to cause damage (200 nematodes/200 mL of soil). Fungal isolations were done from rotting roots and *Helminthosporium*-like fungi isolated (Table 1). Identification of this fungus to species level is currently underway using DNA barcoding techniques.

Fig. 1. Sir Walter showing yellowing symptoms.

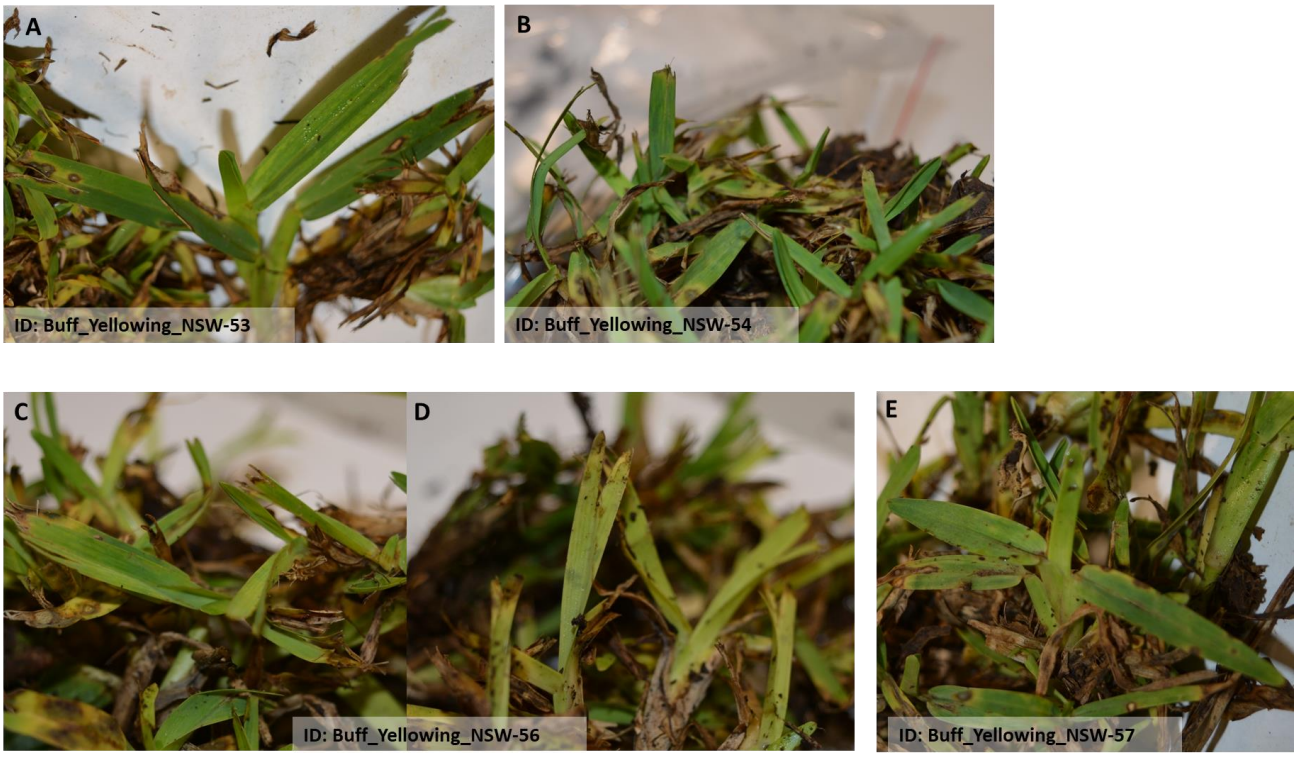

Fig. 2. Sir Walter sampled to test for panicum mosaic virus and sugarcane mosaic virus. A-C, and E: Mosaic symptoms, D – Interveinal yellowing.

Further information:

This project is a scoping study and will lead to recommendations for further research. Unfortunately a management program for the disease syndrome has yet to be devised.

If you would like to discuss the results, please do not hesitate to contact us: A/Prof. Andrew Geering, mobile 0407035941, email a.geering@uq.edu.au; Dr Nga Tran, n.tran3@uq.edu.au.

Yours sincerely,

Andrew Geering

Associate Professor

Table 1. Diagnostic test results on buffalo grass samples collected from a turf farm.

Sample laboratory identifier	Variety	Symptoms	GPS coordinates	Test results			
				PMV [a]	SCMV [b]	Nematodes [c] (Species/number per 200 mL of soil)	Fungal isolation [d]
Buff_Yellowing_NSW-53	Sir Walter	Mosaic		Negative	**Positive**	n/a	n/a
Buff_Yellowing_NSW-54 [c]	Sir Walter	Mosaic and interveinal yellowing		Negative	**Positive**	Ring (*Criconemella* sp.)/99	n/a
Buff_Yellowing_NSW-55 [c]	Sir Walter	Interveinal yellowing		n/a [e]	n/a	Ring (*Criconemella* sp.)/90	n/a
Buff_Yellowing_NSW-56	Sir Walter	Interveinal yellowing		Negative	Negative	n/a	n/a
Buff_Yellowing_NSW-57	Sir Walter	Mosaic and interveinal yellowing		Negative	**Positive**	n/a	*Helminthosporium*-like.

[a] PMV = Panicum mosaic virus, tested by ELISA using kit from Creative Diagnostics.
[b] SCMV = Sugarcane mosaic virus, tested by ELISA using kit from Creative Diagnostics, samples tested positive are in bold font.
[c] Soil core samples were collected to test for nematodes at the Nematology Diagnostic Laboratory of Queensland Department of Agriculture and Forestry, Dutton Park Qld 4102. To extract nematodes from the soil, two hundred mL of soil from each sample was set up in a Whitehead tray over 3 days. The resulting solutions were sieved using a 38 um sieve and then examined under the microscope for the presence of all plant-parasitic nematodes. Damage threshold for ring nematodes is 200 (nematodes/200 mL of soil) (Stirling, G., Interpreting the results of nematode analyses on turf grass in Australia, October 2012, unpublished).
[d] Fungal isolation was done from roots.
[e] n/a = not applicable.

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Sugarcane mosaic virus infects *Stenotaphrum secundatum* in Australia

Nga T. Tran[1] · Ai Chin Teo[1] · John E. Thomas[1] · Kathleen S. Crew[1,2] · Andrew D. W. Geering[1]

Received: 28 September 2020 / Accepted: 2 November 2020

Abstract

This study presents the first report of sugarcane mosaic virus (SCMV) infecting *Stenotaphrum secundatum* (buffalo grass) in Australia, from a turf farm in the Hunter Valley, New South Wales. The plant displayed mosaic symptoms and contained flexuous, filamentous virions of 700–750 × 10–11 nm typical of members of the genus *Potyvirus.* Infection of the sample by SCMV was confirmed by double antibody sandwich ELISA and RT-PCR amplification of the coat protein coding region of the viral genome. In a phylogenetic analysis, the buffalo grass isolate was sister to a clade of maize-infecting isolates of SCMV from eastern Africa and was 75.8% and 79.4% identical to the exemplar isolate of SCMV at nucleotide and amino acid levels, respectively.

Keywords Potyvirus · Buffalo grass · St. Augustinegrass · Nucleotide sequence

Stenotaphrum secundatum (buffalo grass in Australia or St. Augustinegrass in the USA) is a hardwearing and vigorous warm season turfgrass species, thought to be endemic to the Atlantic coasts of the Americas and Africa (Sauer 1972; Busey 2003). It is the most important turf species in Australia based on the value of its production (Hort Innovation 2019). Sugarcane mosaic virus (SCMV), an aphid-transmitted virus in the genus *Potyvirus*, was first identified as a pathogen of *S. secundatum* in the Canal Point-Pahokee-Belle Glade sugarcane growing region of Florida in 1963 (Todd et al. 1964). SCMV was not considered a serious problem to the turf industry in Florida until an outbreak of a lethal necrosis disease occurred in 2013 on 50 residential lawns within a 10 km radius of the Bayway Isles community in south St. Petersburg (Harmon 2014; Harmon et al. 2015). Aside from the severity of the symptoms, this disease outbreak was notable as it represented the first record of SCMV on *S. secundatum* outside of the sugarcane growing region of southern Florida, and also occurred on cv. 'Floratam', the most popular cultivar in the State.

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SCMV has a worldwide distribution and infects several economically important monocotyledonous crops including maize (*Zea mays*), sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum officinarum*) (Yang and Mirkov 1997; Wu et al. 2012). In Australia, there are three described strains of SCMV that are named after their primary natural hosts, namely sugarcane (*S. officianarum*), Sabi grass (*Urochloa mosambicensis*) and Queensland blue couch grass (*Digitaria didactyla*) (Teakle and Grylls 1973; Gough and Shukla 1981). The known natural host ranges of these SCMV strains in Australia are limited to single plant species, except for the Queensland blue couch strain, which has also been found in *Axonopus compressus*, *Digitaria adscendens*, *Paspalum dilatatum* and *Z. mays* (Teakle and Grylls 1973). All validated Australian records of SCMV are from Queensland (Teakle and Grylls 1973; Handley et al. 1996, 1998), although this most likely reflects limited surveillance and diagnostic efforts rather than true geographic range.

In June 2019, a sample of *S. secundatum* cv. 'Sir Walter' from a turf farm in the Hunter Valley, New South Wales, Australia was submitted for disease diagnosis (Queensland Department of Agriculture and Fisheries Plant Virus Collection isolate 5599). A mosaic pattern was present on the leaves, suggestive of a viral infection (Fig. 1a). Flexuous, filamentous virions of 700–750 × 10–11 nm were observed in negatively contrasted sap extracts of symptomatic leaf tissue using a JEOL JEM-1400 transmission electron microscope, consistent with the plant being infected with a potyvirus. Supporting this hypothesis, a positive reverse

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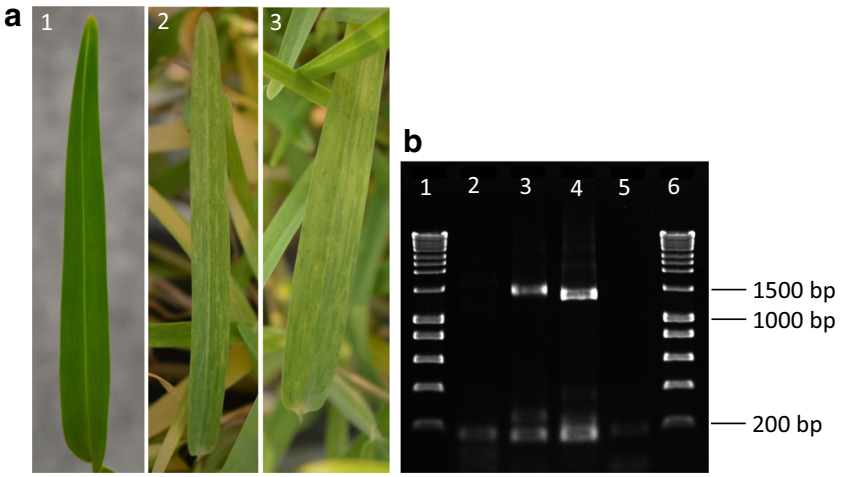

Fig. 1 (**a**) *Stenotaphrum secundatum* cv. 'Sir Walter': (1) healthy and (2, 3) infected by virus isolate 5599; (**b**) Reverse transcription-PCR detection of sugarcane mosaic virus (SCMV) using primers to amplify the entire coat protein coding region of the viral genome: lanes 1 and 6, HyperLadder 1 kb (Bioline); lane 2, healthy cv. 'Sir Walter'; lane 3, virus isolate 5599 from cv. 'Sir Walter'; lane 4, SCMV positive control, virus isolate 5610 from *Urochloa mosambicensis*; lane 5, no template control

transcription (RT)-PCR result was obtained when a total nucleic acid extract from symptomatic tissue, prepared using a Biosprint 15 DNA Plant Kit (QIAGEN, Hamburg, Germany) as per the manufacturer's instructions but without the RNase A digestion step, was tested with the universal potyvirus primer set U341/D341 (Langeveld et al. 1991) using a MyTaq One-Step RT-PCR Kit (Bioline Meridian Bioscience, Memphis, USA). The RT-PCR thermocycling conditions were: one cycle each at 45 °C for 20 min and 95 °C for 1 min; 40 cycles of 95 °C for 10 s, 50 °C for 10 s and 72 °C for 30 s each; followed by a final extension at 72 °C for 2 min. The amplicon was then electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Sequencing of the amplicon at Macrogen Incorporated (South Korea) using an AB 3730xl DNA Analyzer (Applied Biosystems, Foster City CA, USA) narrowed the diagnosis to SCMV. The sample was then tested using a SCMV double-antibody sandwich ELISA kit (Creative Diagnostics, Shirley, NY 11967, USA) following the manufacturer's instructions but with half volumes and A_{405nm} values that were 67 times greater than the mean of two healthy 'Sir Walter' replicates were obtained.

To sequence the coat protein (CP) coding region of the viral genome, primers SCMV-NIb-F1 (5′ ATWGCMGARACAGC ACTCCG 3′) and SCMV-UTR-R1 (5′ CCTGTRTCCTGCAG ACTGG 3′) were designed by aligning sequences of the nuclear inclusion protein (NIb) and 3′ untranslated regions of the genomes of SCMV isolates that had the highest scoring matches from a BLASTN search of the NCBI Nucleotide Database using the previously derived cDNA sequence as the query. RT-PCR and sequencing was done as described previously except that a primer annealing temperature of 65 °C was used. The amplicon (Fig. 1b), excluding the primers, was 1165 nucleotides long and its sequence has been deposited in GenBank under accession MW026606. The CP of the virus was identifiable after conceptual translation of the cDNA sequence, and its N-terminus was defined by a conserved serine protease cleavage site (EEVTHQ/SGT) (Adams et al. 2005). Interestingly, the CP contained two copies of the DAG motif associated with aphid transmission, one at amino acid position 5 (proximal) and one at position 112 (distal) from the N-terminus of the CP. Nigam et al. (2019) reported this feature in 77 out of 91 SCMV isolates examined.

Phylogenetic analysis of the CP nucleotide sequence placed virus isolate 5599 as sister to a clade of maize-infecting isolates of SCMV from eastern Africa, and in a different clade to a sugarcane isolate from Australia and a *S. secundatum* cv. 'Floratam' isolate from Florida (Fig. 2). Neither the Queensland blue couch nor the Sabi grass strains of SCMV from Australia have been sequenced, so comparisons were not possible. Pairwise sequence comparisons using Geneious Prime (version 2020.0.5, Biomatters, Auckland, New Zealand) showed that the CP of virus isolate 5599 was 75.8% and 79.4% identical to the exemplar isolate of SCMV (GenBank accession AJ297628) at nucleotide and amino acid levels, respectively.

