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ORIGINAL RESEARCH ARTICLE

Development of Specific Diagnostic Assays for the Eleven Main Viruses Infecting Garlic (*Allium sativum*)

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Abstract

Commercially grown garlic is usually infected by complex mixtures of carla-, poty-, and allexiviruses. Specific detection of these viruses is difficult, particularly as some such as the allexiviruses exhibit serological cross-reactivity, and intraspecific nucleic acid sequence diversity is relatively large. While next generation sequencing shows much promise as a diagnostic method, it is not yet widely used for routine virus screening, especially in developing countries. The main objective of this study was to develop reverse transcription (RT)-PCR assays for each of the most common viruses associated with garlic. Primer specificity was examined in silico using sequences available on GenBank. The assays were then validated using virus isolates of known infection status, and application of the assays demonstrated by testing plants representing forty garlic cultivars grown in a major germplasm collection held at a government research station in South-east Queensland.

Keywords Allexivirus · Carlavirus · Potyvirus · RT-PCR · Specific primers · *Allium*

Introduction

Most commercial garlic cultivars (*Allium sativum*) are vegetatively propagated, a practice that has contributed to the accumulation and global spread of viruses (Cafrune et al. 2006; Conci et al. 2003; Lot et al. 1998). Commonly observed symptoms of infection include mosaic patterns, general yellowing, yellow striping, and leaf malformation and curling (Pérez-Moreno et al. 2014). Members of the genera *Potyvirus*, *Allexivirus* and *Carlavirus* are the most common viruses infecting garlic and they can occur in various combinations (Barg et al. 1994; Cafrune et al. 2006; Lunello et al. 2007). The current tally of viruses that are commonly recorded from garlic includes two potyviruses, viz. onion yellow dwarf virus (OYDV) and leek yellow stripe virus (LYSV); six allexiviruses, viz. garlic virus A to E and X, and shallot virus X (GarVA to E, GarVX and ShVX); and two carlaviruses, viz. shallot latent virus (SLV) and garlic common latent virus (GCLV). An additional allexivirus, garlic mite-borne filamentous virus (GarMbFV), is officially recognized by the ICTV although it has been argued that this species should be synonymised with GarVA (Geering and McTaggart 2019).

Diagnosis of viral infections in garlic has been a long-standing problem. Before the advent of molecular detection tools, correct diagnosis was nearly impossible because traits such as virion morphology, host range, vector specificity and serological cross-reactivity were incapable of recognizing and differentiating the full diversity of viruses infecting garlic. Consequently, there is considerable error in the earlier literature as to the cause of the various diseases in allium crops (Walkey 1990). Antisera are available for many garlic-infecting viruses (Dovas et al. 2001a; Lunello et al. 2000; Paduch-Cichal and Bereda 2016) but not all are commercially available and there is the additional problem of serological cross-reactivity, particularly between some allexivirus species (Lu et al. 2008). More recently, diagnosis has become much easier with the adoption of methods such as RT-PCR. Although many virus-specific RT-PCR assays, including multiplex assays, have been described for garlic

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viruses (Dovas et al. 2001b; Chodorska et al. 2014; Hu et al. 2015; Koczor et al. 2024; Melo Filho et al. 2004; Mituti et al. 2015, Nam et al. 2015; Paduch-Cichal and Bereda 2016; Park et al. 2005), primer design has been generally based on limited sequence datasets, and/or subsets of viruses. There is a need to frequently review and update methods as new species and sequence variants are constantly being discovered, particularly because of the application of high throughput sequencing (HTS) technologies. For example, 61% of the 514 OYDV sequences and 69% of the 484 LYSV sequences on GenBank have only become available in the last 10 years. RT-PCR assays designed many years ago based on limited datasets are still frequently used (e.g. Amir et al. 2024; Gontijo et al. 2025; Mang et al. 2022; Neupane et al. 2025), even though they fail to accommodate the full range of sequence variation currently described. Also, there are limited reports on the detection of allexiviruses, even though these viruses were first reported around 20 years ago (Chen and Chen 2002).

One of the most significant challenges in designing RT-PCR assays for the garlic-infecting viruses is the relatively large amount of intraspecific sequence variation. For example, within the genus *Allexivirus*, isolates of GarVC share as little as 72.8% nucleotide identity within the coat protein gene and isolates of GarVA are similarly divergent (Geering and McTaggart 2019). While it is relatively simple to find conserved motifs of sequence for primer design, the problem lies with finding motifs that are conserved within species but divergent between species to allow specific detection.

High throughput sequencing methods provide the broadest coverage yet for diagnosis and do not require prior knowledge of the viruses that may be present. Although there are many reports of the use of HTS for virus discovery (Peng et al. 2023), new records (Nurulita et al. 2022) and virus characterization (Prajapati et al 2022; Karavina et al. 2023), these methods have not yet been adopted for routine diagnostics, particularly in laboratories with limited resources. This study aimed to develop reliable and robust RT-PCR assays for 11 common garlic-infecting viruses using all publicly available garlic virus sequences. The assays were then applied to the screening of a germplasm collection containing imported and local accessions.

