Eur J Plant Pathol
https://doi.org/10.1007/s10658-024-02832-1

Characterization of macadamia ringspot-associated virus, a novel *Orthotospovirus* associated with *Macadamia integrifolia* in South Africa

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Accepted: 9 February 2024

Abstract South Africa is the largest producer of macadamias in the world, producing an estimated 61,288 tons nut-in-shell in 2022. In order to ensure the sustainability of the industry, it is important that research focuses on the control and eradication of economically important pests and diseases. Macadamia trees in the Mpumalanga Lowveld of South Africa have for some time shown severe chlorosis, which coincides with a significant drop in production, with losses of up to 60% being recorded. The disease has since been coined Macadamia Chlorosis Disease (MCD). In an attempt to determine whether MCD may be associated with a virus, high-throughput Illumina sequencing was performed on RNA extracted from both diseased and healthy trees collected from farms in Mpumalanga. Subsequent data analyses could not link a specific virus to MCD, however, reads spanning the full genome of a novel virus belonging to the *Orthotospovirus* genus were obtained. An RT-PCR assay was optimized for the detection of this virus and subsequent surveys linked the virus to ringspot symptoms which are commonly observed on different macadamia cultivars. The virus has to date been identified from orchards in Mpumalanga, Limpopo and KwaZulu-Natal. Other viruses described in the genus are known to cause severe crop losses and it is therefore important that the virus, provisionally named macadamia ringspot-associated virus (MRSV), be further studied to determine whether association with this virus can lead to yield losses, and whether appropriate control strategies must be implemented to prevent the spread of MRSV.

Keywords *Orthotospovirus* · Macadamia · Ringspot · High throughput sequencing

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-024-02832-1.

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Introduction

South Africa is the world leader in macadamia production with 61,288 tonnes nut-in-shell macadamia

Published online: 06 March 2024

being produced in 2022, having an annual production value of ZAR 4.8 billion (approximately 300 million USD) (SAMAC). Macadamia is mainly grown in Limpopo, Mpumalanga and KwaZulu-Natal provinces, with some production occurring in the Eastern- and Western Cape provinces. The main industry is export-based, with 98% of macadamias being exported internationally, primarily to East and Southeast Asia. In order to ensure the continuation of the industry, it is important that research focuses on the control and eradication of economic important pests and diseases. This becomes challenging in the face of new and emerging pests and diseases. The emergence of new diseases in plants is typically triggered by modern agricultural practices, which places large numbers, of often exotic plant species, in direct contact with potential pathogens, with viruses accounting for ~ 50% of these (Bernardo et al., 2017). Microbial diseases of macadamia in South Africa have, until now, primarily been associated with a variety of fungal species. Among these are *Cladosporium cladosporioides*, which causes raceme blight (van den Berg et al., 2008), as well as *Colletotrichum gloeosporioides* and *Phomopsis spp.*, which have been associated with husk rot (Akinsanmi & Drenth, 2016).

Since 2016, macadamia producers have noticed a decline in the production of trees which are showing yellowing of leaves on single branches, which would eventually spread throughout the tree canopy. Various tests have been conducted to determine whether this yellowing is caused by abiotic factors or by the presence of an unknown fungal or bacterial pathogen. None of these tests could however give conclusive answers. The yellowing symptoms observed, as well as the spread of this symptom in orchards, would suggest that the causal agent associated with this symptom is biological in nature. The name, 'macadamia chlorosis disease' (MCD), has since been used to describe the yellowing symptoms observed on macadamia trees.

Infections by viruses can often result in the expression of similar symptoms in host plants (Matus et al., 2008), such as decline, chlorosis, necrosis and leaf roll (Scholthof et al., 2011). A large diversity of viruses are associated with fruit tree species (Umer et al., 2019) with a few being documented on nut trees. Hazelnuts have been shown to be infected with the ilarviruses, apple mosaic virus (Grimova et al., 2016) and prunus necrotic ringspot virus (Sokmen et al., 2005), whereas the *Nepovirus,* cherry leaf roll virus, has been found to be associated with blackline disease of walnut (Ferretti et al., 2017). Whereas in macadamia specifically, the *orthotospovirus,* watermelon silver mottle virus (WSMoV) have been associated with necrotic leaf symptoms in China (Zhang et al., 2021). This is the only known detection of a virus from members of the *Proteaceae* (Summerell, 2018).