To our knowledge, this is the first host record for SCMV in *S. secundatum* in Australia, and the first location record for this virus in New South Wales. Previously, Pares and Gillings (1990) reported a Johnsongrass strain of SCMV infecting a *S. secundatum* plant collected from the Hawkesbury Valley, west of Sydney. However, following taxonomic revisions, this record would now be considered that of Johnsongrass mosaic virus (JGMV), a close relative of SCMV (Shukla et al. 1989). Although the identification was only based on differential trapping of virions by immunosorbent electron microscopy using antibodies against SCMV and JGMV, the record can

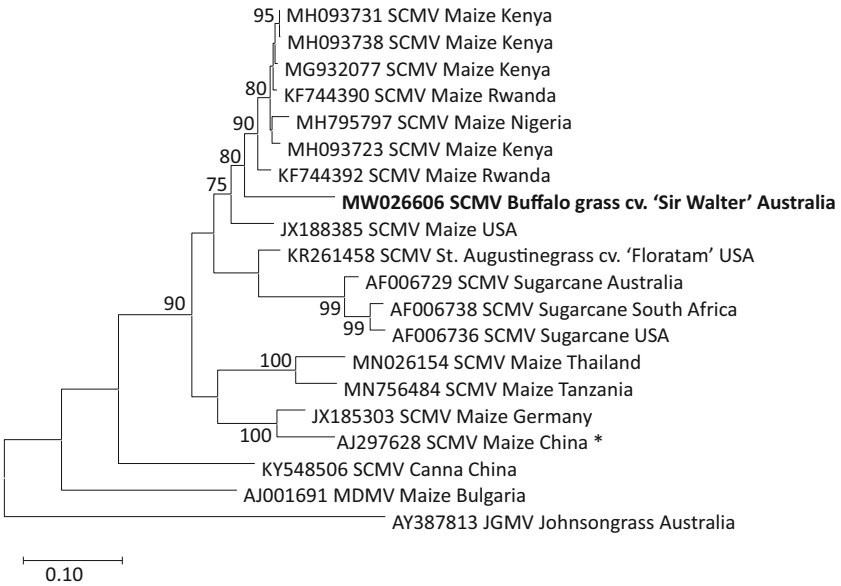

Fig. 2 Phylogram showing inferred evolutionary relationships of virus isolate 5599 from cv. 'Sir Walter' (GenBank accession MW026606, bold font) with other sugarcane mosaic virus (SCMV) isolates from around the world, based on coat protein nucleotide sequences. The tree was constructed using the Maximum Likelihood method implemented in MEGA X. Bootstrap values calculated from 1000 replicates are shown in branch nodes, and only values greater than 70% are included. Virus isolates are listed by GenBank accession followed by virus acronym, host plant and country of origin. The exemplar isolate of SCMV is indicated by an asterisk (*). Outgroups are maize dwarf virus (MDMV) and Johnsongrass mosaic virus (JGMV). The scale bar represents numbers of substitutions per site

be considered reliable as the two virus species are serologically distantly related (Shukla et al. 1989).

Recent experience from Florida suggests that SCMV has the potential to be an economically important pathogen for the Australian turf industry. SCMV is non-persistently transmitted by aphids and the use of insecticides is not recommended for control as the aphids can acquire and transmit the virus in a matter of seconds, before the insecticide kills the aphid, and transmissions are mostly by migratory, winged forms of the aphid, not those colonizing the plant (Harmon 2014). Disease control methods recommended in Florida include sanitation of equipment when mowing multiple lawns and the use of tolerant cultivars such as 'Palmetto' and 'Bitterblue' (Harmon 2014). SCMV has a wide range of wild and cultivated hosts in the *Poaceae* but the host range varies according to the particular strain of the virus (Xiao et al. 1993; Yang and Mirkov 1997) and this aspect of the epidemiology of SCMV on *S. secundatum* in Australia needs to be investigated. Virus-like symptoms have been observed on a range of cultivars of *S. secundatum* in other turf farms nearer to Sydney by the authors and expanded surveys for SCMV as well as Panicum mosaic virus (Thomas and Steele 2011) are therefore warranted.

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The association of plant viruses with buffalo grass yellowing in Australia

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Dr. Nga Tran was the co-supervisor for conceptualising the project sample collections, guidance in glasshouse trials potting, RT-PCR result troubleshooting, demonstrator of archiving sample, RT-PCR, ELISA and RNA extractions methodology, provided guidance in result analysis, and analysis related to Illumina sequencing results.

Results presented in this thesis are the summary of the experimental works undertaken by myself under the supervision of the advisory team listed above. The final report was written by me with feedback and editorial suggestions from my supervisors.

Signed, Ai Chin Teo (Student ID: 45447325)

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Keyword list

Buffalo grass, *Stenophrum secundatum*, Sugarcane mosaic virus, SCMV

Abstract

This study investigated the association of plant viruses with buffalo grass yellowing in Australia. Symptomatic buffalo grass samples were collected from turf farms across Queensland (QLD), Western Australia (WA) and New South Wales (NSW). Sugarcane mosaic virus (SCMV) and panicum mosaic virus (PMV) were found associated with buffalo grass yellowing using reverse transcription PCR and further confirmed by Sanger sequencing. SCMV was widely distributed in NSW, followed by QLD and WA with assorted cultivars, however PMV was detected in NSW and restricted in cv. 'Palmetto'. Phylogenetic analysis on SCMV coat protein coding region showed that SCMV in buffalo grass was grouped into two clades. Clade 1 is genetically closely related to SCMV in maize from Mexico and East African countries with high bootstrap supports. Clade 2 is clustered with the blue couch and sabi grass strains, which showed that SCMV isolates in this study are genetically diverse, suggestive of introduction multiple sources. In contrast, all the PMV isolates were in the same clade with the previously published Australian PMV buffalo grass strain (GenBank accession HM017843), sharing 98.2-99.8% and 98.1-100% of identity at nucleotide and amino acid level, respectively. This result showed that all the Australian PMV isolates from buffalo grass were genetically closely related, likely indicating a single introduction of virus to the host. Host specificity study of PMV showed that this virus was restricted to buffalo grass cv. 'Palmetto'. A novel species, provisionally named grass nepovirus belonging to subgroup C of the *Nepovirus*, was also detected from buffalo grass using Illumina high-throughput sequencing. Its RNA 1 Pro-RdRp and RNA 2 coat protein shared 57.8% and 32.4% amino acid identity with cherry leaf roll virus, respectively. The nepovirus was found to infect buffalo grass alone or with SCMV. A recently detected novel panicovirus member, bermuda grass latent virus (BGLV), previously only reported in United States (GenBank accession KX758441), was detected in this study. It shared 96.7% and 100% of nucleotide and amino acid identity to the exemplar isolate, respectively. It was speculated to be involved in viral lethal necrosis with SCMV in Florida. Buffalo grass is the Australian turf of choice and planted by most of the turf farmers, thus the detection of SCMV, PMV, grass nepovirus and BGLV is of concern to turf growers. Therefore, assessing the

economic impact and genetic diversity of mosaic viruses and uncharacterised viruses to turf industry has a high priority.

Publications during Honours

Peer-reviewed papers

Tran, N.T., Teo, A.C., Thomas, J.E., Crew K.S. and Geering A.D.W. (2020) Sugarcane mosaic virus infects *Stenotaphrum secundatum* in Australia. Australasian Plant Dis. Notes **15,** 41. https://doi.org/10.1007/s13314-020-00410-y

Thomas, J.E., Raymond M., Tran N.T., Crew K.S., Teo A.C., Geering A.D.W. (2020) Complete genome sequences and properties of Spartina mottle virus isolates from *Cynodon transvaalensis* × *Cynodon dactylon*. Plant Pathology (accepted with minor revisions).

Teo A.C., Tran N.T., Thomas, J.E., Crew K.S, Geering A.D.W. (2020) Genome characterisation of bermudagrass latent virus in Australia. (In preparation)

Conference abstract and presentation

Teo A.C., Tran N.T., Thomas J.E., Geering A.D.W. (2020) Characterization of bermuda grass latent virus in Australia. QAAFI Annual Research Meeting, December 1, Brisbane, Australia.

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Abbreviations

aa	Amino acid
AGRF	Australian Genome Research Facility
ArMV	Arabis mosaic virus
BG	Buffalo grass
BGLV	Bermuda grass latent virus
bp	Base pair
BRSV	Beet ringspot virus
BRV	Blackcurrant reversion virus
cDNA	Complementary DNA
CLRV	Cherry leaf roll virus
CMMV	Cocksfoot mild mosaic virus
CNSV	Cycas necrotic stunt virus
CP	Coat protein
cv.	Cultivar
DAS-ELISA	Double antibody sandwich enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
GARSV	Grapevine anatolian ringspot virus
GBLV	Grapevine Bulgarian latent virus
GCMV	Grapevine chrome mosaic virus
GDefV	Grapevine deformation virus
GFLV	Grapevine fanleaf virus
JGMV	Johnsongrass mosaic virus
JTT	Jones-Thornton-Taylor
KH_2PO_4-K_2HPO_4	Potassium phosphate buffer
MDMV	Maize dwarf mosaic virus
MP	Movement protein
nib	Nuclear inclusion b
NSW	New South Wales
nt	Nucleotide
NTP	Nucleoside triphosphate
PBRSV	Potato black ringspot virus
PCR	Polymerase chain reaction
PMV	Panicum mosaic virus

PPV1	Panicum papanivirus 1
Pro	3C-like proteinases
PVB	Potato virus B
QDAF	Queensland Department of Agriculture and Fisheries
QLD	Queensland
RACE	Rapid Amplification of cDNA Ends
RdRp	RNA-dependent RNA polymerase
RT-PCR	Reverse transcription polymerase chain reaction
SAD	Saint Augustine Decline
SCMV	Sugarcane mosaic virus
SDV	Satsuma dwarf virus
TBRV	Tomato black ring virus
TNAE	Total nucleic acid extracts
ToRSV	Tomato ringspot virus
TRSV	Tobacco ringspot virus
TPAV	Thin paspalum asymptomatic virus
US	United States
UTR	Untranslated region
VPg	Viral protein genome-linked
WA	Western Australia

Introduction and literature review

This honours project aimed to investigate the causes of buffalo grass yellowing on Australian turf farms. It was hypothesized that buffalo grass yellowing is a result of infection by plant viruses. In this literature review, an overview of the Australian turf industry and buffalo grass in particular is provided and knowledge of viral pathogens of buffalo grass summarized. Finally, the aims of this study are listed.

The Australian turf industry

The turf industry is a multi-million-dollar industry in Australia, comprising about 183 turf farmers located in all States (IBISWorld, 2020). When compared with other agricultural commodities, turf is unusual in that it is a living product and requires ongoing maintenance after it has left its place of production and therefore supports many secondary layers of business. The turf industry is continuing to expand with an average growth rate of 1.8% from 2015 to 2020 (IBISWorld, 2020).

Turf provides many services to the community. Planting turf is beneficial for groundwater reserves (Menzel, 1991), reducing contamination of rainwater runoff (Schuyler, 1987) and cooling urban areas, as air temperatures above turf are 10°C lower than above concrete (Ow et al., 2020). Turf also plays an important role in bioremediation and carbon sequestration (Gladon et al., 1993).

Buffalo grass

Stenotaphrum secundatum (Buffalo grass in Australia or St Augustinegrass in the USA) is a robust perennial grass species (Sauer, 1972). By area of production, it ranks second to green couch grass (*Cynodon dactylon*) in importance in Australia but surpasses this species in value of production (Hort Innovation, 2019). The estimated production of buffalo grass and average total farm gate turf revenue per farm per annum is approximately 12 million square metres and $AUD 890,000, respectively (Hort Innovation, 2019). Buffalo grass is widely planted in subtropical and warm temperate regions of the world, such as in Australia (Sauer, 1972). The first recorded planting of

buffalo grass was in 1880 on A. M. Reed's Mulberry Grove plantation, Florida (cited in Busey, 1995).

Buffalo grass yellowing

In Australia, one of the major constraints to turf production is a disease called 'buffalo grass yellowing'. This disease is characterized by generalised chlorosis of the leaves and weaker root systems, which causes problems for harvest as the turf mat is prone to fall apart when cut (Geering et al., personal communications, February 2020). Buffalo grass yellowing is sporadic appearance and diseased turf appears to recover when transplanted to a new location (Geering et al., personal communications, February 2020). The cause of buffalo grass yellowing is unknown and there may be several independent causes, as well as a strong influence of weather and cultivation practices on disease expression. Panicum mosaic virus (PMV) and sugarcane mosaic virus (SCMV) have been reported to cause yellowing of buffalo grass in the United States of America (USA) (Cabrera and Scholthof, 1999; Todd et al., 1964) but the associations of these viruses to buffalo grass yellowing in Australia are unknown.

Viral diseases of buffalo grass

Saint Augustine Decline (SAD)

SAD is caused by PMV alone or in combination with Panicum papanivirus 1 (PPV1; syn. satellite Panicum mosaic virus) (Buzen Jr et al., 1984). SAD is mainly a problem in the Gulf Coast states of the USA, except for Florida (Cabrera and Scholthof, 1999). At early stages of infection, SAD is characterised by by chlorotic stippling and mottling on the leaves. As the disease progresses, plants develop more generalised chlorosis that resembles nitrogen or iron deficiency, and the turf mat thins, allowing invasion of weeds. The only country other than the USA where PMV is found is Australia, where it was first detected in cv. 'Palmetto' from the Hawkesbury Valley, New South Wales, in 2008 (Thomas and Steele, 2011).

PMV is the type member of genus *Panicovirus*, family *Tombusviridae*. The virus has 30 nm diameter icosahedral virions and a monopartite, positive-sense single-stranded RNA genome about 4,300 nucleotides (nts) in size (Turina et al., 1998). PMV was first found

on switch grass (*Panicum virgatum*) in Kansas (Sill Jr and Pickett, 1957), then on buffalo grass (McCoy et al., 1970) and centipedegrass (*Eremochloa ophiuroides*) (Haygood and Barnett, 1992). The PMV strain in switch grass is serologically distinct from the strain of PMV in buffalo grass (Holcomb et al., 1989; King et al., 2011). The buffalo grass strain of PMV is host specific (King et al., 2011). There are no known vectors of PMV but it is mechanically transmissible (Haygood and Barnett, 1992; Holcomb et al., 1989), hence control can be achieved by treating mower blades with 10% sodium hypochlorite or by steam sterilisation (McCoy et al., 1970). Planting PMV-resistant cultivars such as 'Floratam', 'FA-108', 'TX-33', 'Seville', 'FA-2002' and 'Raleigh' has provided effective control in the USA (Reinert et al., 1980).

PPV1 is the sole member of the genus *Papanivirus* and has a 17 nm diameter isometric virion and single-stranded RNA genome of 824 nts. It is regarded as a satellite virus as it is dependent on PMV for replication and transmission (Turina et al., 2000; Turina et al., 1998). In switchgrass, co-infection with both viruses results in significantly more severe symptoms than infection by PMV alone (Qiu and Scholthof, 2004) but this is not the case in buffalo grass (Cabrera and Scholthof, 1999).

Mosaic Disease

Mosaic disease, caused by SCMV, was first reported in buffalo grass in the Canal Point Pahokee-Belle Glade sugarcane growing region of Florida in 1963 (Todd et al., 1964). Symptoms range in severity from mild yellowing and mottling to more extensive yellow or brown necrotic patches, leaf sheath discoloration and growth retardation (Abbott and Tippett, 1966). SCMV was considered of minor importance until an outbreak of lethal necrosis occurred in 2013 on cv. 'Floratam', which is the most popular cultivar in the State (Harmon et al., 2015). The first report of SCMV in Australia in buffalo grass cv. 'Sir Walter' is very recent (Tran et al., 2020). Several aspects of the biology and epidemiology of mosaic disease, including sources of inoculum and genetic diversity of the viral pathogen, in turf production in Australia is understudied.

SCMV is a positive-sense single-stranded RNA virus in the genus *Potyvirus*, family *Potyviridae* (Matthews, 1982). The *Potyviridae* is the largest and most economically important family of plant viruses (Scholthof et al., 2011). The genome of SCMV is 9.6 nts in size (Gell et al., 2015) and it infects plants in the Poaceae family including sorghum, maize, and sugarcane (Shukla et al., 1989; Tosic and Ford, 1972). SCMV has non-enveloped, filaments flexuous particles about 700-760 nm long and 13-14 nm wide

(Teakle and Grylls, 1973). SCMV can also causes maize dwarf mosaic disease in maize similar to maize dwarf mosaic virus, which leads to significant yield losses (Ford et al., 1989; Gan et al., 2010; Toler, 1974). SCMV buffalo grass strain host range study on different varieties of sugarcanes had proven it was an additional host of SCMV (Todd et al., 1964). SCMV is non-persistently aphid transmissible (Singh et al., 2005). Hence, control of aphid vector would not be efficient, but planting resistant cultivars has been the most efficient way in SCMV control in maize (Quint et al., 2002). Besides, it is also transmissible via the movement of infected root cane, but seed transmission was not one of the transmission modes (Singh et al., 2005). Two years later, a study done by Li et al. (2007), showed that it is possible for seed transmission to happen by mating infected female maize with healthy or infected male maize (infection rates were 5.64 % and 4.50 %, respectively). SCMV is also transmissible by mechanical sap-inoculation (Singh et al., 2005).