Table 1 Primer sequences, amplification targets and thermal cycling conditions

Virus target	Primer sequences (5′–3′)	Amplicon size (bp)	Amplification region[a]	cDNA synthesis temperature	Annealing temperature	Perfect matches to target virus[b]
Onion yellow dwarf virus (OYDV)	OYDV-F: AAYATGAAAKCRGCAGTYGG OYDV-R: ATICCAACIGTTGAIGGTAT	305	Partial NIb	50 °C	59 °C	76/105 (72%) 79/105 (75%)
Leek yellow stripe virus (LYSV)	LYSV-F: CATATGCAGTGATGTTTCG LYSV-R: AAGGATAGGATACTACGGGTT	464	3′ UTR	45 °C	53 °C	144/153 (94%) 130/132 (98%)
Garlic common latent virus (GCLV)	GCLV-F: GACAAYATGACIGCRGGTGA GCLV-R: TCTGCRTTGTTGGATCCAT	713	CP	45 °C	62 °C	91/102 (89%) 95/107 (89%)
Shallot latent virus (SLV)	SLV-F: CCITTTGGKTCACTTTAGG SLV-R: TACATRCAIATRTCCCARAA	433	Partial CP	45 °C	53 °C	116/126 (92%) 148/152 (97%)
Garlic virus A (GarVA)	GarVA-F: GCRCACTCRGARCTCAARGA GarVA-R: TCYRAYTGAGCRCGTGA	640	Partial CP and partial NABP	45 °C	57 °C	158/196 (80.6%) 123/140 (87.9%)
Garlic virus B (GarVB)	GarVB-F: TAGCCTGTYTACWATTTGCTCA AllexiNAB-R: CCYTTCAGCRTRTAGCTTARC	287	Partial NABP	45 °C	53 °C	84/116 (72%) 195/196 (99.5%)
Garlic virus C (GarVC)	GarVC-F: CCWCCIAARCCYCTCATGYTRAC AllexiNAB-R: CCYTTCAGCRTRTAGCTTARC	356	Partial CP and partial NABP	45 °C	57 °C	77/83 (93%) 195/196 (99.5%)
Garlic virus D (GarVD)	GarVD-F: ATGAGRTTYAARCCCACYGA GarVD-R: CCYTTCAGCATATAGCTTA	476	Partial CP and partial NABP	50 °C	52 °C	256/270 (94.8%) 231/234 (98.3%)
Garlic virus E (GarVE)	GarVE-F: GTCCAGGAAAGGCTACCA GarVE-R: AGYCTACGTAATTTACATTCAGT	374	Partial CP and partial NABP	50 °C	56 °C	13/17 (76%) 15/17 (88%)
Garlic virus X (GarVX)	GVX-F: TGCCYTCYGAAAATGAC GVX-R: GCCRTAGCGYTTYGCTCTG	782	Partial CP and partial NABP	50 °C	57 °C	59/69 (86%) 70/75 (93%)
Shallot virus X (ShVX)	ShVX-F: AGYGACGTKGAGTCCAATTC ShVX-R: GATGCATCAGAAAGTTATCAT	561	Partial CP and partial NABP	45 °C	59 °C	15/19 (79%) 14/18 (77%)

[a]NIb: nuclear inclusion body, CP: coat protein, NABP: nucleic acid binding protein, UTR: untranslated region. [b]Proportion of sequences retrieved from GenBank

Materials and Methods

Design of Specific Primers

Specific primer pairs were designed for 11 viruses from three genera, i.e. *Carlavirus* (GCLV and SLV), *Potyvirus* (OYDV and LYSV), and *Allexivirus* (GarV-A to -E, -X, and ShVX). All available sequences for these viruses were obtained from GenBank (https://www.ncbi.nlm.nih.gov). The amplification target area was selected based on alignment of conserved regions using MUSCLE (Edgar 2004). Primer sequences were selected with the aid of GPRIME (Gibbs et al. 1998) and refined by visual assessment of the alignment. Resultant specific primer pairs are provided in Table 1.

Reference Virus Isolates and Healthy Controls

To optimise each RT-PCR assay, garlic virus isolates were chosen from the Queensland Department of Primary Industries Plant Virus Collection to act as positive controls (Table 2). The infection status of these virus isolates had previously been determined using either a high throughput sequencing approach or by conventional RT-PCR and amplicon sequencing (Yue Duan and Visnja Steele, unpublished). Virus-free seedlings of shallot or chives were used as negative controls, as virus-free garlic was unavailable.

Field Samples

In 2017, bulked samples comprising single leaves from each of ten field plants per accession were taken from a garlic germplasm collection growing at the Gatton Research Facility, Queensland Department of Primary Industries. This collection is composed of subtropical cultivars sourced locally, as well as cultivars imported from the World Vegetable Centre (previously known as Asian Vegetable Research and Development Center (AVRDC)), Taiwan (Table 3). Samples were freeze-dried and kept at −20 °C for long-term storage.

Total Nucleic Acid Extractions

Total nucleic acids were extracted using a BioSprint 15 Plant DNA Kit (QIAGEN Pty Ltd, Hilden, Germany) following the manufacturer's protocol, except that RNase A was not added to the buffer RLT.