The detection and diagnosis of viruses and virus-like diseases are often confounded by the lack of universal marker genes (Sullivan, 2015). High-throughput sequencing (HTS), unlike ELISA, PCR and LAMP, provides a non-targeted approach for the sequence of the entire complement of DNA or RNA present in a sample. It has been used to great effect in the discovery of plant viruses (Barba et al., 2014). The ability to detect the majority of potential pathogens within a plant sample is the greatest advantage of using HTS, as well as the potential to expedite the development of routine diagnostic techniques (Maree et al., 2018).

Within the current study, HTS technologies were employed to determine whether a virus is associated with MCD in South Africa. Furthermore, we describe the occurrence of a novel *orthotospovirus* associated with concentric ringspot symptoms being observed in Macadamia orchards in South Africa.

Materials and methods

Sample collection 2019 and high throughput sequencing analyses (HTS)

During 2019, samples were collected from five different macadamia farms, and one commercial nursery around the Mpumalanga Lowveld, South Africa (Table 1). From each farm, leaves from field trees showing yellowing or chlorosis symptoms as well as visually healthy control samples, originating from the same orchard, were collected. At farms where nurseries trees are kept, samples were collected from nursery trees as well. Each sample was assigned a unique accession number and the cultivar, age, symptoms observed and GPS position of each tree, was recorded. A photo was taken of each sample collected.

From these samples, total RNA was extracted from the midribs and petioles using the method described by

Table 1 Number of samples collected from Mpumalanga during 2019 with information on tree age, cultivar and symptoms recorded

Farm name	Tree age (year)	Cultivar	Number of trees collected	Leaf symptoms recorded
Kudu Farms	Nursery	'Nelmak 2'	6	Chlorosis
			1	Healthy
	Nursery	'H2'	3	Chlorosis, Stunting
			1	Healthy
	14	'HAES788'	4	Chlorosis, Die back
			1	Healthy
	15	'Beaumont'	3	Chlorosis, Die back, Necrosis
			1	Healthy
	23	'A16'	3	Chlorosis, Mottle
			1	Healthy
	24	'HAES816'	3	Chlorosis, Die back, Mottle
			2	Healthy
Esperia	8	'Beaumont'	3	Chlorosis, Mottle, Necrosis
	11	'Beaumont'	3	Chlorosis, Mottle, Necrosis
			1	Healthy
Stellenrust	1	'Beaumont'	3	Chlorosis, Stunting
			1	Healthy
	25	'Beaumont'	4	Chlorosis, Mottle, Necrosis
			1	Healthy
	25	'HAES788'	4	Chlorosis, Mottle, Necrosis
			1	Healthy
Friedenheim	15	'Nelmak 2'	1	Healthy
	20	'Beaumont'	3	Chlorosis, Mottle, Necrosis
			2	Healthy
Schagen	12	'Beaumont'	3	Chlorosis, Mottle, Necrosis, Stunting
			1	Healthy
	16	'Beaumont'	2	Chlorosis, Mottle
	25	'Beaumont'	1	Chlorosis, Mottle, Necrosis, Stunting
			1	Healthy
Commercial Nursery	Nursery	'Beaumont'	2	Chlorosis, Mottle
			1	Healthy
		'Nelmak 2'	1	Healthy
		'816'	1	Healthy
		'HAES716'	1	Healthy

White et al. (2008). Each RNA sample was assessed using a Qubit 2.0 with the RNA broad range reagent (Invitrogen, Waltham, MA, USA) and NanoPhotometer N60 (Implen, Munich, Germany). Extracted RNA meeting the minimum quality criteria (20 ng/⌈l; 260/280=2, 260/230=2.2) was used to prepare RNAtag-seq libraries according to Shishkin et al. (2015), with ribodepletion performed using RiboZero Plant Leaf (Illumina, San Diego, CA, USA). The resulting libraries were sequenced on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) at the Agricultural Research Council – Biotechnology Platform, Pretoria, South Africa. Datasets for 63 individual samples were demultiplexed using Je (Girardot et al., 2016). Assemblies were performed using metaSPAdes 3.14.0 (Nurk et al., 2017) and putative viral contigs identified using the NCBI viral refseq and the viral fraction of the nr database, using blastn and blastx, respectively (Altschul et al., 1990).