Johnsongrass mosaic virus (JGMV) is also a member of genus *Potyvirus* and has been found once in buffalo grass at Windsor, NSW (Pares and Gillings, 1990). JGMV was first reported in Australia as maize dwarf mosaic virus (MDMV) (Taylor and Pares, 1968) and later as the johnsongrass strain of SCMV (Teakle and Grylls, 1973). Using characteristics of the coat protein as a taxonomic marker, JGMV was finally determined to be a distinct potyvirus species (Shukla et al., 1992). The JGMV genome is 9,800 nts in length and it has flexuous particles 12 nm wide and 750 nm long (Gough et al., 1987). The known distribution of JGMV is Australia, USA, Colombia, Venezuela, Nigeria, and Brazil (Brunt, 1996; Garrido and Trujillo, 1993; Morales et al., 1996; Seifers et al., 2005; Shukla and Teakle, 1989; Silva et al., 2013). JGMV causes mosaic and red stripe diseases in sorghum and mosaic disease in maize (Teakle et al., 1970). The virus is also known to infect other members of the *Poaceae* family, including sweet and grain fodder (Gough and Shukla, 1993). Johnsongrass was known as the reservoir of JGMV in NSW (Penrose, 1974). JGMV has been found once in buffalo grass at Windsor, NSW (Pares and Gillings, 1990).

Lethal necrosis

Lethal necrosis is a new and devastating disease that was first observed in a canal residential estate of St Petersburg, Florida (Harmon et al., 2015). Initially it was thought that lethal necrosis was caused by a more virulent strain of SCMV but next generation sequencing results suggest that it is a result of synergistic interaction between this virus

and bermuda grass latent virus (BGLV), a novel member of the genus *Panicovirus* (Buhlman et al., 2020). BGLV was first discovered in *Cynodon dactylon* (Green couch in Australia or Bermuda grass in USA) and is only known from the USA (Tahir et al., 2017).

Aims of the research project

The aims of this honours project were to:

i) Investigate if SCMV and PMV were associated with the buffalo grass yellowing;

ii) Analyse strain variation, host susceptibility to virus isolates and host range of the virus isolates recovered from buffalo grass; and

iii) Determine if any unrecognized viruses were involved in buffalo grass yellowing in Australia.

Methodology

Plant materials

In order to investigate the association and distribution of viruses in buffalo grass in Australia, a total of 97 symptomatic buffalo grass samples were selected for analysis from collections made by Drs ADW Geering, JE Thomas and N Tran from turf farms in NSW, Queensland (QLD) and Western Australia (WA). Apart from buffalo grass, five samples of blue couch (*Digitaria didactyla*), two samples each of green couch (*Cynodon dactylon*), johnsongrass (*Sorghum halepense*), summer grass (*Digitaria sanguinalis*), and one sample each of broadleaf carpet grass (*Axonopus compressus*) and sabi grass (*Urochloa mosambicensis*) were analysed. Sample collection details are listed in **Appendix I**.

Reverse transcription-polymerase chain reaction (RT-PCR)

To prepare template for RT-PCR, total nucleic acid extracts (TNAE) were prepared from 100 mg of lyophilised leaf tissue using a BioSprint 15 Plant DNA Kit (Catalog number: 941517, QIAGEN) as per the manufacturer's instructions but omitting the RNase A digestion step. All assays were done using a MyTaq^{TM} One-Step RT-PCR Kit (Catalog number: BIO-65049, Bioline Meridian Bioscience), with reactions containing 1 μL of TNAE, 5 μL of One-Step Mix (2x), 0.4 μL of each primer (10 μM), 0.2 μL Ribosafe Inhibitor and 0.1 μL reverse transcriptase and water to a final reaction volume of 10 μL. The thermocycling conditions were one cycle each at 45°C for 20 min and 95°C for 1 min, followed by 40 cycles of 95°C for 10 s, 55-65°C for 10 s, 72°C for 30 s and finally once cycle of 72°C for 2 min. PCR primers for each target virus are listed in **Table 1**, together with primer annealing temperatures and expected amplicon sizes.

To validate *de novo* sequence assemblies of novel viral genomes that were identified by Illumina sequencing, overlapping amplicons were generated using the general RT-PCR method described above and the primers listed in **Appendix II**.

RT-PCR products were separated on a 1.5% agarose gel in 0.5 × TBE, run at 110 V for 50 min, and visualized by staining with ethidium bromide.

Polymerase chain reaction (PCR)

To test for mastreviruses, which have a single-stranded DNA genome, a PCR was done using MyTaq^{TM} Red DNA Polymerase (Catalog number: BIO-21108, Bioline Meridian Bioscience) and a combination of Mastrevirus-F1a and Mastrevirus-R1 primers set (**Table 1**). Each reaction contained 1 μL of total nucleic acid extracts, 2.5 μL of MyTaq Red reaction buffer (5x), 0.13 μL of each primer (10 μM), 0.13 μL of MyTaq HS DNA polymerase and water to a final volume of 25 μL. PCR thermocycling conditions were: one cycle of 95°C for 1 min, followed by 35 cycles of 95°C for 20 s, 54°C for 20s, and 72°C for 30s and a final extension cycle of 72°C for 5 min. Amplified product was analysed using electrophoresis as described before.

Table 1. Primers used in polymerase chain reaction (PCR) and reverse transcription PCR for virus diagnosis.

Virus	Primer name	Sequence 5' to 3'	Annealing temperature (°C)	Expected amplicon (bp)
JGMV[a]	JGMV-F1	ATGGTRAGCAACAAAGCCATAC	55	367
	JGMV-R1	ACGGCTCATCCAAATTTCTCAT		
Grass nepovirus	Grassnepo-F1	GAGACTATGCGTCATTTGATG	53	350
	Grassnepo-R1	CAGCGATTAAATTATCATCTCC		
Mastrevirus	Mastrevirus-F1a	GCC AGT CYT CRT CCT CGT T	54	250
	Mastrevirus-R1	ACIGGIAAGACIWCTTGGGC		
Panicovirus	PanicoRdRp-F2	TGCGCWATWGGATTYGAYGC	65	350
	PanicoRdRp-R1	CCTCATCATCCWCCGAGCA		
PMV[b]	PMV-F1	AGAAGTTATCCCACGAGGA	64	590
	PMV-R1	CGGTCCTAGCCTGTGATA		
SCMV[c]	SCMV-F1	TCTGGACGGAAATGTCGG C	65	253
	SCMV-R1	CGGGTRTCCTGCAGACTGG		
	SCMV_Nib_F2	ATWGCMGARACAGCACTCCG	63	1235
	SCMV-R1	CGGGTRTCCTGCAGACTGG		

[a] JGMV = johnsongrass mosaic virus.

[b] PMV = panicum mosaic virus.

[c] SCMV = sugarcane mosaic virus.

Sanger sequencing

Direct Sanger sequencing of the PCR products was done at Macrogen (Seoul, Korea), using the PCR primers to prime the reactions.

Illumina sequencing

Total RNA was extracted from 100 mg of leaf tissue using TRIzol™ Reagent (Catalog number: 15596018, Invitrogen) (1:10 ratio of mass tissue: Trizol) in combination with PureLink™ RNA Mini Kit (Catalog number: 12183020, Invitrogen) as per the manufacturer's instructions. The RNA was resuspended in 15 μL of RNase-Free water by incubating at room temperature for 5 min and yield quantified using a Multiskan Sky Microplate Spectrophotometer. The RNA samples were then submitted to Australian Genome Research Facility (AGRF) (Melbourne, Australia) for complementary DNA (cDNA) library preparation. Prior to Illumina sequencing, ribomosomal RNA was removed by using a Ribo-Zero Plant kit (Catalog number: RS-122-2402, Illumina) with 500 ng of input RNA. Illumina MiSeq sequencer with a 300 cycles kit and a total library size average of 300 base pairs were used for 150-bp paired-end sequencing.

Bioinformatics

To analyse Illumina sequencing data, the reads were paired, and the adaptor and primer sequences trimmed with the BBDuK Geneious plugin in Geneious Prime version 2020.1.1 software package (Biomatters Ltd., Auckland). Trimming conditions were a minimum quality of 30 and reads below 30 nucleotides (nt) were removed. Removal of duplicate reads and merging of paired reads were all implemented in Geneious Prime. Reads of grass nepovirus isolate were mapped to host chloroplast reference genome and SCMV reference geome (buffalo grass, RefSeq accession NC_036704.1; SCMV, RefSeq accession NC_003398.1). Thereafter, unmapped reads *were de novo* assembled with default settings and maximum 1,000 contigs were produced. Generated contigs were subjected to BLAST search. The contig with greatest number of reads and depth of coverage was obtained. The Illumina sequencing data were then confirmed by Sanger sequencing except the termini.

De novo assembled sequence reads were manually corrected where needed and analysed using Geneious Prime. BLAST search was performed using the available sequences. Reference sequences of SCMV and PMV were downloaded from NCBI and imported into Geneious. Nucleotide and amino acid (aa) sequence alignments were done on MUSCLE algorithm basis. Phylogeny was created using Maximum Likelihood method in MEGA X (Kumar et al., 2018), based on Tamura-Nei matrix-based model (Tamura and Nei, 1993) of evolution for nt sequences, Jones-Thornton-Taylor (JTT) model for aa sequences and 1000 bootstrap replicates. Sequences determined in this research project and used for phylogenetic analysis were to be submitted to GenBank.

Mechanical inoculations

To obtain the grass nepovirus in pure culture without SCMV and examine host range of BGLV, mechanical inoculations were attempted on two maize cultivars; cv. 'Enterprise' which is resistant to JGMV and cv. 'Prelude' is susceptible to JGMV. Viruses were mechanically inoculated onto maize plants on 13 days after sowing, at 3- to 4-leaf stage. Inoculum was prepared in 0.1 M potassium phosphate buffer (KH_2PO_4-K_2HPO_4) pH 7.1, with 0.1 g of infected leaf and ground using pestle and mortar (1:10 ratio of mass tissue: inoculation buffer). Inoculum was then rubbed onto carborundum-dusted young seedlings. Inoculated plants were evaluated for virus symptoms 4 to 5 days post inoculation (dpi) and daily observation continued for at least 2 weeks. Inoculated maizes were harvested after 14 dpi for virus indexing purpose.

Buffalo grass cvs. 'Palmetto', 'Grand Royal', 'Prestige' and 'Sir Walter' were inoculated on day 23 after potting as previously described method. Daily observations continued for 19 dpi and inoculated buffalo grass was sampled after 26 dpi for virus indexing.

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

DAS-ELISAs were done for SCMV and PMV using reagents obtained from Creative Diagnostics (Catalog numbers: DEIAPV226 and DEIAPV200, respectively) as per the manufacturer's instructions but using half volumes. Leaf samples were ground in

extraction buffer (0.03 M sodium phosphate buffer, pH 7.3) at ratio of 1 g per 10 mL, briefly centrifuged at 10,000 *g* to clarify the extract and added to the wells of a 96 wells microplate. Absorbance values were measured using a Multiskan Sky Microplate Spectrophotometer at 405 nm and values greater than three times the means for the healthy control were considered as ELISA positive for the tested virus (Guadie et al., 2019).

Virus minipreps

Small-scale, partial virus purifications (virus minipreps) were done using a modified method of Lockhart (1986). Two grams of fresh leaf tissue were ground with a pestle and mortar and acid-washed sand in 6 mL of extraction buffer (0.2 M potassium phosphate buffer pH 7.0, 15 mM ethylenediaminetetraacetic acid (EDTA), 2% polyvinylpyrollidone average molecular weight 40,000, 2% polyethylene glycol, 0.4% sodium sulphite). To remove particulate matter, the leaf extract was filtered through muslin and then centrifuged at 12,000 *g* for 15 min at 5°C. The supernatant was then decanted into a 10 mL R70.1 ultracentrifuge tube, 120 µL of 33% Triton X-100 mixed in, the solution underlaid with 1 mL of 30% sucrose in 0.2 M potassium phosphate buffer pH 7.0 and subjected to ultracentrifugation (230,000 *g* for 35 min at 5°C). The high-speed pellet was resuspended in 100 µL of 10 mM potassium phosphate buffer pH 7.0 and transferred to a microcentrifuge tube. Chloroform (30 µL) was added and vortexed to mix before centrifugation (10,000 – 15,000 *rpm*). The supernatant phase was stored at -80°C. Virus preparations were visualised under a transmission electron microscope (TEM) after negatively contrasting with 1% ammonium molybdate pH 5.8 or used as template for 5'/3' Rapid Amplification of cDNA Ends (RACE).

5′/3′ RACE

5′ RACE was done using a 5′/3′ RACE Kit, 2nd Generation (Catalog number: 03353621001, Roche Applied Science) as per the manufacturers' instructions and the virus-specific primers listed in **Table 2.** When necessary, the 3′ end of the viral RNA was polyadenylated using *E. coli* poly(A) polymerase (Catalog number: M0276S, BioLabs) and cDNA prepared using Superscript IV reverse transcriptase (Catalog number:

18090010, Invitrogen) and an oligo dT primer Poty1 (Gibbs and Mackenzie, 1997) and followed by PCR with genome specific primers.

Table 2. Virus specific primers used in 5′/3′ RACE for grass nepovirus sequencing.

Virus RNA	**Primer name**	**Sequence 5′ to 3′**	**Annealing temperature (°C)**
5′ RACE RNA 1	RNA1-SP1-R	CCATAGGCTCTCCTGTGAGC	63
	RNA1-SP2-R	TCACTGGTGCTAAGCATCAAAGC	63
	RNA1-SP3-R	CGTAGCAGCAAAGCTCACTGGC	66
3′ RACE RNA 1	RNA1-SP5-F1	TGGTCGATTACCTACAGAAGCGT	55
	unk2_F0	AGG GAT ATG GCG CCG TAT	56
5′ RACE RNA 2	RNA2-SP1-R	TATGCCTCACCACGCTTCTGGTT	65
	RNA2-SP2-R	CCACAATAATGGCACCTGGCAT	62
	RNA2-SP3-R	TGCAGCCTTGGAGAACACTCGTT	65
3′ RACE RNA 2	RNA2-SP5-F1	GGCAAAGCATCCTTGAGGTCAA	62
	RNA2-SP5-F2	GTAGTTTCCTCGCAGTGGGGTTG	66

Results and discussion

Virus surveys

A large collection of diseased buffalo grass samples from around Australia was assembled, all of which had some degree of chlorosis and many had mosaic patterns or chlorotic streaks that were suggestive of a virus infection. Of the 97 samples that were tested by PCR or RT-PCR, 53 were positive for one of the viruses (**Table 3**) that were tested for, as outlined below.

Table 3. Detection of viruses in grass samples.

Virus[a]	Grass species	No. of samples	Location[b]
SCMV	*Stenotaphrum secundatum*	25	NSW
SCMV	*Digitaria sangunalis*	1	
PMV	*S. secundatum*	4	
PMV	*D. sangunalis*	1	
JGMV	*Sorghum halepense*	1	
SCMV + PMV	*S. secundatum*	2	
SCMV	*S. secundatum*	9	QLD
SCMV	*Urochloa mosambicensis*	1	
SCMV	*Digitaria. didactyla*	1	
JGMV	*S. halepense*	1	
BGLV	*Cynodon dactylon*	1	
Mastrevirus	*D. didactyla*	1	
SCMV	*S. secundatum*	3	WA
SCMV	*D. didactyla*	2	

[a] Virus acronyms: sugarcane mosaic virus (SCMV); Panicum mosaic virus (PMV); johnsongrass mosaic virus (JGMV); bermudagrass latent virus (BGLV).