RT-PCR

Virus-specific sequences were amplified from sample extracts using a MyTaq™ One-Step RT-PCR Kit (Bioline Meridian, Memphis, USA). Each amplification reaction consisted of 1 × MyTaq One-Step mix, 0.4 µM forward primer, 0.4 µM reverse primer, 0.2 U/µL RiboSafe Inhibitor, 0.1 U/µL reverse transcriptase, and nuclease-free water added to a final volume of 25 µL. The RT-PCR thermocycling conditions were: one cycle each at 45/50 °C for 20 min (Table 1) and 95 °C for 1 min; 40 cycles of 95 °C for 10 s, 53–62 °C (Table 1) for 10 s and 72 °C for 30 s; followed by a final extension at 72 °C for 2 min. The amplicons were electrophoresed through a 1.5% TBE-agarose gel, stained with ethidium bromide, and photographed under UV light using a Gel Doc XR + System (Bio-Rad Laboratories Inc., Hercules, USA).

Sequencing and Bioinformatics

The amplicons were sent to Macrogen Inc. (South Korea) for direct Sanger sequencing. Nucleotide sequences were edited, trimmed, and analysed using Geneious Prime version 2020.1.1 software package (Biomatters, Ltd., Auckland, New Zealand). Virus identities were confirmed by sequence similarity searches of the GenBank database using BLASTN

Table 2 Viruses detected in accessions and positive and negative controls for the RT-PCR assays. Positive control used for each virus target is highlighted in pink and negative control in green

Sample[a]	Carlavirus		Potyvirus		Allexivirus						
	GCLV	SLV	OYDV	LYSV	GarVA	GarVB	GarVC	GarVD	GarVE	GarVX	ShVX
5152	-[b]	+[c]	+	+	-	-	-	-	-	-	-
5304	-	-	-	-	+	-	+	+	-	+	n/a
5308	+	n/a[d]	n/a	+	n/a	n/a	+	+	n/a	n/a	n/a
5327	+	+	+	+	+	+	+	+	+	+	n/a
Q4766	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	+
TSS cv. 'Tuktuk'[e]	-	-	-	-	-	-	-	-	-	-	n/a
Chives	n/a	n/a	-	-	-	n/a	n/a	n/a	n/a	n/a	n/a

[a]Accession number in the Queensland Department of Primary Industries Plant Virus Collection

[b]Virus not detected

[c]Virus detected

[d]n/a: not assessed

[e]TSS: true seed shallot, cv.: cultivar

Table 3 Viruses that were detected in the Gatton garlic germplasm collection using the eleven RT-PCR assays

Sample[a]	Cultivar	Country origin	Carlavirus		Potyvirus		Allexivirus						
			GCLV	SLV	OYDV	LYSV	GarVA	GarVB	GarVC	GarVD	GarVE	GarVX	ShVX
5702	AVRDC-1 (VFTA294M3-1)	Philippines	-	+	+	+	+	+	+	-	-	-	-
5703	AVRDC-2 (VFG178HP2)	China	-	+	-	+	+	+	+	+	-	+	-
5704	AVRDC-3 (VFTA347M11)	Indonesia	-	+	+	+	+	+	+	+	-	-	-
5705	AVRDC-4 (VFTA275m6)	Philippines	-	+	-	+	+	+	+	+	-	+	-
5706	AVRDC-5 (VFG180_3-1)	Taiwan	+	+	-	+	+	+	+	+	-	+	-
5707	AVRDC-6 (VFTA302m4)	Philippines	+	+	+	+	+	+	+	+	-	-	-
5708	AVRDC-7 (VFTA305m4)	Philippines	-	+	-	+	+	+	+	-	-	-	-
5573	AVRDC-8 (VFTA287m7)	Philippines	+	+	+	+	+	+	+	-	+	-	-
5709	AVRDC-9 (VFTA158m5)	Jamaica	-	+	+	+	+	+	+	+	-	-	-
5710	AVRDC-10 (VFTA350m3)	Indonesia	-	+	-	+	+	+	+	+	-	-	-
5711	AVRDC-11 (VFG77-6m3)	Taiwan	-	+	-	+	+	+	+	-	-	-	-
5712	AVRDC-12 (VFG174m9-1)	Not known	-	+	-	+	+	+	+	+	-	-	-
5713	AVRDC-13 (VFG43m3)	Taiwan	-	+	-	+	+	+	+	+	-	-	-
5714	AVRDC-14 (VFG176m9)	Taiwan	-	+	+	+	+	+	+	+	-	-	-
5715	AVRDC-15 (VFTA280m10)	Philippines	-	+	+	+	+	+	+	+	-	-	-
5716	AVRDC-16 (VFG76m2-2)	Taiwan	-	+	+	+	+	+	+	+	-	-	-
5717	AVRDC-17 (VFG29m1)	Taiwan	+	+	-	+	+	+	+	+	-	-	-
5718	AVRDC-18 (VFG35m5)	Taiwan	+	+	+	+	+	+	+	+	-	-	-
5719	AVRDC-19 (VFG173S2)	Not known	+	+	+	+	+	+	+	+	-	-	-
5720	AVRDC-20 (VFTA547_2-1)	Philippines	-	+	+	+	+	+	+	-	-	-	-
5721	AVRDC-21 (VFG180_4-12)	Taiwan	+	+	+	+	+	+	+	+	-	-	-
5722	AVRDC-22 (VFTA336m3)	Indonesia	-	+	-	+	+	+	+	+	-	-	-
5723	AVRDC-23 (VFG196m11)	China	+	-	+	+	+	+	+	+	-	-	-
5724	AVRDC-24 (VFG34m3)	Taiwan	-	+	+	+	+	+	+	+	-	-	-
5725	AVRDC-25 (VFG176m6-3)	Taiwan	-	+	+	+	+	+	+	-	-	-	-
5726	AVRDC-26 (VFTA288m2)	Philippines	-	+	+	+	+	+	+	+	-	-	-
5727	AVRDC-27 (VFG141m2)	Brazil	+	+	-	+	+	+	+	+	-	-	-
5728	AVRDC-28 (VFTA301m4)	Philippines	-	+	+	+	+	+	+	+	-	-	-
5729	Kenlarge −16–1	Australia	-	+	+	+	-	+	+	+	-	+	-
5730	Kenlarge −16–3	Australia	+	+	+	+	-	+	+	+	-	+	-
5731	Kenlarge −16–4	Australia	-	+	-	+	-	+	+	+	-	+	-
5732	Kenlarge −16–5	Australia	-	+	+	+	-	+	+	-	-	+	-
5733	Kenlarge −16–6	Australia	-	+	+	+	-	+	+	+	-	+	-
5574	PremResel2	Australia	+	+	+	+	+	+	+	+	+	+	-
5734	Columbian Red small	Australia	+	+	+	+	+	+	+	+	-	+	-
5735	Southern Glen Small	Australia	-	+	+	+	-	+	+	+	-	-	-
5736	Italian Red small	Australia	+	+	+	+	+	+	+	+	-	-	-
5575	Glen 10 small	Australia	+	+	+	+	+	+	+	+	+	+	-
5737	17-SG-JO	Australia	+	+	+	+	-	+	+	-	-	-	-
5738	Italian Red Premium	Australia	+	+	-	+	+	+	+	+	-	-	-