Phylogeny of novel *orthotospovirus*

Open reading frames (ORFs) for the various proteins encoded by their respective genomic segment were determined using ORF finder (https://ncbi.nlm.nih.gov/orffinder). The amino acid sequences obtained from ORF finder were aligned with recognized orthotospoviruses listed on ICTV as well as the updated revision done in 2019 (Abudurexiti et al., 2019) using MAFFT version 7 (https://mafft.cbrc.jp/alignment/server). Datasets were subsequently trimmed using BioEdit version 7.2.5 (Hall, 1999), so cognate sequences could be assessed. The three genomic segments (i.e. L, M and S) were also aligned and trimmed in this manner. The percentage similarity of nucleotide sequence for each genomic segment and corresponding protein sequences per genomic segment from datasets were determined (Table 2). For each protein dataset, the best fit evolutionary model was determined and phylogenies of cognate amino acid sequences were conducted using Mega X (Kumar et al., 2018) (Fig. 1).

Detection of novel *orthotospovirus* by RT-PCR

To verify the presence of the novel virus, primers against the aligned L-, M- and S-segments, as obtained by HTS, were designed using Primer blast (ncbi.nlm.nih.gov/tools/primer-blast/) (Table 3). A one-step RT-PCR reaction for each primer set was set up by the addition of 100 ng of RNA to a 50 µl master mix containing 10 U M-MLV reverse transcriptase (Promega, USA), 5 µl of 10X Biotaq Buffer (Meridian Bioscience, USA), 0.5 U Biotaq DNA polymerase

Table 2 Nucleic acid (nt) and amino acid (aa) similarities between the novel virus identified from this study and other described members of the *Orthotospovirus* genus as recognized by the ICTV

Orthotospovirus	L-segment		M-segment			S-segment	
	Whole L % nt	RdRp % aa	Whole M % nt	NSm % aa	Gn/Gc % aa	Whole S % nt	NC % aa
Alstroemeria necrotic streak	47.0	35.6	39.9	35.8	28.4	28.7	19.6
Alstroemeria yellow spot	47.3	36.2	40.8	37.8	27.8	30.4	18.6
Bean necrotic mosaic	44.3	35.1	36.8	34.6	25.8	26.2	19.1
Calla lily chlorotic spot	46.8	36.0	40.2	34.2	28.3	29.7	21.4
Capsicum chlorosis	47.3	35.8	40.4	36.8	28.3	26.4	21.1
Chrysanthemum stem necrosis	46.8	35.7	40.6	35.2	27.8	27.0	20.9
Groundnut bud necrosis	47.0	36.2	39.6	37.5	27.7	28.6	20.0
Groundnut chlorotic fan-spot	65.0	65.0	58.9	68.3	55.1	n/a	37.2
Groundnut ringspot	46.7	35.7	38.7	36.2	28.9	28.1	20.0
Groundnut yellow spot	n/a	n/a	n/a	n/a	n/a	45.3	39.6
Hippeastrum chlorotic spot	47.1	36.2	40.0	37.8	28.4	29.4	18.9
Impatiens necrotic spot	46.8	35.7	38.9	32.1	28	27.7	21.1
Iris yellow spot	47.0	35.8	40.0	37.5	27.6	28.9	20.0
Melon severe mosaic	46.1	35.3	37.2	37.8	27.6	26.3	20.4
Melon yellow spot	46.7	35.7	39.3	34.5	28.1	26.9	19.5
Mulberry vein banding	47.1	36.5	39.8	37.8	27.9	24.9	19.5
Pepper chlorotic spot	46.9	36.6	39.1	37.0	26.9	27.6	18.6
Polygonum ringspot	46.2	36.2	39.9	38.2	28.4	28.1	18.9
Soybean vein necrosis	45.0	35.0	37.0	32.3	24.1	28.4	20.7
Tomato chlorotic spot	46.9	35.1	38.2	35.8	29.2	27.7	18.8
Tomato spotted wilt	46.7	35.6	39.4	34.2	28.4	27.7	18.8
Tomato yellow ring	47.1	36.3	39.5	37.8	28.2	31.7	18.2
Tomato zonate spot	47.1	36.1	40.7	33.8	28.2	25.6	18.8
Watermelon bud necrosis	46.8	36.1	39.6	36.5	27.9	26.7	18.9
Watermelon silver mottle	47.2	36.0	40.1	34.3	27.5	26.3	18.9
Zucchini lethal chlorosis	46.5	35.3	38.6	32.5	28.4	25.5	19.4