[b] Location: New South Wales (NSW); Queensland (QLD); Western Australia (WA).

SCMV

The diagnostic assay that was used for SCMV produced an amplicon of 253 bp, covering parts of the coat protein (CP) and 3′ untranslated region. SCMV was detected in 39/97 buffalo grass samples, and was the most broadly distributed, occurring in all States that were surveyed (**Table 3**). SCMV was notably prevalent in NSW, occurring on 10/13 turf farms in the Hawkesbury Valley and 2/5 farms in the Hunter Valley. In contrast, SCMV

was only detected in buffalo grass in 1/12 and 2/8 farms in Queensland and Western Australia, respectively. Various symptoms were associated with SCMV infection, including chlorotic stippling, striations and spindle-shaped lesions (**Fig. 1**).

Figure 1. Buffalo grass cv. 'Sir Walter' **(A)** healthy, **(B)** sugarcane mosaic virus-infected leaves, showing different intensities and contrasts of chlorotic mottling symptoms and shades of green.

To investigate strain variation, a new RT-PCR assay was developed to amplify the entire CP-coding region of the genome, as variation in this protein is a species-demarcation criterion for potyviruses. SCMV_Nib_F2 was located at the end of nuclear inclusion b (NIb) coding region and SCMV-R1 within the 3' untranslated region (UTR), producing an expected amplicon size of 1,300 bp. A subsample of SCMV isolates was used for this study, ensuring that there was at least one representative infected sample from each cultivar of each farm. Isolates of SCMV from blue couch and sabi grass were also included in this study to investigate alternative hosts of the virus (**Table 4**). In total, CP sequences were obtained for 17 isolates of SCMV, comprising 10 from NSW, four from Queensland and three from WA.

Table 4. Description of 17 isolates of sugarcane mosaic virus (SCMV) in Australia.

States	Virus accession[a]	Grass species	Cultivar	Length (nts)[b]
NSW	5613	*Stenotaphrum secundatum*	Sapphire	1006
	5599	*S. secundatum*	Sir Walter	1035
	5614	*S. secundatum*	Shade Master	985
	5615	*S. secundatum*	Palmetto	985
	5616	*S. secundatum*	Maltida	985
	5617	*S. secundatum*	Sir Walter	985
	5618	*S. secundatum*	Sapphire	985
	5619	*S. secundatum*	Sir Walter	985
	5620	*S. secundatum*	Sir Walter	985
	5621	*S. secundatum*	Sir Walter	985
QLD	5622	*Digitaria didactyla*	wild type	964
	5623	*S. secundatum*	Prestige	985
	5624	*S. secundatum*	Prestige	985
	5610	*Urochloa mosambicensis*	n/a	963
WA	5625	*S. secundatum*	Palmetto	985
	5626	*Digitaria didactyla*	Queensland blue	985
	5627	*S. secundatum*	Palmetto	985

[a] Queensland Department of Agriculture and Fisheries (QDAF) Plant Virus Collection

[b] Length of coat protein coding region.

The isolates of SCMV from buffalo grass in Australia were diverse, sharing 84.0-97.4% nucleotide identity within the CP gene (data not shown). There was also variation in the gene's length: the modal length was 985 nts but a range from 963 to 1035 nts was observed. The variation in length occurred at the N-terminus of the protein.

When a phylogenetic analysis was done, the virus isolates from buffalo grass formed two distinct clades (1 and 2) that were poorly correlated with either host cultivar or geographical origin (**Fig. 2**). Each of the clades contained virus isolates from NSW and Queensland and virus isolates from the popular cultivar 'Sir Walter' were also distributed over the two clades. This result suggests many independent introductions of SCMV into buffalo grass from alternative hosts rather than a single introduction and then movement of the virus by trade of living turf.

In Queensland, the isolates of SCMV within sabi grass and blue couch are regarded as distinct strains based on the differences in host range and serological relationships (Teakle and Grylls, 1973). However, virus isolates from these two plant species grouped within

the buffalo grass clade 2 (Blue), hence they are genetically closely related, especially the blue couch strain.

Interestingly, the isolates of SCMV from buffalo grass in Australia were distantly related to the Florida isolate of SCMV from cv. 'Floratam' (GenBank accession KR261458). SCMV is mainly a problem of turf in the southern, sugarcane-growing region of Florida and the isolate of SCMV in buffalo grass groups with sugarcane isolates of the virus, suggesting transmission between the two plant species (Todd et al., 1964).

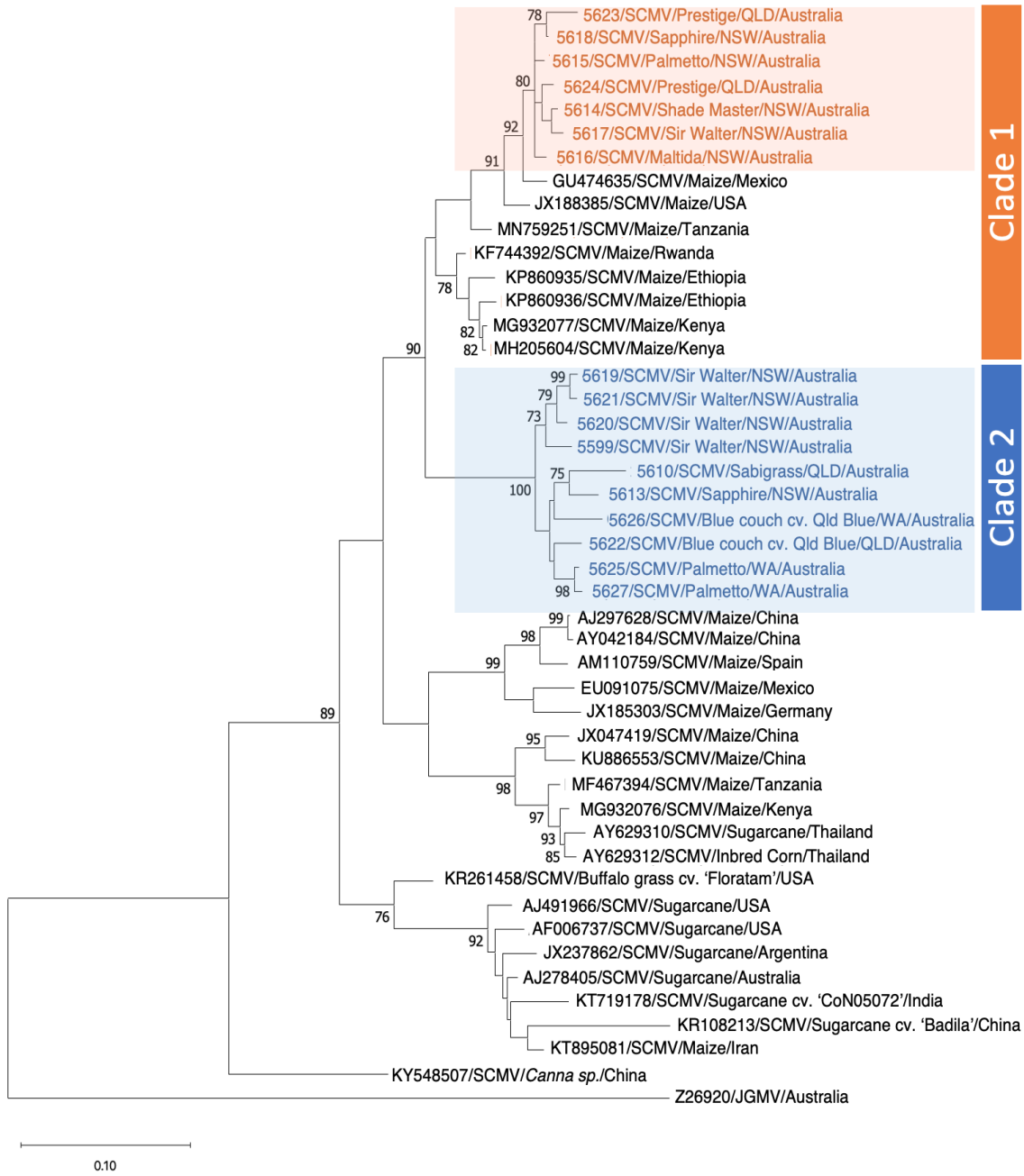

Figure 2. Phylogram showing inferred evolutionary relationship of sugarcane mosaic virus (SCMV) isolates obtained from the present study (highlighted in orange and blue) with other SCMV isolates from around the world, based on coat protein coding region of the viral genome. Virus isolates are listed as

accession number/virus acronym/host plant/place of origin. The exemplar isolate of SCMV is AJ297628 from China and the outgroup is johnsongrass mosaic virus (JGMV). The scale bar represents the number of substitutions per nucleotide site.

Studies of the genetic diversity of SCMV from various countries showed clustering in relation to the original host and geographical distribution (Li et al., 2013; Perera et al., 2009; Viswanathan et al., 2009). The spread of SCMV in buffalo grass in Australia maybe through multiple introductions by geographically distinct overseas strains, and from local blue couch grass and further spread in buffalo grass turf, but more survey data are needed before firm conclusions can be drawn. SCMV isolated from sugarcane in Australia were not closely related to the SCMV collected in this study.

Panicovirus

To be able to simultaneously detect PMV and BGLV, a universal panicovirus RT-PCR assay was designed by aligning the RNA-dependent RNA polymerase coding regions of all four officially recognized panicovirus species. Primer-annealing temperatures were optimised using an isolate of panicum mosaic virus in cv. 'Palmetto' as the control, and a single amplicon of the expected size, 590 nts, was obtained.

All buffalo grass samples were tested, including those with SCMV infection, to determine if there were mixed infections. Positive diagnostic results only obtained with seven samples of cv. 'Palmetto' from three turf farms in the Hawkesbury Valley. Direct sequencing of the amplicon confirmed that these plants were infected with PMV. Symptoms of infection included chlorotic stippling and mottling (**Fig. 3**).

Figure 3. Buffalo grass cv. 'Palmetto' **(A)** healthy and **(B)** panicum mosaic virus-infected leaves showing chlorotic mottling and yellowing symptoms.

The isolates of PMV in Australia were closely related (98.0-99.8% nucleotide sequence identity) yet each was distinct. All recently collected virus isolates were also closely related to the published buffalo grass isolate of PMV from Australia (GenBank accession HM017843) with nt and aa identities of 98.2-99.8% and 98.8-100%, respectively. A phylogenetic analysis grouped all the Australian PMV isolates together, including the published one (GenBank accession HM017843) into the same clade which was distinct to the PMV exemplar isolate from pearl millet (GenBank accession U55002) with high bootstrap support (**Fig. 4**). Phylogenetic analysis result showed that PMV buffalo grass strain is potentially a new species, but there are no panicovirus species demarcation criteria available. Hence, full genome and more work have to be done before drawing a firm conclusion.

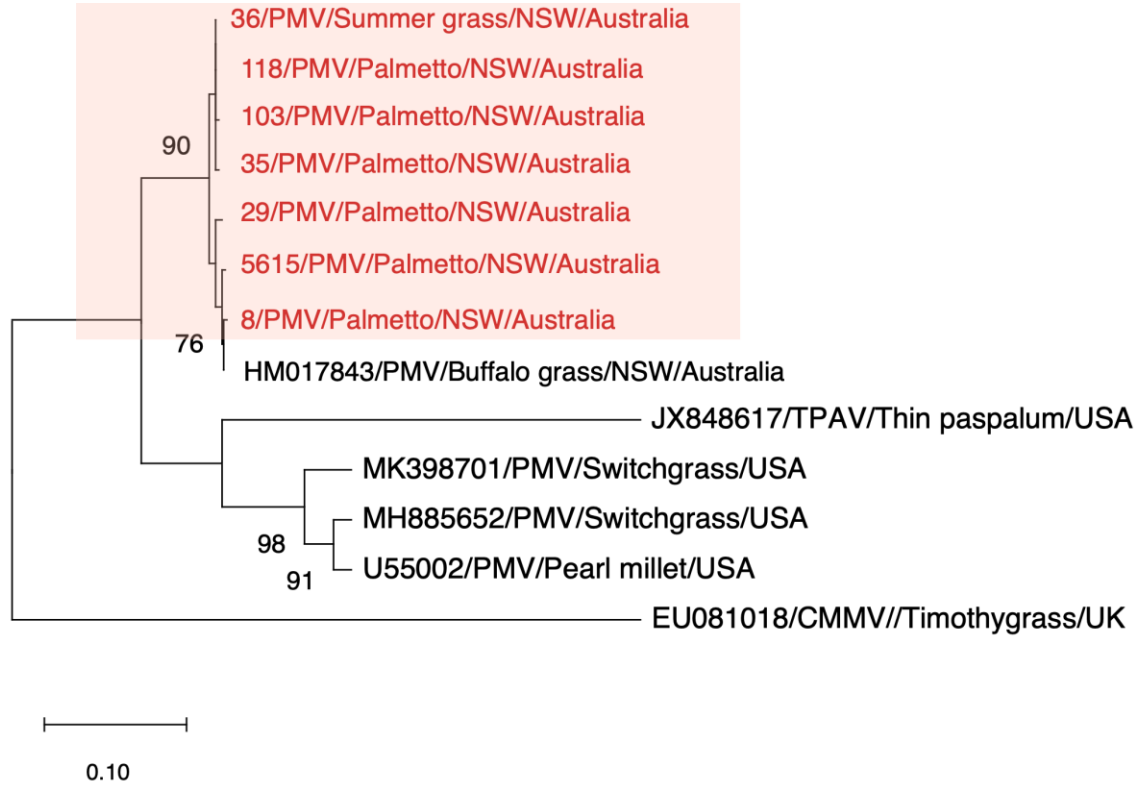

Figure 4. Phylogram showing evolutionary relationships of Panicum mosaic virus (PMV) isolates collected in this study (highlighted in red) constructed with published PMV isolates, based on partial coat protein sequences (489 nts). The phylogram was inferred using the Maximum likelihood method implemented in MEGA. Bootstrap values from 1000 replicates are shown in the nodes of the branches and only values greater than 70% are included. Virus isolates are listed by accession number/virus acronym/host plant/location of origin. The pearl millet strain of PMV is the exemplar isolate; Cocksfoot mild mosaic virus (CMMV) and thin paspalum asymptomatic virus (TPAV) are included as outgroups. The scale bar represented numbers of substitution per nucleotide site.

Pairwise comparisons on the basis of MUSCLE alignment between partial PMV CP sequences in this study with PMV switchgrass strains (GenBank accessions MK398701 and MH885652) shared 83.6-84.4% nt identity (data not shown). Pearl millet strains (GenBank accession U55002) sharing nt identity of 83.5-83.9% with PMV CP sequences in this study (data not shown). The aa identities between PMV in this study and publicly available PMV strains were 90.0-100.0% (data not shown). Although previous literature of PMV characterisation showed the existence of at least six serotypes of the virus (Buzen Jr et al., 1984), only four sequences of PMV available currently. Hence, the sequence variation between six PMV serotypes (Buzen Jr et al., 1984) was not possible.

The phylogenetic analysis suggested a single introduction of PMV in buffalo grass into NSW, Australia. It could then have spread locally through mechanical transmission and vegetative propagation (Haygood and Barnett, 1992; Holcomb et al., 1989). In this study,

PMV was only detected in NSW and in buffalo grass cv. ‘Palmetto’. PMV infected cv. ‘Palmetto’ was planted in the same farms with various buffalo grass cultivars but there were no other cultivars infected by PMV.