[a]Accession number in the Queensland Department of Primary Industries Plant Virus Collection

(https://blast.ncbi.nlm.nih.gov/). Multiple sequence alignments for each target virus were then constructed using CLUSTAL Omega. The alignments for each virus comprised the test samples, selected sequences from GenBank, and an outgroup species from a related species in the same genus. Phylogenetic trees were built using the RAxML version 8.2.11 program (Stamatakis 2014), as implemented in Geneious Prime, with 1,000 bootstrap replicates.

Results

In Silico *Evaluation of the Virus-Specific Primer Pairs*

To test the theoretical specificity of the PCR primers that were designed, in silico analyses were performed using the downloaded viral sequences. The aims of these analyses were twofold: to investigate whether the primer sequences matched all described sequence variants of the nominated virus species, and to identify matches to non-target virus species. The results of these analyses are presented in Table 1.

In most cases, species-specific forward and reverse PCR primers were able to be designed but for GarVB and GarVC, a universal allexivirus primer had to be used as the reverse primer and for GarVD, a slightly modified version of this universal primer. The species-specificity of these assays was engendered by the forward primer.

All primers had perfect matches to at least 72% of the sequence variants for each virus and in most cases, the figure was 85% or greater. Where mismatches occurred, this was often at just a single nucleotide site within the primer sequence and never at the 3' end, and these primers were most probably still functional.

The OYDV forward primer matched a number of non-target potyviruses. However, amplification of these non-target viruses would not be expected to occur as the paired reverse primer was OYDV-specific, with a single exception of a match to Mediterranean ruda virus. However, there was no match between the latter virus and the OYDV forward primer. Additionally, these non-target viruses were not known to infect garlic.

A potential issue was identified with GarvA and GarVD if both viruses were present in the same sample. In a GarVD-specific RT-PCR, the GarVD -R primer would potentially bind to 43/138 GarVA genomes with no mismatches and to a further 95/138 GarVA genomes with a single mismatch. However, GarVD-F primer matched only 7/190 GarVA genomes and so the chance or producing an amplicon was minimal and