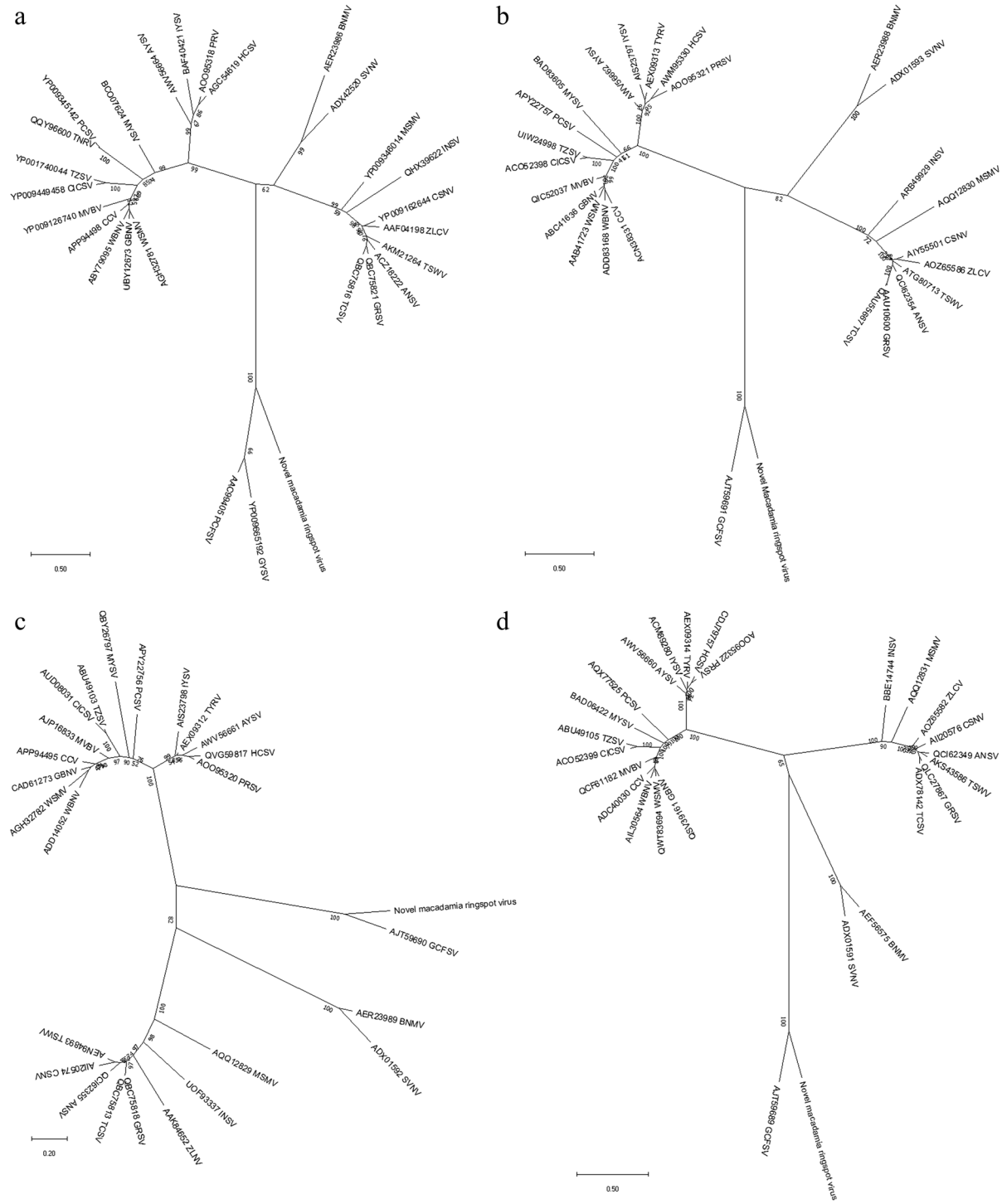

Fig. 1 Maximum-likelihood phylogeny of cognate *orthotospovirus* **a**) nucleocapsid (NC), **b**) glycoprotein (Gn/Gc), **c**) non-structural protein (NSm) and **d**) RNA-dependant RNA-polymerase (RdRp) amino-acid sequences. The evolutionary history was inferred using the Le & Gascuel, 2008 model and a discrete gamma distribution was used to model evolutionary rate differences among sites. Bootstrap values are displayed at branch nodes and the Genbank accessions of sequences used are given. The trees are drawn to scale, as indicated

(Meridian Bioscience, USA) 0.5 µM of each primer and dNTP mix, 0.01 M DTT, 1.5 mM $MgCl_2$ and nuclease free water. Reactions were performed under the following conditions, cDNA synthesis at 42°C for 45 min, followed by 94°C for 2 min, 35 cycles of 94°C for 20 s, 58°C for 20 s and 72°C for 30 s and final extension at 72°C for 5 min. Amplification products were viewed under UV on a 1% agarose gel following electrophoresis. The viral positive samples as identified by HTS as well as healthy and no template controls were included in each reaction. The specificity of the primers was subsequently confirmed by direct sequencing and phylogenetic analyses of the amplification products as previously discussed. The remaining samples from the 2019 survey were screened for the novel virus using the optimized RT-PCR assay.

Association of concentric ringspot symptom with novel virus

During 2020, the farms from the 2019 survey were revisited and only samples showing concentric ringspot symptoms were collected (Fig. 2). For the 2021 season, samples showing concentric ringspot symptoms were collected by farmer participation from farms in Mpumalanga, Limpopo and the southern coast of KwaZulu-Natal. Samples collected during 2020–2021 were subjected to total RNA extraction using the method of White et al. (2008). All samples collected from 2020–2021 were subjected to screening of the virus using the S-segment primer set only, as described above.

Results

During 2019, a total of 69 samples was collected, consisting of 52 trees which were considered "diseased" and 17 which were seemingly healthy. Total RNA was extracted from all samples but only 63 passed QC analyses and were used to prepare

Fig. 2 Concentric ringspot symptom associated with MRSV on mature 'Beaumont' leaves collected from the Mpumalanga Lowveld