BGLV in *Cynodon dactylon*

A sample of *C. dactylon* with a mild mottle was collected from a turf farm in South East Queensland. This sample tested positive using the universal panicovirus RT-PCR assay, yet was negative for PMV by ELISA, suggesting the presence of an unrecognized panicovirus species. Sequencing of the amplicon suggested that the plant was infected with BGLV and this conclusion was supported by observations of virions under the electron microscope (**Fig. 5**). Phylogenetic analysis based on BGLV partial RdRp sequence alignment with representatives of panicoviruses in family *Tombusviridae* was completed using nt and aa sequences. Both phylograms had identical topologies, hence only one phylogram is illustrated (Fig. 6). The Queensland isolate of BLGV clustered together with the exemplar isolate from the USA (KX758441) with high bootstrap support. Sequenced BGLV amplicons had significant matches to KX758441 with a nt identity of 96.7% and aa identity of 100% (data not shown). This record of BLGV in Australia is only the second report of this virus, worldwide.

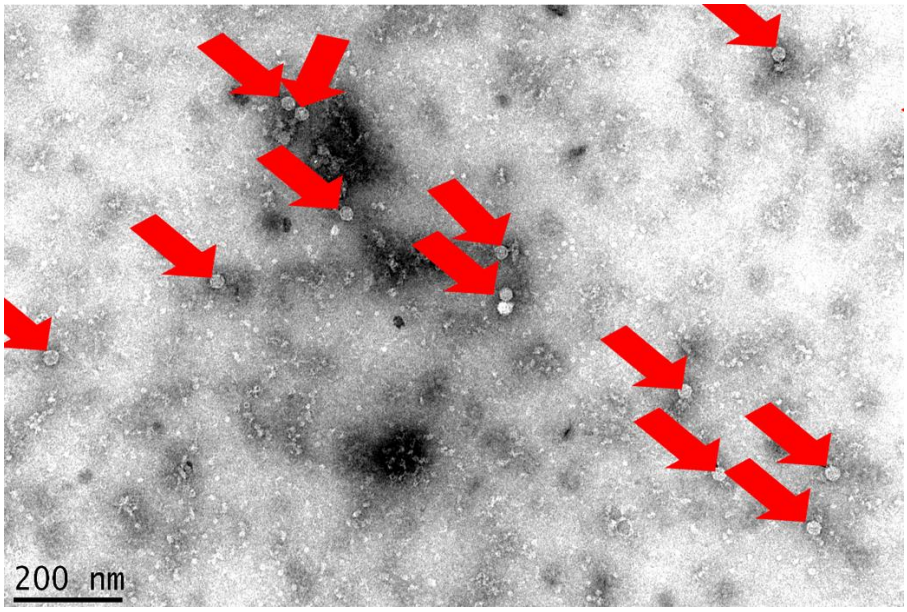

Figure 5. Electron micrograph of bermuda grass latent virus negatively stained with 1% ammonium molybdate pH 5.8, showing isometric virions with an average diameter of 27 nm, typical of members of the genus *Panicovirus*.

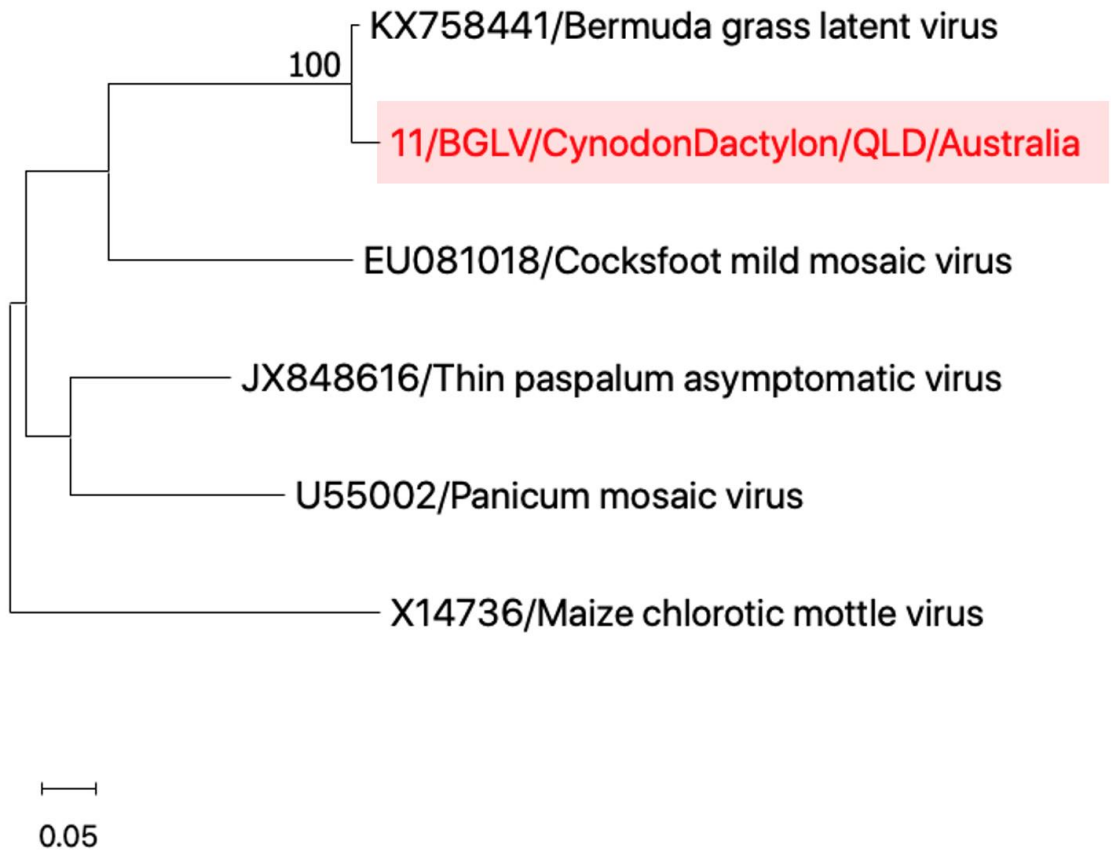

Figure 6. Phylogram based on the alignments of panicovirus partial RNA-dependent RNA polymerase sequences (590 nts). The phylogram was inferred using the Maximum likelihood method implemented in MEGA. Bootstrap values from 1000 replicates are shown in the nodes of the branches and only values greater than 70% are included. Isolate 11 obtained from the present study is highlighted in red. Bermuda grass latent virus (BGLV) exemplar isolate is accession KX758441. Panicovirus included in this three were cocksfoot mild mosaic virus (accession EU081018), panicum mosaic virus (accession U55002) and thin paspalum asymptomatic virus (accession JX848616). Maize chlorotic mottle virus (accession X14736) was used as outgroup. The scale bar represented numbers of substitution per site.

Although BGLV is asymptomatic in green couch (Tahir et al., 2017), it was suspected to cause lethal viral necrosis in buffalo grass when co-infecting with SCMV (Buhlman et al., 2020). The synergistic relationship between these two viruses and the mechanism in causing severe disease were remained unknown. The co-existence of SCMV and BGLV in Australia might cause a huge threat to turf industry without proper control. Therefore, investigating the distribution of BGLV in Australia should be prioritised in the future.

Mastrevirus and JGMV

Using primers Mastrevirus-F1a/R1, an amplicon approximates 250 bp had been produced from one blue couch sample (**Table 3**). However, there was no mastreviruses been detected in buffalo grass. Similarly, two johnsongrass samples were shown to be infected by JGMV (**Table 3**) by JGMV-specific PCR, but JGMV was not detected in any buffalo grass samples.

In summary, SCMV was widely distributed in NSW, followed by QLD then WA. PMV was only detected in NSW buffalo grass cv. 'Palmetto'. After the sample screening, JGMV was not detected in buffalo grass. One green couch sample infected by BGLV but the screening of BGLV showed that there were no buffalo grass infected. Previously, there were JGMV (Pares and Gillings, 1990), PMV (Thomas and Steele, 2011) and SCMV (Tran et al., 2020) were detected in buffalo grasses sampled from NSW. The available results showed that SCMV was actively thriving and causing buffalo grass yellowing in Australia. This detection established an additional host of SCMV in Australia.

Detection of a novel nepovirus

A buffalo grass sample cv. 'Sir Walter' with severe disease symptoms from the Hawkesbury Valley, NSW was observed to have flexuous and 30 nm isometric virions under the electron microscope (**Fig. 7**). The flexuous virions were those of SCMV based on RT-PCR and Sanger sequencing of the amplicon. However, isometric virions were not detected by any primers involved this study. Hence, this sample was submitted for Illumina sequencing.

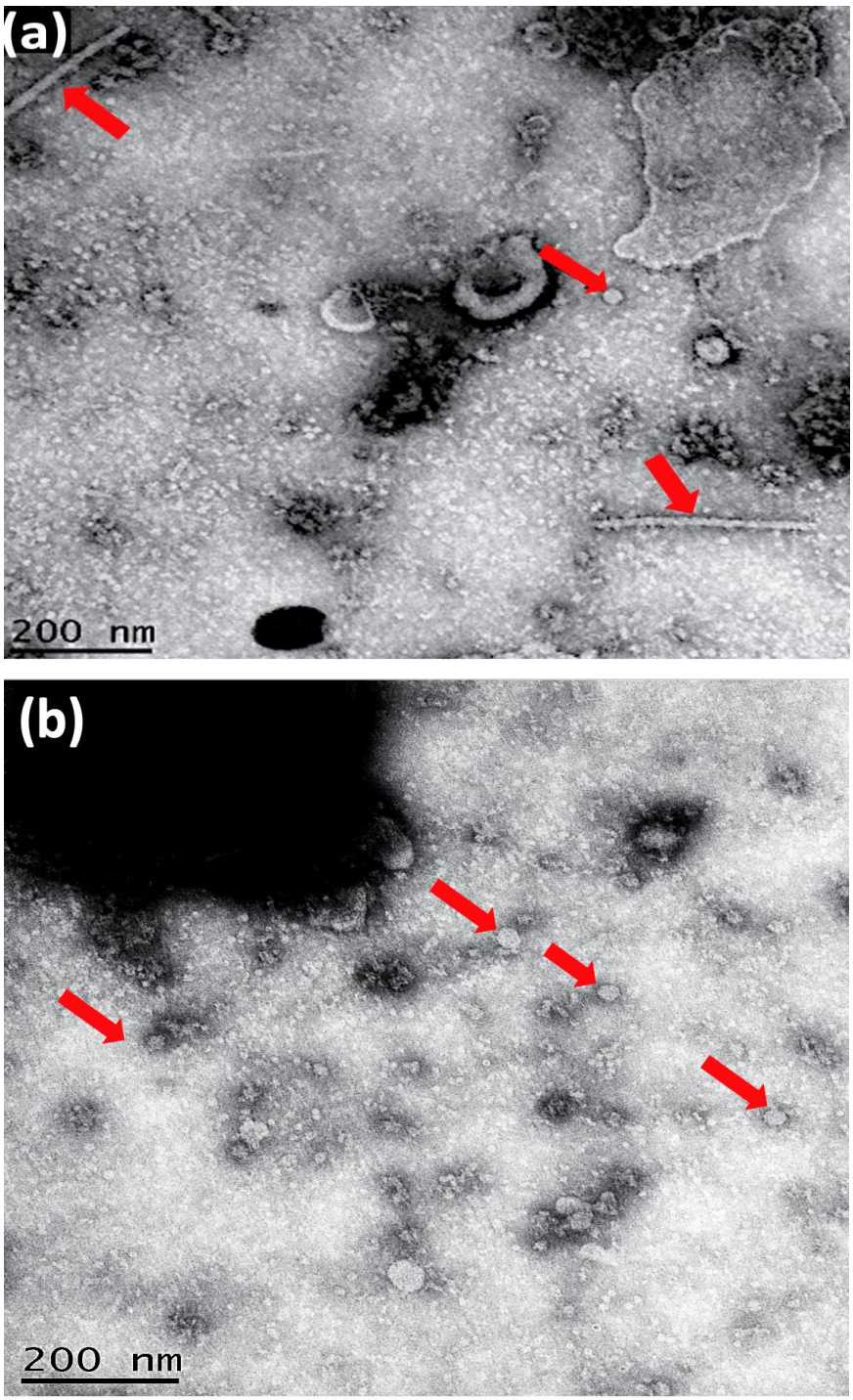

Figure 7. Electron micrograph of a leaf sap preparation made from buffalo grass cv. 'Sir Walter' showing **(a)** isometric virions approximately 30 nm in diameter and (b) flexuous virions. Virions are negatively stained with 1% ammonium molybdate pH 5.8.

Assembly and analysis of grass nepovirus genome

In order to detect new viruses without *a prior* knowledge of sequence, RNA extracts of samples were subjected to Illumina sequencing. It was used to obtain the full-length genome of a novel member of the genus *Nepovirus*. The full-length genome assemblies were confirmed by Sanger sequencing (including 5'/3' RACE). The presence of three

genera *Comovirus*, *Fabavirus*, and *Nepovirus* grouped in one subfamily *Comovirinae*, hence subfamily demarcation criterion was not defined. Nonetheless, genus and species demarcation criteria was available and listed in **Table 5** (Thompson et al., 2017). The virus followed the genomic organisation of the genus *Nepovirus* which comprises of bipartite linear single-stranded RNAs with a viral protein genome-linked (VPg) at a 5' end and a 3′ poly(A) tail (Thompson et al., 2017). The RNA 1 segment encodes nucleoside triphosphate (NTP)-binding protein, VPg, 3C-like proteinases (Pro) and RNA-dependent RNA polymerase (RdRp), and RNA 2 encodes a movement protein (MP) and single CP (Thompson et al., 2017). This virus was provisionally named "grass nepovirus". Grass nepovirus having 2 segments with 7,814 nts for RNA 1 and 7,089 nts for RNA 2 (excluding poly(A) tails), which is consistent with nepovirus genome content (RNA 1: 7,200-8,400 nts; RNA 2: 3,700-7,300 nts) (Thompson et al., 2017). Grass nepovirus RNA 1 had closest nt identity of 38.1% with cherry leaf roll virus (CLRV, Refseq accession NC_015414) in subgroup C. However, grass nepovirus RNA 2 shared the closest nt identity with blackcurrant reversion virus (BRV, RefSeq accession NC_003502) in subgroup C which was 25.0%. The protein domains of grass nepovirus RNA 1 and RNA 2 were predicted following the known cleavage sites of subgroup C viz. Q/S, Q/G and D/S (Thompson et al., 2017) (**Fig. 8**).

Table 5. Genus and species demarcation criteria of *Secoviridae* family (Thompson et al., 2017). Criteria met by grass nepovirus were highlighted.

Genus demarcation criteria

No.	Genus	*Comovirus*	*Fabavirus*	*Nepovirus*
1.	Number of genomic RNAs	2	2	2
2.	Number of protein domains / processing sites within polyprotein	8	8	7/8 (depends on subgroups)
3.	Number of coat proteins (CP)	2	2	1
4.	Presence of additional open reading frames (ORF) / subgenomic RNAs	-	-	-
5.	Clustering as a single branch in phylogenetic trees derived from amino acid (aa) sequence based on the conserved proteinase and RNA-dependent RNA polymerase (Pro-RdRp) region.			