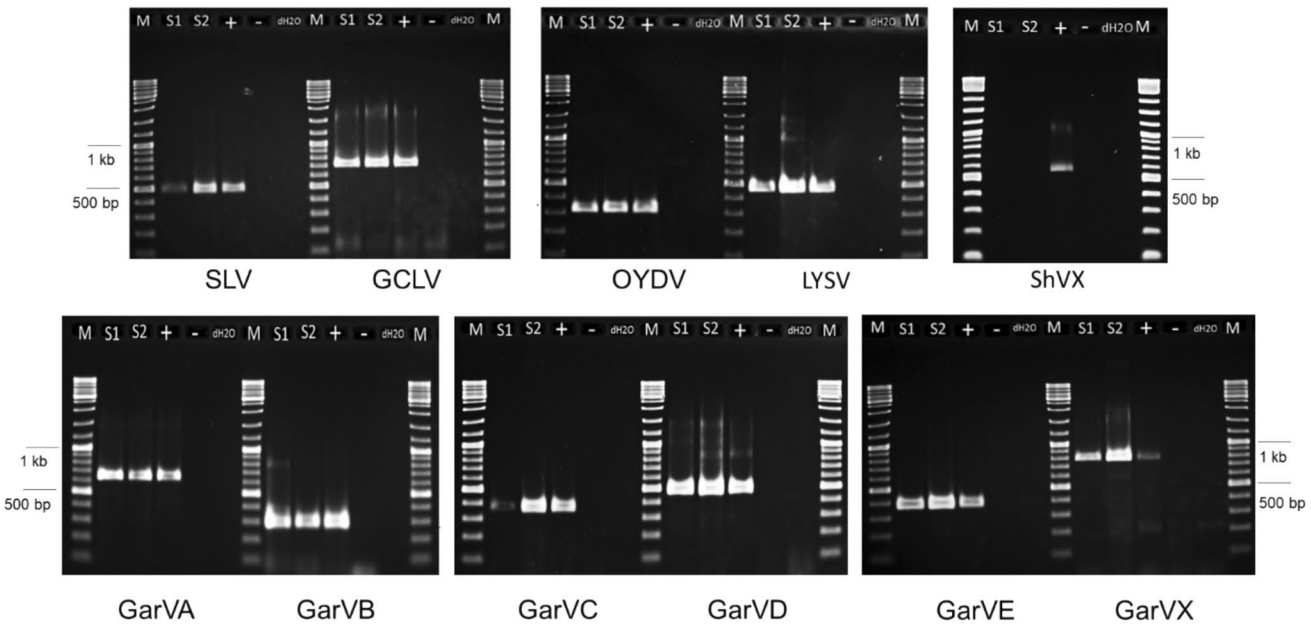

Fig. 1 Visualization of RT-PCR products from isolates representing eleven species of garlic-infecting viruses. Amplification of each target was carried out using eleven specific primer pairs (Table 1) with different templates and controls. M: GeneRuler 100 bp plus DNA ladder mix (Thermo Fisher Scientific – US); S1: test sample 1, S2: test sample 2,+: positive control using a sample infected with the target virus (Table 2), -: negative control using a sample not infected with the target virus (Table 2), NT: no template control using nuclease free water. S1 and S2 were, respectively, samples 5718, 5719 (SLV); 5706, 5707 (GCLV); 5702, KL5 (OYDV); 5702, KL5 (LYSV); 5703, 5704 (GarVA); 5706, 5573 (GarVB); 5702, KL5 (GarVC); 5703, 5707 (GarVD); B12-8, Prem-2 (GarVE); 5705, KL6 (GarVX). Positive controls were SLV 5327 (LC731763.1), GCLV 5327 (LC731764.1), OYDV 5327 (LC731766.1), LYSV 5308 (LC731779.1), ShVX Q4766 (LC731768.1), GarVA 5327 (LC731769.1), GarVB 5327 (LC731769.1), GarVC 5327 (PX931686), GarVD 5308 (LC731775.1), GarVE 5327 (LC480303.2), GarVX 5327 (LC731778.1). No test samples were available for ShVX and the positive control used was DSMZ isolate PV-0622 (MW854280.1)