RNAtag-seq libraries. A total of 231,476,718 reads were generated from two multiplexed libraries. Raw reads are available as an NCBI sequence read archive under BioProject number PRJNA887330. Plant viral reads were obtained from five samples (datasets) based on blast analyses. The number of reads associated with individual datasets (shown to be associated with a novel virus), together with the sequence length of each segment and average coverage is presented in supplementary Table 1. The genome of the novel virus identified, consists of three segments, typical of orthotospoviruses, with the average lengths of the L, M and S segments being 8,715, 4,779 and 2,935 nucleotides, respectively. No additional plant viruses were detected from the metaviromic data.

The genomes showed highly conserved inverted complementarity in the terminal nine nucleotides of each segment (5' – AGAGCAATC – 3'), which is

Table 3 Primer sequences for detection of novel *Orthotospovirus*

Genome Target	Size (bp)	Forward Primer	Reverse Primer
L segment	300	GGCACTGTCATCCATCCTCC	GGTGCCTCCGTATTGCAGAA
M segment	453	ATCACCCGTGCAAGTTGAGT	GAGCTCTACAATGCCCATGC
S segment	434	CCTCCTGCTATGCACTTGAAC	ACCAAGGCTAAGAGTGATGC

typical of members of the genus, *Orthotospovirus* (Oliver & Whitfield, 2016). While RACE was not carried out for any of the genome segments, the presence of these inverted repeats in the terminal nucleotides of each segments indicates that the genome segments presented here are complete. The proteins encoded as well as the full nucleotide sequences for each genome segment were compared to known orthotospoviruses and the percentage similarity is presented in Table 2. Across all datasets, the novel virus shared the greatest similarity to groundnut chlorotic fanspot virus (GCFSV) for both nucleotide and amino acid datasets. For the proteins, RNA-dependent RNA polymerase (RdRp), non-structural protein encoded by the M-segment (NSm), glycoprotein (Gn/Gc) and nucleocapsid protein (NC), the novel virus shared 65%, 68.3%, 55.1% and 37.2% amino acid identity to GCFSV, respectively. Whereas it shared 65% and 58.9% nucleotide identity to the L- and M-segments of GCFSV. The S-segment could not be compared as there is no sequence for this genome segment of GCFSV available on Genbank. The novel virus also shared high amino acid identity and sequence identity with groundnut yellow spot virus (GYSV), with which it shared 39.6% amino acid identity with its NC protein and 45.3% nucleotide identity across the S-segment. No other sequence or amino acid information is available for GYSV on Genbank and further comparisons could not be made.