Species demarcation criteria

No.	Criterion
1.	CP aa sequence less than 75% identity
2.	Conserved Pro-Pol region aa sequence with less than 80% identity
3.	Differences in antigenic reactions
4.	Host specificity
5.	Vectors
6.	Absence of cross-protection
7.	Absence of re-assortment between RNA 1 and RNA 2

Note: Not all the criteria needed to be met

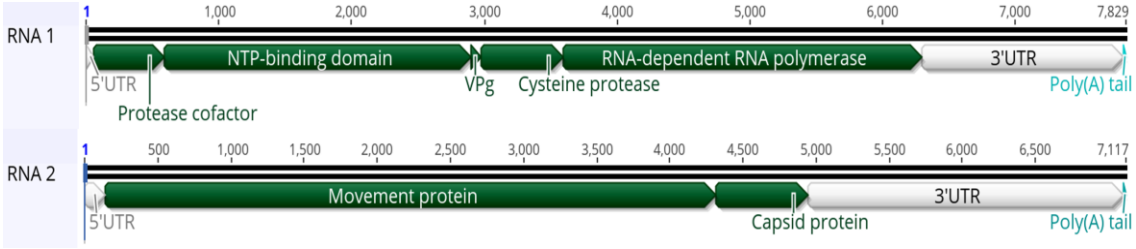

Figure 8. Genome organisation of the grass nepovirus **(a)** RNA 1 and **(b)** RNA 2, each of which encodes a single polyprotein that is potentially cleaved into the protein shown in dark green in the figure above. RNA 1 encodes protease cofactor, nucleoside triphosphate (NTP)-binding protein, viral protein genome-linked (VPg), cysteine protease and RNA-dependent RNA polymerase. RNA 2 polyprotein contains movement protein and capsid protein. Each of the RNA 5' untranslated region (UTR), 3' UTR, and poly(A) tail are indicated in grey and turquoise, respectively.

Phylogenetic analyses were completed based on the RNA 1 Pro-RdRp region and the RNA 2 CP region from the reference sequence subgroup A, B and C of nepoviruses and grass nepovirus obtained in this project (**Fig. 9**). Phylogram revealed that both RNA 1 Pro-RdRp region and RNA 2 CP region were clustered together with CLRV (RefSeq accession NC_015415), nepovirus subgroup C with high bootstrap support (**Fig. 9**). The grass nepovirus RNA 1 Pro-RdRp region shared 57.8% aa identity with CLRV. RNA 2 CP region shared aa identity of 32.4% with CLRV, respectively. Thus, grass nepovirus met the genus demarcation of one CP and 2 genomic RNA, which then grouped into genus *Nepovirus*. It also met the species demarcation by having CP and Pro-Pol region aa identity lower than 75% and 80%, respectively. Thus, it is a novel species. Electron microscopy had confirmed the presence of empty and full 30 nm isometric virus particles, consistent with the presence of a nepovirus, in a preparation made from a buffalo grass sample co-infected SCMV (**Fig. 7**)

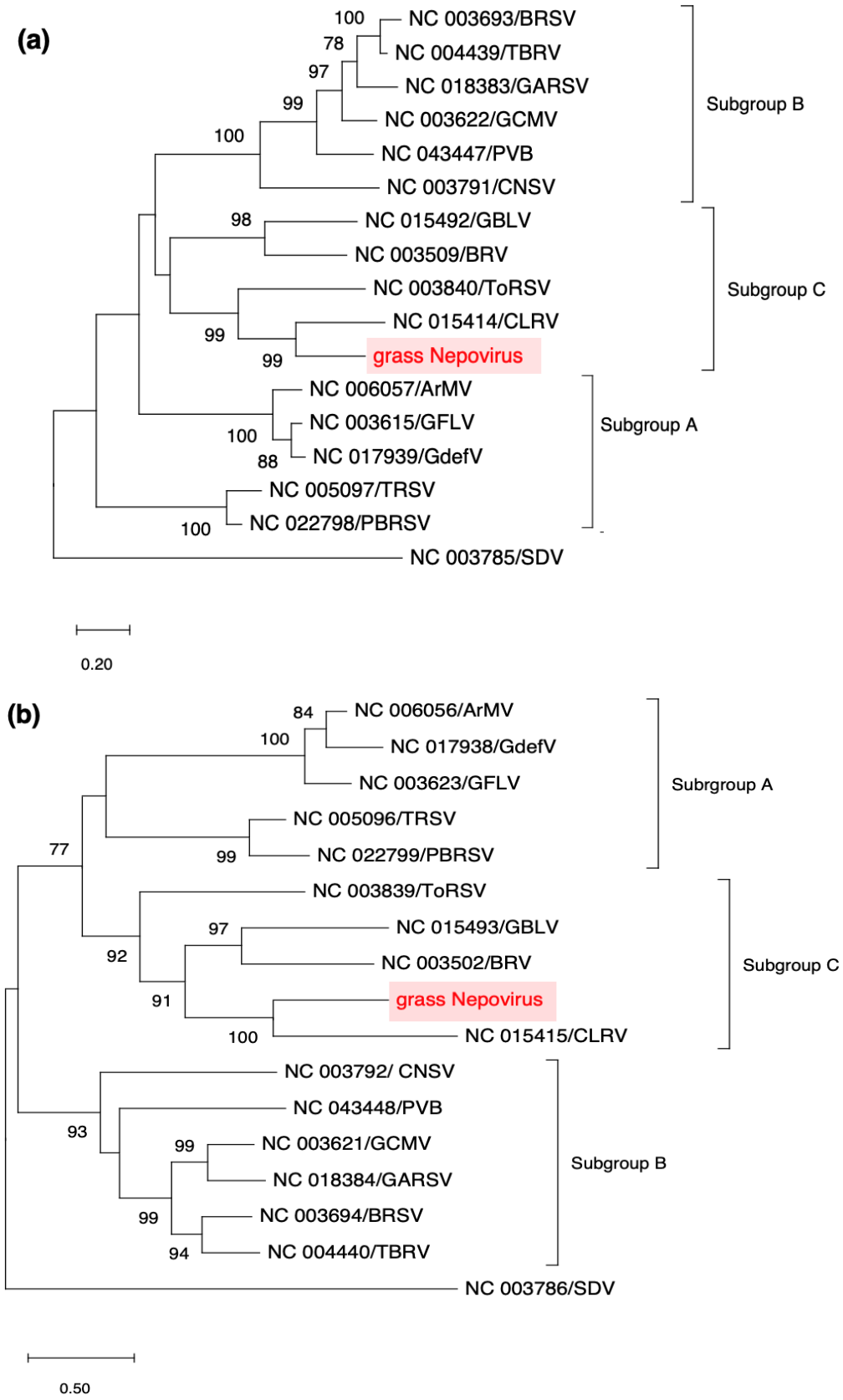

Figure 9. Phylogram based on the alignments of **(a)** RNA 1, 3C-like proteinases (Pro) and RNA-dependent RNA polymerase and **(b)** RNA 2, capsid protein encoding regions of the grass nepovirus and published nepoviruses in subgroups A, B and C. The phylogenetic tree was inferred using the Maximum likelihood method implemented with MEGA, bootstrap value from 1000 replicates greater than 70% were shown in branch nodes. Nepoviruses included in this phylogram were beet ringspot virus (BRSV), tomato black ring

virus (TBRV), grapevine anatolian ringspot virus (GARSV), grapevine chrome mosaic virus (GCMV), potato virus B (PVB), grapevine Bulgarian latent virus (GBLV), cycas necrotic stunt virus (CNSV), blackcurrant reversion virus (BRV), tomato ringspot virus (ToRSV), cherry leaf roll virus (CLRV), arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), grapevine deformation virus (GDefV), tobacco ringspot virus (TRSV) and potato black ringspot virus (PBRSV). Satsuma dwarf virus (SDV) was used as outgroup. The scale bar represented numbers of substitution per nucleotide site.

Biological purification of grass nepovirus from mixed infection of SCMV

A JGMV-resistant cultivar of maize was speculated to have resistance to SCMV. Thus, mechanical inoculations of SCMV and grass nepovirus (mixed infection) were conducted to study disease symptoms on maize and taking the advantage of maize JGMV-resistant cultivar, to biologically purify the grass nepovirus. For viral disease symptoms evaluation, mock inoculated (Pot 1 JGMV-resistant cv. 'Enterprise' and Pot 2 JGMV-susceptible cv. 'Prelude'), SCMV inoculated maize (Pot 3 JGMV-resistant cv. 'Enterprise' and Pot 4 JGMV-susceptible cv. 'Prelude') and grass nepovirus inoculated maize (Pot 5 JGMV-resistant cv. 'Enterprise' and Pot 6 JGMV-susceptible cv. 'Prelude') were remained asymptomatic after 6 dpi and 13 dpi, respectively. SCMV inoculated maize cv. 'Prelude' was showing yellow or lighter shades of greens mottling symptoms on leaf blade and younger leaves after 7 dpi (**Fig. 10**). Grass nepovirus inoculated maize JGMV-resistant cv. 'Enterprise' observed with prominent broad chlorotic streak symptoms after 14 dpi. At the same time, mock inoculated maize and SCMV inoculated maize cv. 'Enterprise', were remained asymptomatic. As the disease progresses, the yellowing and mottling was intensified but no symptoms were observed from the stalk. The height of the SCMV inoculated maize cv. 'Prelude' were significantly shorter compared to SCMV inoculated maize cv. 'Enterprise' while the height difference of the mock inoculated was not obvious (personal observation).

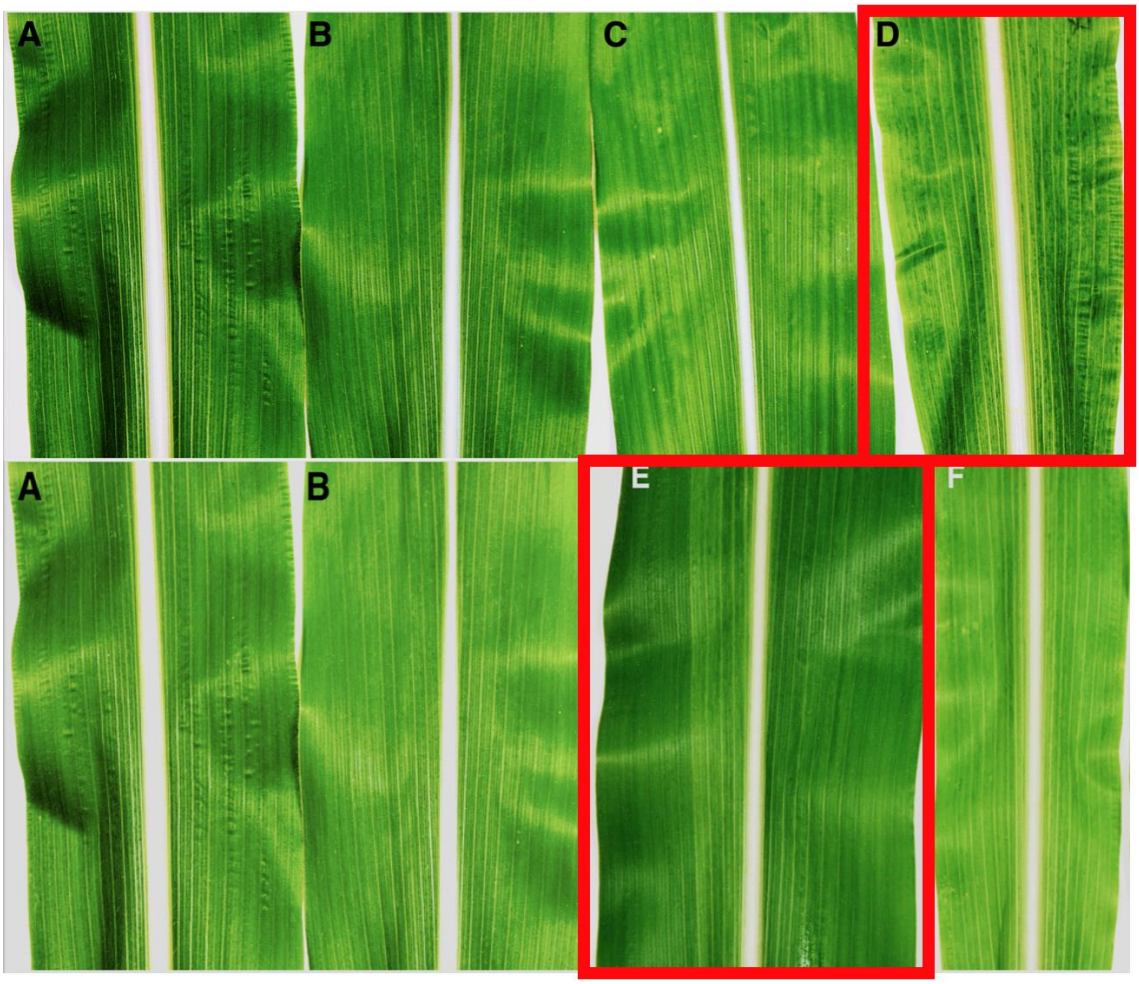

Figure 10. Host range studies of sugarcane mosaic virus (SCMV) on maize johnsongrass mosaic virus-(JGMV) susceptible cultivar (cv.) 'Prelude' and JGMV-resistant cv. 'Enterprise'. Maize **A** (Pot 1), **C** (Pot 3), **E** (Pot 5) cv. 'Enterprise', maize **B** (Pot 2), **D** (Pot 4), **F** (Pot 6) cv. 'Prelude'. Maize **A** and **B** were mock inoculated, maize **C** and **D** were SCMV inoculated, maize **E** and **F** were grass nepovirus inoculated. Mottling symptoms spotted on **D** SCMV inoculated maize JGMV-susceptible cv. 'Prelude'. Prominent broad chlorotic streak symptoms only spotted on **E** SCMV inoculated maize JGMV-resistant cv. 'Enterprise'.

Indexing by SCMV DAS-ELISA did not detect SCMV in mock inoculated maize (Pot 1 and Pot 2) and SCMV inoculated maize (Pot 3 JGMV-resistant cv. 'Enterprise') (**Table 6**). However, SCMV were detected in Pot 4. Hence, the biological purification of grass nepovirus had failed. Grass nepovirus were not transmitted to maize and it was confirmed by RT-PCR and electron microscopy (**Fig. 11**).

Table 6. Detection of sugarcane mosaic virus (SCMV) by Double Antibody Sandwich-Enzyme-Linked Immunosorbent Assay in maize johnsongrass mosaic virus- (JGMV) susceptible cv. 'Prelude' and JGMV-resistant cv. 'Enterprise' plant inoculation.