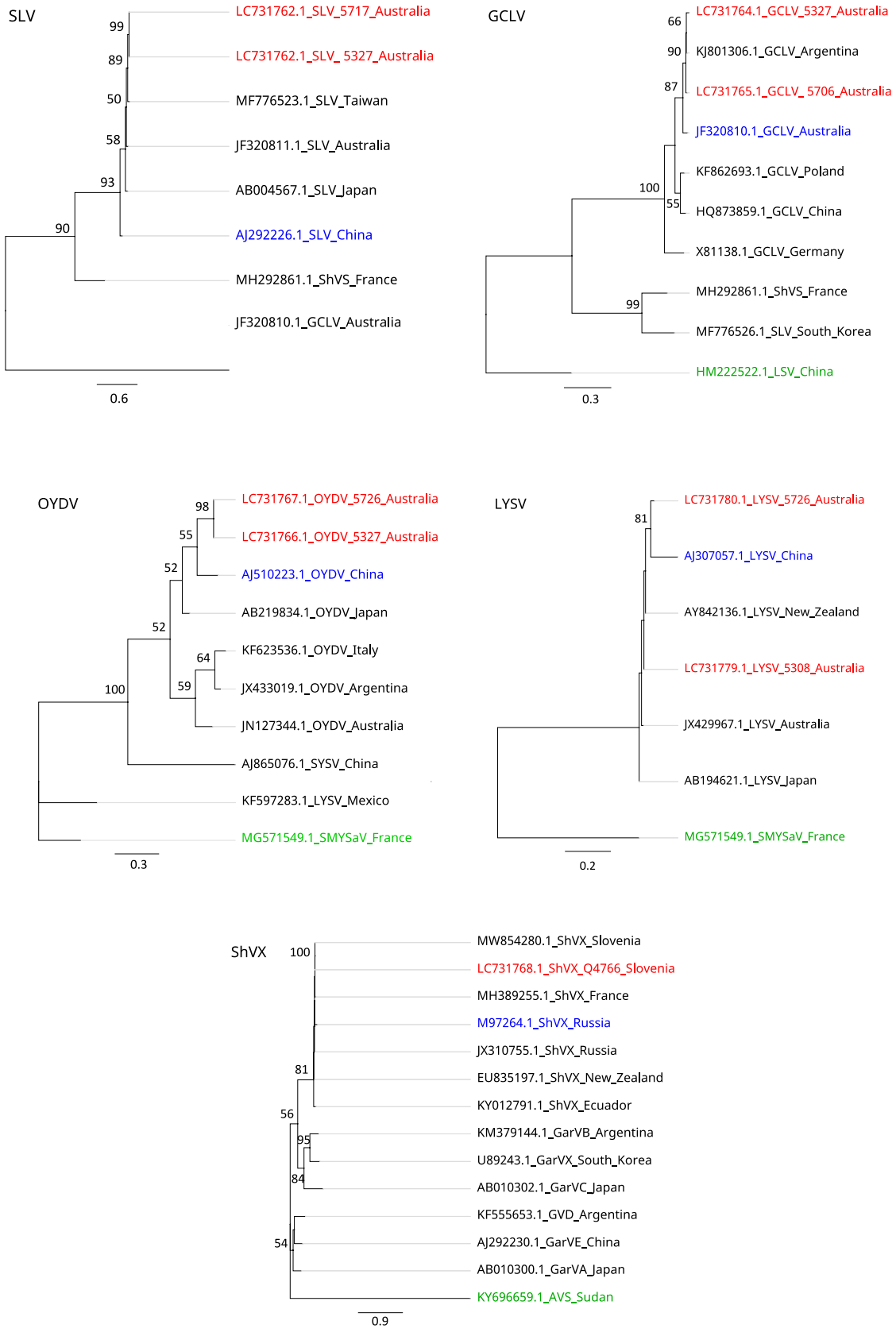
SLV
LC731762.1_SLV_5717_Australia
LC731762.1_SLV_ 5327_Australia
MF776523.1_SLV_Taiwan
JF320811.1_SLV_Australia
AB004567.1_SLV_Japan
AJ292226.1_SLV_China
MH292861.1_ShVS_France
JF320810.1_GCLV_Australia
99
89
50
58
93
90
0.6
GCLV
LC731764.1_GCLV_5327_Australia
KJ801306.1_GCLV_Argentina
LC731765.1_GCLV_ 5706_Australia
JF320810.1_GCLV_Australia
KF862693.1_GCLV_Poland
HQ873859.1_GCLV_China
X81138.1_GCLV_Germany
MH292861.1_ShVS_France
MF776526.1_SLV_South_Korea
HM222522.1_LSV_China
66
90
87
100
55
99
0.3
OYDV
LC731767.1_OYDV_5726_Australia
LC731766.1_OYDV_5327_Australia
AJ510223.1_OYDV_China
AB219834.1_OYDV_Japan
KF623536.1_OYDV_Italy
JX433019.1_OYDV_Argentina
JN127344.1_OYDV_Australia
AJ865076.1_SYSV_China
KF597283.1_LYSV_Mexico
MG571549.1_SMYSaV_France
98
55
52
52
64
59
100
0.3
LYSV
LC731780.1_LYSV_5726_Australia
AJ307057.1_LYSV_China
AY842136.1_LYSV_New_Zealand
LC731779.1_LYSV_5308_Australia
JX429967.1_LYSV_Australia
AB194621.1_LYSV_Japan
MG571549.1_SMYSaV_France
81
0.2
ShVX
MW854280.1_ShVX_Slovenia
LC731768.1_ShVX_Q4766_Slovenia
MH389255.1_ShVX_France
M97264.1_ShVX_Russia
JX310755.1_ShVX_Russia
EU835197.1_ShVX_New_Zealand
KY012791.1_ShVX_Ecuador
KM379144.1_GarVB_Argentina
U89243.1_GarVX_South_Korea
AB010302.1_GarVC_Japan
KF555653.1_GVD_Argentina
AJ292230.1_GarVE_China
AB010300.1_GarVA_Japan
KY696659.1_AVS_Sudan
100
81
56
95
84
54
0.9

Fig. 2 Maximum Likelihood phylogenetic trees for eleven viruses infecting garlic in Australia, performed using RAxML version 8.2.11. Bootstrap scores (1000 replicates) are shown at each node. The tested samples, the exemplar isolate, and the outgroup are written in red, blue, and green letter font, respectively. Virus acronyms are: OYDV, onion yellow dwarf virus; LYSV, leek yellow stripe virus; GCLV, garlic common latent virus; SLV, shallot latent virus; GarVA, garlic virus A; GarVB, garlic virus B; GarVC, garlic virus C; GarVD, garlic virus D; GarVE, garlic virus E; GarVX, garlic virus X; AVS, alfalfa virus S; LSV, lily symptomless virus; ShVS, shallot virus S; SMYSaV, shallot mild yellow stripe associated virus; SYSV, shallot yellow stripe virus

the product would be 427 bp, compared to the GarVA target product of 689 bp. In a GarVA-specific RT-PCR, although GarVA-F primer would bind to c. 65% of GarVD genomes (perfect match in at least the first 17 nt), the GarVA-R primer would not bind to the GarVD genome and hence no amplicon would be expected. The GarVE-F primer had few or no mismatches with 76/196 (39%) GarVA sequences and 60/270 (22%) GarVD sequences, However, the GarVE-R primer did not match these viruses, so in a specific GarVE RT-PCR, no cross-reaction product would be expected.