The percentage similarity between the novel virus and the remaining orthotospoviruses assessed, were lower with the amino acid similarities for RdRp, NSm and Gn/Gc being less than 37%, 38.2%, and 29.2%, respectively, with NC amino acid sequences being less than 21.4%. Nucleic acid similarities between the novel virus and the remainder of the viruses assessed were less than 47.3%, 40.8% and 31.4% for L- M- and S-segments, respectively.

Phylogenetic analyses of the RdRp, Gn/GC, NSm and NC protein sequences confirmed the close relationship of the novel virus to GCFSV and GYSV (Fig. 1). This further indicated that the viral genome obtained from macadamia in South Africa presents a novel virus within the *Orthotospovirus* genus.

RT-PCR detection of novel virus

The primers designed for the detection of the novel virus were able to amplify the correct product from samples associated with contigs of the novel virus, based on size, and no amplification was observed in healthy samples. Phylogenetic analyses of the amplification products of each primer set further confirmed that the primers were specific for their intended targets (data not shown). The primers were then used to re-screen the stored RNA from samples collected in 2019, to determine whether there may be an association between the virus and the chlorotic symptoms observed. Only one nursery sample, in addition to the five samples from which the initial viral contigs were identified, tested positive for the virus. All six samples from which the virus was identified by a combination of HTS and RT-PCR, were 'Beaumont' nursery plants. The symptoms recorded on these nursery trees included yellowing, stunting and rugosity. However, no association between the chlorotic field trees and the novel virus could be established.

Association of novel virus with concentric ringspot symptom

In 2020/2021, sampling focused on leaves showing concentric ringspot symptoms as depicted in Fig. 2. A total of 84 samples was collected and the age, cultivar and locality of these samples are listed in Table 4. All of these samples tested positive for the novel virus, whilst all negative and healthy controls remained negative. The virus was identified from 'Beaumont', 'Nelmak 2', 'H2', 'HAES788', 'A16' and 'HAES816' cultivars ranging in age from nursery trees up to 25 years.

To further demonstrate an association of the virus with the ringspot symptoms, leaves showing different symptoms from a single 'Beaumont' tree were tested for the virus (Fig. 3). Only the concentric ringspot symptom tested positive for the virus.

Discussion

Within the current study, through the use of HTS, a member within the *Orthotospovirus* genus infecting macadamia in South Africa, was characterized. As this virus shares less than 90% amino acid identity (Plyusnin et al., 2012) with other known orthotospoviruses across the NC protein sequence, this virus is considered to be a new member within this genus. We further demonstrated an association of the virus

Table 4 Macadamia samples showing concentric ringspot symptoms collected 2020–2021

Year	Province	Tree age (Year)	Cultivar	Number of trees collected
2020	Mpumalanga	Nursery	'Beaumont'	5
		1	'Beaumont'	4
		3	'A4'	2
		14	'HAES788'	3
		15	'Beaumont'	10
		20	'Beaumont'	3
		25	'Beaumont'	3
2021	Mpumalanga	6	'A4'	5
		15	'HAES814'	3
		21	'Beaumont'	5
		22	'Beaumont'	3
		22	'Nelmak 2'	3
		25	'Beaumont'	3
		25+	'Beaumont'	6
	Southern KZN	Nursery	'Beaumont'	15
	Limpopo	4	'Beaumont'	1
		6	'Beaumont'	3
		13	'Beaumont'	1
		14	'Beaumont'	3
		16	'Beaumont'	2

with ringspot symptoms being observed on macadamia in South Africa and therefore propose the name macadamia ringspot-associated virus (MRSV).