Treatment	Pot	Maize cultivar	Reaction screening
Mock	1	Enterprise	-
	2	Prelude	-
Sugarcane mosaic virus (SCMV)	3	Enterprise	-
	4	Prelude	+
Grass nepovirus (extracted from	5	Enterprise	+
SCMV co-infected sample)	6	Prelude	+

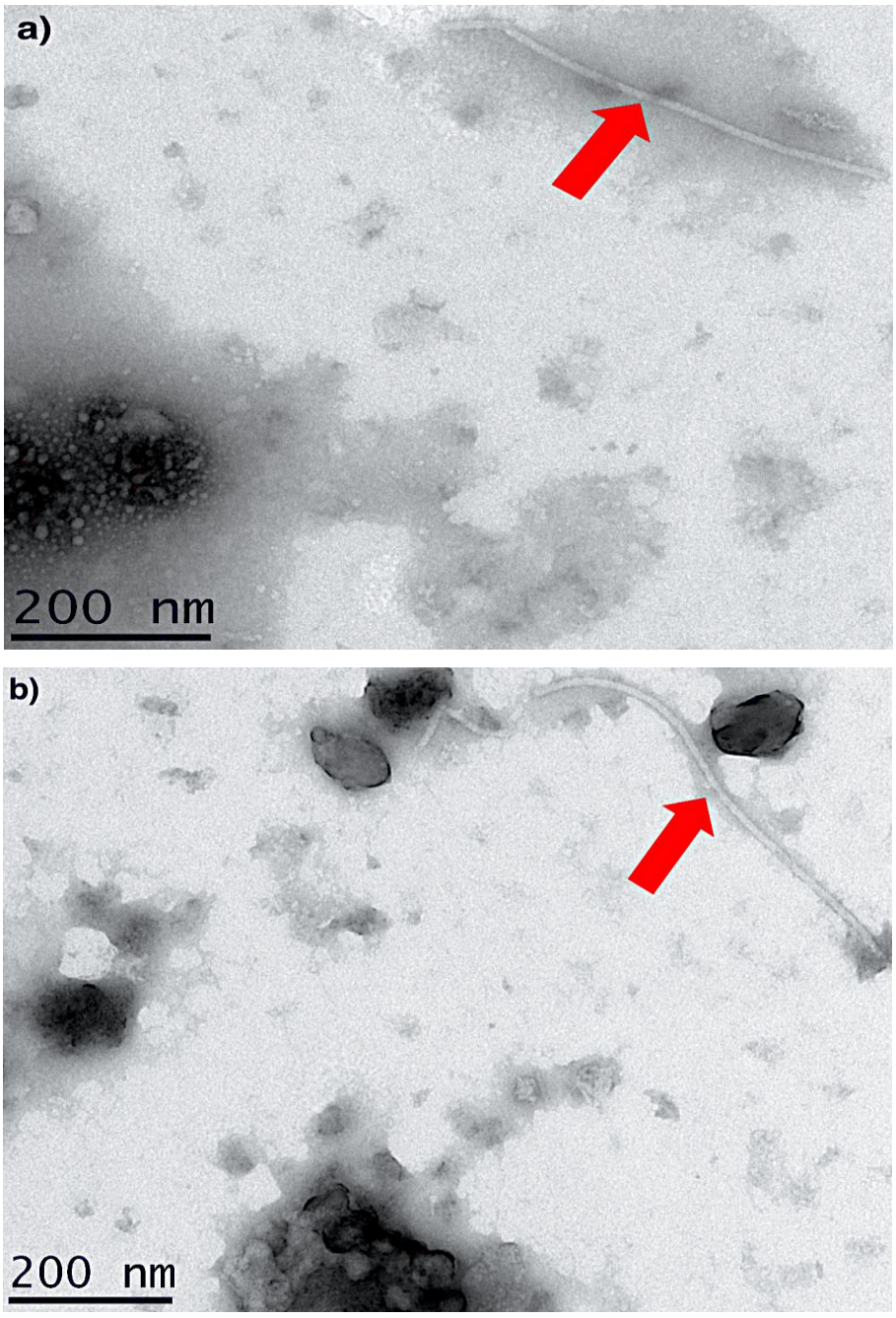

Figure 11. Electron micrographs **(a)** and **(b)** of a sap extract from Pot 5 inoculated maize cv. 'Enterprise' showing flexuous particles of SCMV only (680 – 750 nm long). Preparations were negatively stained with 1% ammonium molybdate pH 5.8.

Grass nepovirus screening

All grass samples, including those with virus infections were tested to determine if there were mixed infections. One blue couch sample and 21 buffalo grasses from eight different cultivars sample were detected prevalently in NSW, QLD and WA. In total, were 22 samples in which the grass nepovirus was detected, both in symptomatic and asymptomatic turf. Therefore, the disease symptoms of novel grass nepovirus was not be definable. The phylogenetic tree of grass nepovirus were inferred with partial RdRp sequence (265-309 nts) (**Fig. 12**). Grass nepovirus isolates detected in this study were closely related to each other, pairwise identity showed that sequences shared 91.1-100% of nt identity and 96.5-100% of aa identity (data not shown). They clustered with grass nepovirus exemplar isolate, sample 6 with high bootstrap support, but sample 29 was genetically more distant (**Fig. 12**). Single infection of grass nepovirus were detected in samples 63, 64, 94, 95, 97, 110 and 111 (**Table 7**).

Table 7. Description of 22 isolates of grass nepovirus in Australia.

States[a]	Accession[b]	Grass species	Cultivar	Virus[c]
NSW	5614	*Stenotaphrum secundatum*	Shade Master	SCMV
	5616	*S. secundatum*	Maltida	SCMV
	6	*S. secundatum*	Sir Walter	SCMV
	7	*S. secundatum*	Palmetto	PMV + SCMV
	27	*S. secundatum*	Sir Walter	SCMV
	29	*S. secundatum*	Palmetto	PMV + SCMV
	35	*S. secundatum*	Palmetto	PMV
	37	*S. secundatum*	Sapphire	SCMV
	41	*S. secundatum*	Prestige	SCMV
	94	*S. secundatum*	Maltida	n/a
	97	*S. secundatum*	Maltida	n/a
	95	*S. secundatum*	Prestige	n/a
	103	*S. secundatum*	Palmetto	PMV
	108	*S. secundatum*	Sir Walter	SCMV
	110	*S. secundatum*	Maltida	n/a
	111	*S. secundatum*	Kings Pride	n/a
	118	*S. secundatum*	Palmetto	PMV
QLD	14	*S. secundatum*	Prestige	SCMV
	89	*S. secundatum*	Sir Walter	n/a
WA	5626	*Digitaria didactyla*	Queensland blue	SCMV
	63	*S. secundatum*	Palmetto	n/a
	64	*S. secundatum*	Neergabby (ST15)	n/a

[a] Location: New South Wales (NSW); Queensland (QLD); Western Australia (WA).

[b] Accession: Queensland Department of Agriculture and Fisheries (QDAF) Plant Virus Collection; Private sample identifier.

[c] Virus acronyms: sugarcane mosaic virus (SCMV); Panicum mosaic virus (PMV).

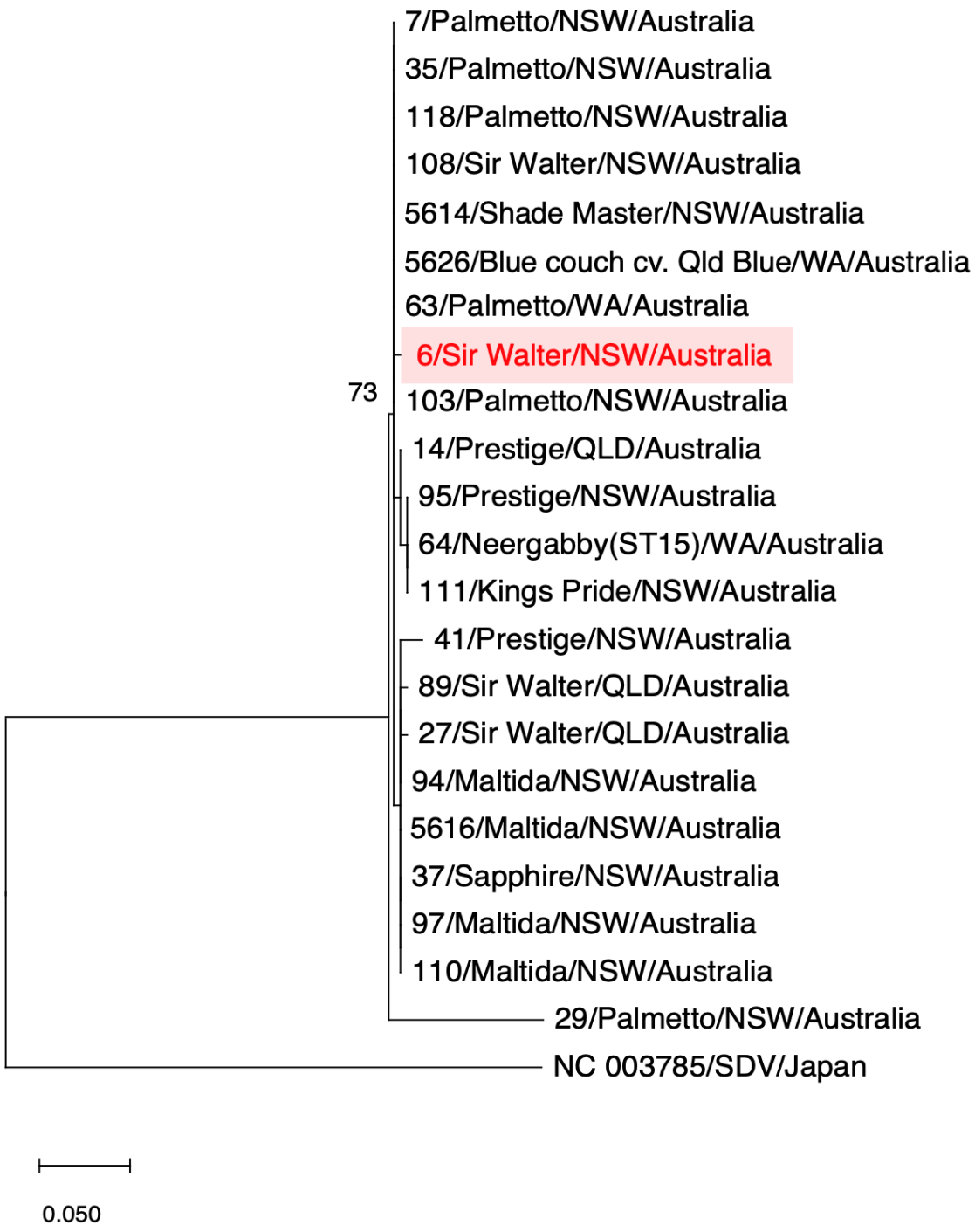

Figure 12. Phylogram showing inferred evolutionary relationships of grass nepovirus, obtained from the present study, based on the alignments of the partial RNA-dependent RNA polymerase sequences (265-309 nts). The phylogenetic tree was inferred using the Maximum likelihood method implemented with MEGA, bootstrap value from 1000 replicates greater than 70% were shown in branch nodes. Satsuma dwarf virus (SDV) was used as outgroup. The scale bar represented numbers of substitutions per site.

The known monocot host of grass nepovirus were buffalo grass and blue couch grass. Previously, the known host range of nepoviruses were dicotyledons (Martelli and Taylor, 1990; Thompson et al., 2017). Nepoviruses are known to have nematode or mite vectors

(Mayo and Robinson, 1996; Thompson et al., 2017; Wellink et al., 2000), though no vector is known for CLRV (Jones et al., 1981). The transmission mode of the grass nepovirus remains unknown and there are no definable disease symptoms as it was detected from both asymptomatic and symptomatic samples. Thus, the association of grass nepovirus in causing buffalo grass yellowing to needs be investigated.

Host susceptibility of panicum mosaic virus

During surveys, PMV was only detected in buffalo grass cv. 'Palmetto', thus host susceptibility of other buffalo grass cultivars to PMV was investigated. Healthy buffalo grass cv. 'Palmetto', 'Sir Walter', 'Prestige', and 'Grand Royal' pots were prepared as 5 replicates. Two replicates were mock inoculated, 3 replicates were inoculated with PMV. Buffalo grass cv. 'Grand Royal' had striations symptoms but all the turfs in this host range study were confirmed to be PMV-free by RT-PCR before the PMV inoculation. Viral disease symptoms observations were made daily for 26 dpi. Viral disease symptoms of yellowing and mottling only observed on cv. 'Palmetto' after 19 dpi (**Fig. 13**). Buffalo grass cv. 'Sir Walter', 'Prestige' and 'Grand Royal' remained asymptomatic.

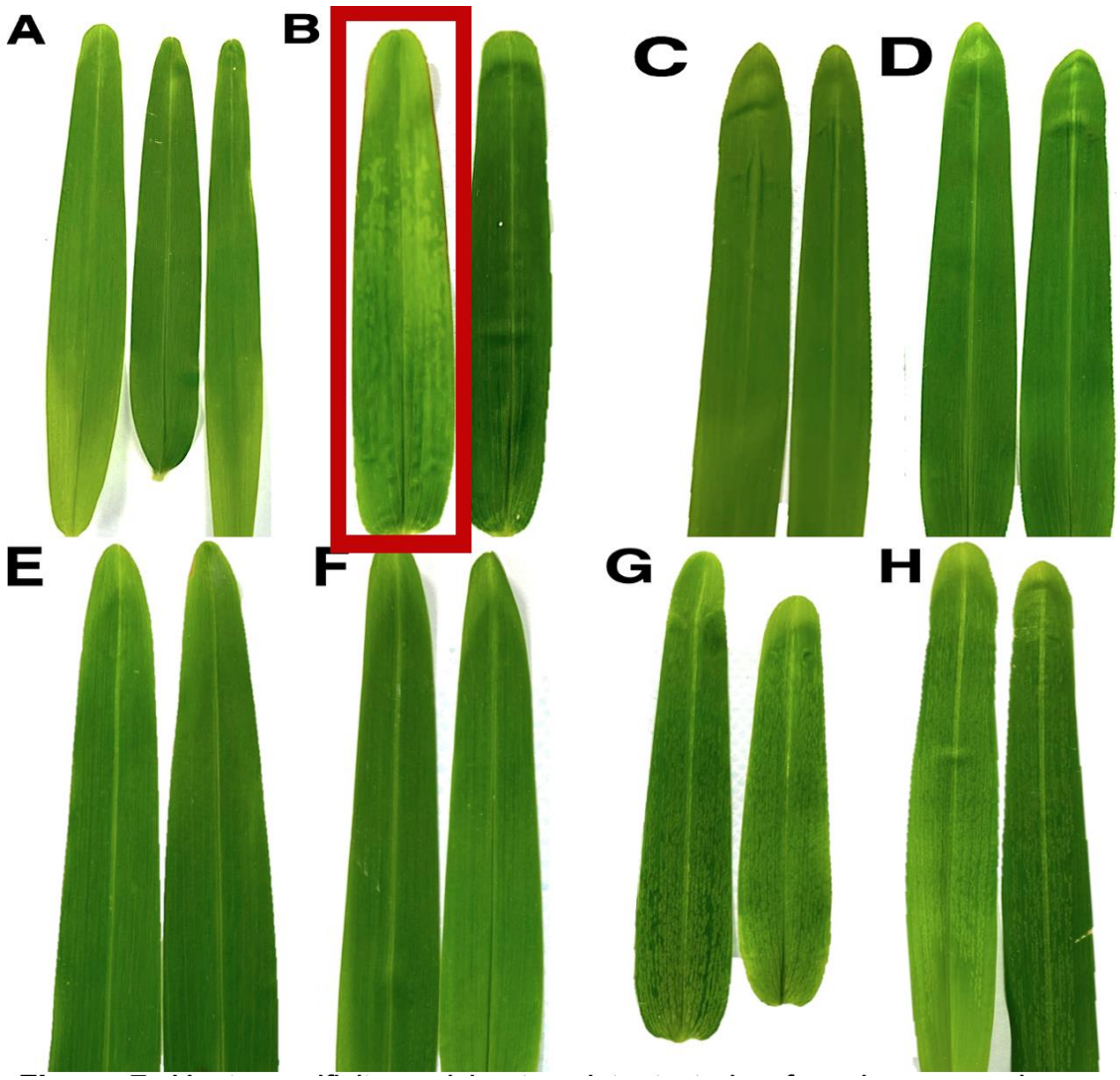

Figure 13. Host specificity studies of panicum mosaic virus (PMV) on buffalo grass cultivar (cv.) 'Palmetto', 'Sir Walter', 'Prestige', and 'Grand Royal'. 'Palmetto' **(A)**, 'Sir Walter' **(C)**, 'Prestige' **(E)**, and 'Grand Royal' **(G)** were mock inoculated. 'Palmetto' **(B)**, 'Sir Walter' **(D)**, 'Prestige' **(F)**, and 'Grand Royal' **(H)** were PMV inoculated. Mottling symptoms and yellowing expressed on cv. 'Palmetto' **(B)** only (red). Striations on cv. 'Grand Royal' **(G)** and **(H)** are not disease symptoms known to associate with PMV.

Virus indexing by PMV DAS-ELISA was conducted after dpi 26 and showed that PMV only infected cv. 'Palmetto' (**Table 8**). PMV was not transmitted to buffalo grass cv. 'Sir Walter', 'Prestige' and 'Grand Royal'. As limited number of plants were inoculated in this experiment, a trial with more cultivars and more replica could be conducted in the future in order to confirm these results.