Verification of Primer Specificity

The specificity of the primers was verified by sequencing the amplicons for two samples from each virus target (Fig. 1). For ShVX, only the positive control was sequenced as the virus was not present in any of the germplasm available (all tested samples were negative). Phylogenetic analyses showed that the amplicon sequences grouped with other isolates of the target virus in all cases (Fig. 2). GenBank accession codes for the sequences of the RT-PCR amplicons of these test samples are presented in Fig. 2.

Application of the Species-Specific RT-PCR Assays for Molecular Indexing of a Garlic Germplasm Collection

Twenty-eight bulked samples of AVRDC cultivars (World Vegetable Center) and 12 bulked samples of Australian cultivars were tested using the 11 species-specific RT-PCR assays (Table 3). LYSV, GarVB, and GarVX were detected in all samples. SLV was found in 96% (27/28) of AVRDC cultivars and 100% (12/12) of Australian cultivars. GarVE was much less common, being found in only 3.5% (1/28) and 16% (2/12) of AVRDC and Australian cultivars, respectively. Among the allexiviruses, GarVA was more frequently found infecting the AVRDC cultivars (100%, 28/28) than the Australian cultivars (41.6%, 5/12). In contrast, GarVX was more common in the Australian cultivars (66%, 8/12) than in the AVRDC cultivars (10.7%, 3/28). In the phylogenetic analyses, the sequences obtained for all virus isolates fell within the expected species clades (Fig. 2).

Discussion

Garlic mosaic disease is caused by a complex of garlic-infecting viruses, predominantly potyviruses, carlaviruses, and allexiviruses (Da Silva et al. 2019; Lunello et al. 2007). This work was undertaken to develop a comprehensive and up-to-date set of virus specific RT-PCR assays for the eleven most common of these viruses. The primer pairs developed in this study were based on comprehensive datasets, to maximise their inclusivity and specificity. However, a decision was made to not account for all documented sequence variability for each virus by adding extra degeneracy to the primers, as this risked reducing the sensitivity of detection of the RT-PCR assay and increasing the amount of non-specific amplification. One problem with the international nucleotide sequence databases is the lack of quality assurance and inability to verify that the rare sequence polymorphisms that have been described are real and not sequencing errors. On rare occasions, virus accessions are also misclassified. Our assays were validated using field samples that originated from commercial crops and an international germplasm collection, and five to eight different viruses were detected in each plant. There was no evidence of misdiagnosis amongst these samples. Although virus sequences were amplified from all our garlic extracts, indicating adequate RNA isolation, consideration could be given to adding an internal RNA extraction control (e.g. Paduch-Cichal and Bereda 2016).

Ten of the eleven virus targets, including five different allexiviruses, were detected from both AVRDC and Australian garlic cultivars. In our assays, each primer pair enabled detection of its homologous target and no misdetection of closely related species. The allexivirus RT-PCR assays represent great improvements on previously described assays that have relied upon the use of degenerate primers (Abraham et al. 2019; Majumder and Baranwal 2014; Nam et al. 2015), followed by Sanger sequencing in the case of Abraham et al. (2019). None of these alternative RT-PCR assays are well-suited for diagnosis of mixed infections. Da Silva et al. (2019) demonstrated the use of five specific allexivirus primer pairs to clarify HTS results obtained from eight Brazilian garlic cultivars. However, GarVE was not present in these samples and the primer pairs were only designed with the intent to detect the five allexivirus sequences that they assembled by HTS, without any consideration of the full spectrum of variability of each virus as represented on GenBank.