From the current study, the virus was positively identified from leaves showing concentric ringspot symptoms and this association was further verified by testing various symptoms and healthy leaves from a single tree for MRSV. The appearance of ringspot symptoms is a common indication of *orthotospovirus* infection shared across host plants (Zhang et al., 2021), which was no different for the symptoms observed on macadamia trees in South Africa. However, the initial nursery plants from which the viral reads were obtained showed a range of symptoms including yellowing, stunting and rugosity. The appearance of these symptoms is likely due to increased susceptibility of nursery trees to viral infection. Therefore, it will be important that the full range of symptoms associated with MRSV be established experimentally, using trees of different ages. Such studies will also shed light into whether MRSV moves systemically within infected plants, which will have an influence on the manner in which vegetative propagation of macadamia is controlled in future.

An *orthotospovirus* has previously been identified from macadamia in China. The identified virus was found to react with antibodies against WSMoV and it was accepted that the virus present belongs to the WSMoV serogroup. The WSMoV-infected macadamia trees were associated with chlorotic and yellow symptoms, with necrotic leaf margins (Fang et al., 2013). This is different to the concentric ringspot symptom associated with the virus identified from South Africa, suggesting that the two viruses are distinct members within the *Orthotospovirus* genus. Unfortunately, no sequence data is available for the virus from China to compare our sequences with and confirm that these two viruses are indeed different.

There are currently five recognized phylogenetic clades within the *Orthotospovirus* genus based on the NC protein sequences. For each clade, it is assumed similar thrips species vector the viruses within a clade and that the geographical origin of these viruses are shared (Oliver & Whitfield, 2016). The obtained MRSV shared the greatest nucleotide and amino acid sequence homologies with GYSV and GCFSV (synonymous with peanut yellow spot virus and peanut chlorotic fan-spot virus, respectively) (Chou et al., 2017), and fell within the GYSV clade based on phylogenetic analyses. The two current members within the GYSV clade are persistently transmitted by the thrips, *Scirtothrips dorsalis* Hood (Chen & Chiu, 1996; Satyanarayana et al., 1998). This vector has not yet been identified from macadamia crops in South Africa, however, 15 species of thrips within the Thripinae subfamily and two species within the Phlaeothripinae subfamily are known from macadamia orchards distributed throughout the Mpumalanga Lowveld. The Thripinae species identified, included one species of *Frankliniella*, one *Scirtotrhips*, and eleven species within the *Thrips* genus, all of which are genera which have previously been identified as vectors of *orthotospoviruses* (Jones, 2005). The most abundant species identified from macadamia in the Mpumalanga Lowveld is *S. aurantii* Faure (Hepburn, 2015), making this species the most likely vector of MRSV, based on the phylogenetic grouping of MRSV. However, further research is required to confirm whether this species of thrips is able to transmit MRSV under

Fig. 3 Symptoms sampled from a single 'Beaumont' tree to determine association of MRSV with ringspot symptom observed. Symptoms tested include **a**) concentric ringspot, **b**) mottling, **c**) chlorotic spots, **d**) necrosis and **e**–**f**) healthy leaves

controlled conditions and whether any of the other species present may transmit the virus.

The majority of the South African macadamia industry is based on 'Beaumont' (34.5%), followed by 'A4' (24.9%) plantings. Within this study, MRSV was detected on both these cultivars at different ages as well as 'Nelmak 2' (10.9% planted), 'HAES816' (10.3% planted), 'A16' (5.1% planted), and 'HAES788' (2.4% planted). Additionally, the virus was identified from the three major macadamia production sites i.e. KwaZulu Natal, Mpumalanga and Limpopo provinces (samac.org.za), it is therefore important that the effect of MRSV on yield be established to determine the cost–benefit of controlling MRSV in South African orchards.

In conclusion, a novel virus was found to be associated with macadamia in South Africa infecting major cultivars at all stages. It will thus be in the best interest of the industry to further determine the effect of the virus on yield to ensure that South Africa remains the world leader in macadamia production.

Acknowledgements We would like to thank Macadamias South Africa NPC (SAMAC) for funding.

Funding Open access funding provided by Agricultural Research Council.

Declarations

Competing interests The authors have no competing interests that are relevant to the content of this article to declare.

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