Table 8. Detection of PMV by Double Antibody Sandwich-Enzyme-Linked Immunosorbent Assay in buffalo grass cv. 'Palmetto', 'Sir Walter', 'Prestige', and 'Grand Royal' turf inoculation.

Buffalo grass cultivar	Reaction screening
Palmetto	+
Sir Walter	-
Prestige	-
Grand Royal	-

BGLV host transmission study on maize

Mechanical inoculations BGLV were conducted to study disease symptoms on maize and host range of BGLV. For viral disease symptoms evaluation, mock inoculated (Pot 1 JGMV-resistant cv. 'Enterprise' and Pot 2 JGMV-susceptible cv. 'Prelude'), BGLV inoculated maize (Pot 7 JGMV-resistant cv. 'Enterprise' and Pot 8 JGMV-susceptible cv. 'Prelude') were remained asymptomatic after 6 dpi and 13 dpi, respectively. BGLV inoculated maize (Pot 7 JGMV-resistant cv. 'Enterprise' and Pot 8 JGMV-susceptible cv. 'Prelude') were still remained asymptomatic throughout the glasshouse trial (**Fig. 14**). BGLV were not transmitted to maize and it was confirmed by RT-PCR.

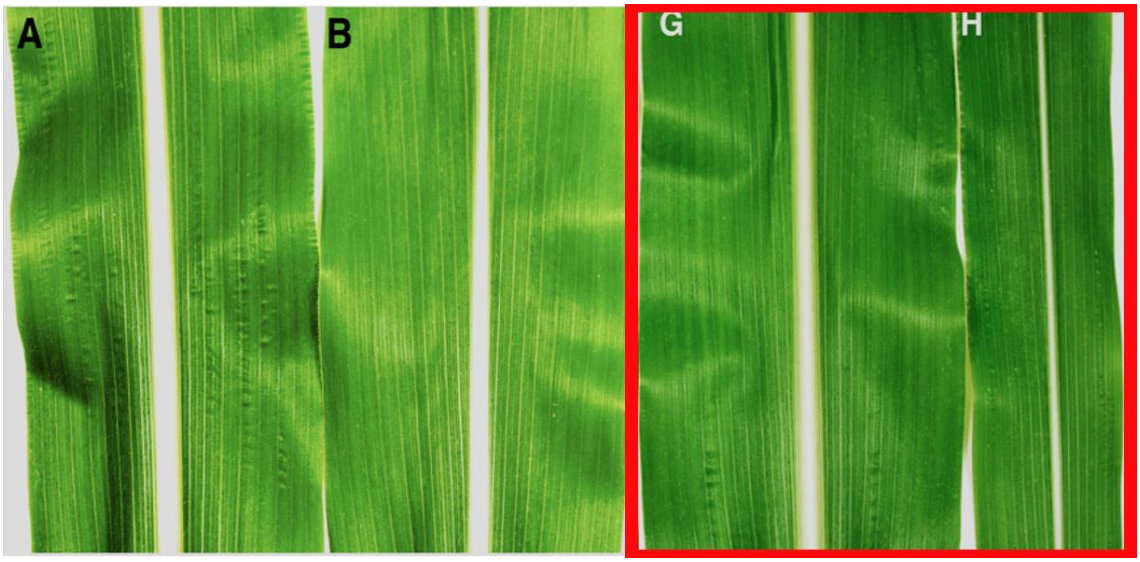

Figure 14. Host transmission studies of bermuda grass latent virus (BGLV) on maize johnsongrass mosaic virus- (JGMV) susceptible cultivar (cv.) 'Prelude' and JGMV-resistant cv. 'Enterprise'. Maize **A** (Pot 1), and **G** (Pot 7) cv. 'Enterprise', maize **B** (Pot 2) and **H** (Pot 8) cv. 'Prelude'. Maize **A** and **B** were mock inoculated, while maize **G** and **H** were BGLV inoculated. No disease symptoms were observed on maize **G** and **H** (red).

Conclusions

Mosaic viruses in this study were widely distributed in NSW, followed by QLD then WA. SCMV and PMV were a major cause in buffalo grass yellowing. Each of the viruses were introduced into Australia from different origins. In this honours project, SCMV in buffalo grass were genetically diverse. It speculated some of the isolates may spread or introduced in planting material from other SCMV strains in Australia. PMV was closely related to the PMV buffalo grass isolate originally detected in Australia and apparently restricted to the buffalo grass cv. 'Palmetto'. It was apparently single introduction and spread through vegetative propagation and local transmission. BGLV were detected in green couch and it was closely related with the BGLV only other reported isolate which was from the USA. A novel grass nepovirus was detected in eight buffalo grass cultivars and one blue couch sample from predominantly from NSW, followed by QLD and WA. Genome of grass nepovirus was characterised but the association with buffalo grass yellowing remains unclear.

Suggestions for future research

Phylogenetic analyses showed that SCMV isolates in this study were closely related to SCMV isolates from Mexican and East African maize, but there is no available sequence of an Australian SCMV isolate from maize. Therefore, surveys of plantation nearby the SCMV infected turf farm should be conducted in the future and it would assist the study of SCMV introduction into buffalo grass. Genome characterisation and more research should be done regarding PMV due to there is no full genome published yet. In addition, further research in PMV susceptibility using assorted buffalo grass cultivar would be beneficial in controlling. Association of buffalo grass yellowing and the novel grass nepovirus shall be determined by mechanically inoculating buffalo grass with or without the combination of SCMV and PMV. More researches regarding relatively new discovered BGLV and grass nepovirus should be done because there is currently very limited information available. Co-existence of SCMV and BGLV in Australia has been proved, hence controlling BGLV from spreading into buffalo grass should be prioritised to prevent lethal viral necrosis in buffalo grass and threatening turf industry. Taking everything into consideration, turf viral disease surveillance on other states should be conducted, since the viruses that cause buffalo grass yellowing are not restricted only in NSW, QLD and WA.

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Appendices

Appendix I. Plant materials details sampling from turf farm or location throughout New South Wales (NSW), Queensland (QLD), and Western Australia (WA). Viral symptoms of sugarcane mosaic virus (SCMV), panicum mosaic virus (PMV) and mastrevirus were applied as part of the criterion for sample collection.

Date (DD/MM/YY)	Farm / No.	States	Turf/cultivars	No. of samples collected	Virus names
24/10/18	1	QLD	Buffalo grass	1	-
	2	QLD	Buffalo grass	2	-
	3	NSW	Buffalo grass / cv. 'Shade master'	1	SCMV
	3	NSW	Buffalo grass / cv. 'Sir Walter'	1	SCMV
	4	NSW	Buffalo grass / cv. 'Prestige'	1	-
04/04/19	4	NSW	Buffalo grass / cv. 'Sir Walter'	1	-
	5	NSW	Buffalo grass / cv. 'Palmetto'	1	PMV, SCMV
	5	NSW	Buffalo grass / cv. 'Sapphire'	1	-
	6	NSW	Buffalo grass / cv. 'Maltida',	1	SCMV
	7	NSW	Buffalo grass / cv. 'Kings Pride'	1	-
18/04/19	2	QLD	Buffalo grass	2	-
11/06/19	8	QLD	Buffalo grass	2	SCMV
09/09/19	9	NSW	Buffalo grass / cv. 'Palmetto'	2	PMV
18/09/19	10	NSW	Buffalo grass / cv. 'Sapphire'	2	SCMV
10/12/19	11	QLD	Broadleaf carpet grass	1	-
11/02/20	12	QLD	Green couch	1	BGLV

	12	QLD	Buffalo grass / cv. ‘Palmetto’	6	-
	12	QLD	Blue couch	1	SCMV
	13	QLD	Buffalo grass / cv. ‘Sir Walter’	2	-
	13	QLD	Johnsongrass	1	-
	14	QLD	Buffalo grass / cv. ‘Sir Walter’	2	-
	14	QLD	Buffalo grass / cv. ‘Sapphire’	1	-
	14	QLD	Buffalo grass / cv. ‘Prestige’	6	SCMV
21/02/20	15	QLD	Buffalo grass / cv. ‘Sapphire’	1	-
	15	QLD	Buffalo grass / cv. ‘Kings Pride’	1	-
	16	QLD	Buffalo grass / cv. ‘Sapphire’	1	-
	17	QLD	Buffalo grass / cv. ‘Sir Walter’	1	-
26/02/20	18	QLD	Sabi grass	1	SCMV
02/03/20	3	NSW	Buffalo grass / cv. ‘Sir Walter’	2	SCMV
	9	NSW	Buffalo grass / cv. ‘Sapphire’	1	PMV
	9	NSW	Summer grass	1	PMV
	10	NSW	Buffalo grass / cv. ‘Sir Walter’	2	SCMV
	10	NSW	Buffalo grass / cv. ‘Sapphire’	1	SCMV
	10	NSW	Buffalo grass / cv. ‘Palmetto’	1	PMV and SCMV
	10	NSW	Johnsongrass	1	JGMV
	10	NSW	Summer grass	1	SCMV
	19	NSW	Buffalo grass / cv. ‘Sir Walter’	2	SCMV
03/03/20	20	NSW	Buffalo grass / cv. ‘Sapphire’	3	-
	20	NSW	Buffalo grass / cv. ‘Sapphire’	1	SCMV

	20	NSW	Buffalo grass / cv. ‘Prestige’	1	SCMV
	21	NSW	Buffalo grass / cv. ‘Sapphire’	2	SCMV
	21	NSW	Buffalo grass / cv. ‘Sapphire’	1	-
	22	NSW	Buffalo grass / cv. ‘Sir Walter’	2	-
	22	NSW	Buffalo grass / cv. ‘Sir Walter’	2	SCMV
04/03/20	23	NSW	Buffalo grass / cv. ‘Sir Walter’	3	SCMV
	23	NSW	Buffalo grass / cv. ‘Sir Walter’	1	-
	24	NSW	Buffalo grass / cv. ‘Sir Walter’	3	SCMV
	24	NSW	Buffalo grass / cv. ‘Sir Walter’	1	SCMV
	25	NSW	Buffalo grass / cv. ‘Maltida’	3	-
	26	NSW	Buffalo grass / cv. ‘Prestige’	1	-
	27	NSW	Buffalo grass / cv. ‘Kings Pride’	1	-
	28	WA	Buffalo grass / cv. ‘Sir Walter’	1	-
	28	WA	Buffalo grass / cv. ‘Palmetto’	5	-
	28	WA	Buffalo grass / cv. ‘Palmetto’	1	SCMV
17/03/20	29	WA	Buffalo grass / cv. ‘Sir Walter’	2	-
	30	WA	Buffalo grass / cv. ‘Sir Walter’	2	-
	30	WA	Blue couch / cv. ‘Queensland blue’	2	SCMV
	31	WA	Buffalo grass / cv. ‘Palmetto’	2	-
	31	WA	Buffalo grass / cv. ‘Palmetto’	1	SCMV
	32	WA	Buffalo grass / cv. ‘Sapphire’	1	-
18/03/20	32	WA	Buffalo grass / cv. ‘Prestige’	4	-
	33	WA	Buffalo grass / cv. ‘Palmetto’	5	-

	33	WA	Buffalo grass / cv. ‘Sir Walter’	1	-
	33	WA	Buffalo grass / cv. ‘New Frontier’	1	-
	35	WA	Buffalo grass / cv. ‘Sir Walter’	1	-
	36	WA	Buffalo grass / cv. ‘Sir Walter’	1	-
20/03/20	31	WA	Buffalo grass / cv. ‘Neergabby (ST15)’	1	-
20/04/20	11	QLD	Blue couch	1	Mastrevirus

Appendix II. Primers used in RT-PCR for virus characterisation.

Virus	Primer name	Sequence 5' to 3'	Annealing temperature (°C)
Grass *Nepovirus* RNA 1	unk2_F1	ATGGCGAACGCATACAACGATTG	61
	unk2_R1	ATTAAAAGCCACCATACCACCACC	
	unk2_F2	GGTTGCTTGTGCAATACAGGTTAG	61
	unk2_R2	GGCAAGATTTCAGCCGTGAGC	
	unk2_F3	AGGTGACTGGCAAACAAGCAG	55
	unk2_R3	CTGCTGATCTCGCTCATGTT	
	unk2_F4	GCAACCTTGTGCCTCTTCACAGGC	57
	unk2_R4	GCAATTGTAAGCGCCTGATGGTAGG	
	unk2_F5	AGTGCAGGATGTGAAGGATAAGGAG	52
	unk2_R5	TCGACAAACTGCTCCTCATCATTGCC	
	unk2_F6	GAAGTCGCTACTGAAATGGTGG	55
	unk2_R6	CTGGTGGAACTCTGCTGCCA	
	unk2_F7	TGAGAAGGGAATGCCACTTACC	55
	unk2_R7	CTATCGTGGTGGAGAGCTC	
	RNA1-F1	ACTCTTACCTCTTTACCCTGTGTC	62
	RNA1-R1	GAGTCTCTTCATCTGGCGTACC	
	RNA1-R2	CATGTGTAATATTGGCTCATTGGC	58
	unk2_F5	AGTGCAGGATGTGAAGGATAAGGAG	
	RNA1-F3	ATCAAGGAGCCTGCTATCTTATCCG	68
	RNA1-R3	CGCTTGTTCCTTCCTTAAGGCGC	
	RNA1-F4	GATAGTTGCAATGTGTGGCCGC	71
	RNA1-R4	CCTTGTTCTGGCACGACGACATGC	
	RNA1-F5	GTCGATTACCTACAGAAGCGTG	58
	unk2_R7	CTATCGTGGTGGAGAGCTC	
Grass *Nepovirus* RNA 2	BRV2_F0	TGCAGCCTTGGAGAAACACTCGTT	63
	BRV2_R0	CATTGCGAGGTGGTTCGGCTA	
	BRV2_F1	GGAATTCCCACTGGTGCTAATGC	67
	BRV2_R1	CTAGGATTTAGAGACTCGTAACGC G	
	BRV2_F2	CCAGCACACTACAACATGTTG	64
	BRV2_R2	GTCTAAGGAGCGTGCACAC	
	BRV2_F3	CCAGTATCATCATCTACAATCCATGC	64
	BRV2_R3	GCCACTAGAACAGCATACTG	
	BRV2_F4	GCATACATACCAAGCTTCGCC	67
	BRV2_R4	TCGCGGGTTCGTGATCGTT	
	BRV2_F5	CTTGGGAGGACATTGTAAGG	58
	BRV2_R5	ACAACCCGCGTTCAATCATTCC	
	BRV2_F6	GGCTCCTCAACCTCACAATA	61
	BRV2_R6	AACGCCGATAGATGGCAC	

	BRV2_F7	CAGCAGAGTTCTCCAATATAGG	57
	BRV2_R7	CGGATTCCTCCTTGAGTGTTC	
	RNA2-F1	AACGAGTGTTCTCCAAGGCTGCA	62
	RNA2-R1	CGAACTCCTGTCTCTGCGTGAGA	
	RNA2-F2	GAATGCCCTCATATGAGGGC	63
	RNA2-R2	CCAATGTGCAGTGGTGCAGG	
	RNA2-F3	TGCTCTCATCGACCTGTGAAGG	62
	RNA2-R3	GGAAGTACGTTGCTAACACC	
	RNA2-F4	ACACCAATGAGTCACCTGCT	65
	RNA2-R4	TAAGCAGAGGTAGCCATGTATG	
	RNA2-F5	GGTTTCGGGTACTGAGGATGA	62
	RNA2-R5	TGGGCCACTGGACTAACTTA	
	RNA2-F6	TACTTCATGCCAATGTTGCCAC	59
	RNA2-R6	CTCACTACTCAGTTCGGTAGG	
	RNA2-R7	CAGAGCAGTGTTAATTCAAAGG	60
	RNA2-SP5-F1	GGCAAAGCATCCTTGAGGTCAA	