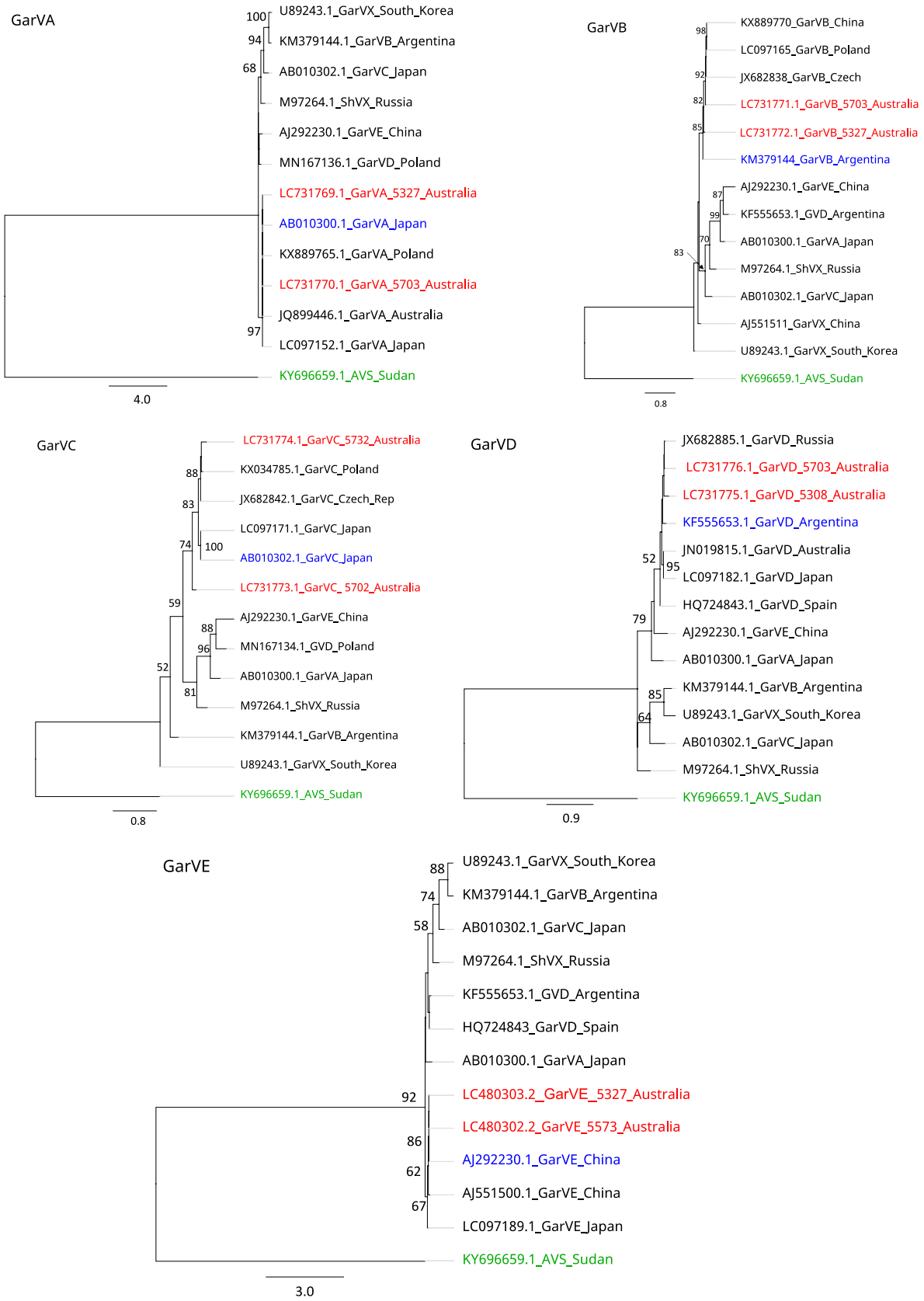

Fig. 2 (continued)

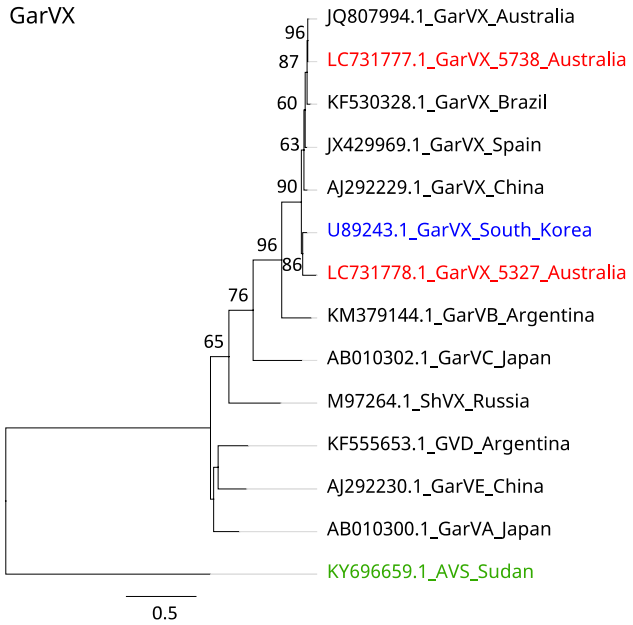

Fig. 2 (continued)

Germplasm screening based on RT-PCR detection showed the domination of SLV, OYDV, LYSV, and GarVA to D infections in both Australian and AVRDC garlic cultivars (Table 3). Prior to this study, GarVD had only been detected in a garlic plant purchased from a shop in the Perth suburb of Batemen, Western Australia ((Wylie et al. 2012), and this virus has not been reported yet in commercial garlic crops (Cremer et al. 2021). Moreover, the detections of GarVE, which are described in more detail elsewhere (Nurulita et al. 2020), represent the first records of this virus anywhere in Australia. It could be expected that GarVD and E are more widely distributed within the Australia garlic industry, particularly as these viruses were found within the collection of local Australian cultivars.

One notable result from the screening of the garlic germplasm collection is that two AVRDC cultivars (5708 and 5811) were infected with a smaller diversity of viruses (five positive from 11 virus targets) than the other cultivars (Table 3). To maintain this relatively high health status, it would be prudent to separate these cultivars from the main germplasm collection. Furthermore, different clonal lineages of the cultivar 'Kenlarge' had different virus profiles. This result suggests inconsistent vertical transmission of the viruses between plant generations or alternatively, vector-mediated spread of the viruses between plants in the germplasm collection.

In summary, the RT-PCR assays described in this paper provide an inexpensive and rapid method to screen for the presence and absence of the viruses in garlic, particularly in basic diagnostic laboratories. HTS remains the 'gold standard' for virus detection but RT-PCR still has a place for high throughput, large volume applications such as epidemiological studies or verifying the healthy status of plants following virus elimination experiments. There is also the option to combine RT-PCR with HTS if information on sequence diversity is needed.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical Approval No applicable.

Conflict of interest The authors declare that they have no conflict of interest.

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