Novel genus-specific broad range primers for the detection of furoviruses, hordeiviruses and rymoviruses and their application in field surveys in South-east Australia

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1. Introduction

A number of viruses from the genera Furovirus, Hordeivirus and Rymovirus are known to infect and damage four of the major temperate cereal crops: wheat, barley, sorghum and oats. The genus Furovirus belongs to the family Virgiviridae and consists of six virus species, all of which are plasmidiphorid-transmitted, rod-shaped, single stranded positive sense bipartite RNA plant viruses that infect graminaceous crop plants such as wheat, barley, rye, oat and sorghum. Typical symptoms of a furovirus infection include yellow to green mosaics on leaves and stunting of plants (Lapiere and Signoret, 2004). Furoviruses can cause severe yield losses of up to 80% of grain production, depending on variety, climatic conditions and time of sowing (Fauquet et al., 2005), with up to 48% grain yield loss in wheat reported in Italy (Vallega et al., 1999) and up to 70% grain yield loss in China (Janping, 1993). Some furoviruses, including the type species Soil-borne wheat mosaic virus (SBWVM), are seed transmitted (Budge et al., 2008). SBWVM is the most widespread species of the group, with infections reported from almost all continents of the world, except Australia (Table 1). However, the biosecurity risks of furoviruses increased with the recent discovery of the protozoan viral vector, Polyvexa graminis, in Queensland, Australia (Thompson et al., 2011). The genus Hordeivirus is not assigned to any virus family to date, and consists of four confirmed species (Table 1). The hordeiviruses have a tripartite genome, consisting of positive sense single stranded RNA, are mechanically, seed and pollen transmitted, and have no

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known vectors (Lapiere and Signoret, 2004). Symptoms of infection include mild mosaic, yellow or white chlorotic striping and sometimes necrosis. The type species, Barley stripe mosaic virus (BSMV) has a world-wide distribution and is the most economically important virus in the group that can cause up to 60% yield losses in barley (Lapiere and Signoret, 2004). In Australia, BSMV is found in Queensland but largely confined to barley cv. Cape (Greber, 1971). BSMV was also recorded in Victoria, New South Wales, Tasmania and Western Australia, but no new records have been reported since 1989, when it was last detected in the Western Australian barley germplasm collection (Shivas et al., 1989). The genus Rymovirus belongs to the family Potyviridae and all members of this genus have a linear, single molecule of positive sense single-stranded RNA that is approximately 9.5 kb in length and are vectored by mites (Fauquet et al., 2005). The natural host range of rymoviruses is restricted to species of the family Poaceae and symptoms of an infection include systemic chlorotic and/or necrotic striping and mosaic of varying severity on hosts. There are three confirmed species in this group with the type species being Ryegrass mosaic virus (RGMV). RGMV is a significant and widespread virus affecting fodder grasses (Lapiere and Signoret, 2004) and is the only rymovirus present in Australia. Reports of RGMV occurrence are from pastures of Tasmania and Victoria (Guy and Sward, 1988; Eagling et al., 1992). Agropyron mosaic virus (AgMV) can cause severe stunting and significant yield losses in wheat, and in co-infection with Wheat streak mosaic virus (WSMV) can cause up to 85% yield reduction (Lapiere and Signoret, 2004). The third member of the group, Hordeum mosaic virus (HoMV) has little economic impact on crops and it is known to occur only in Canada (Lapiere and Signoret, 2004).

Currently, there is no active testing for these exotic viruses in post-entry quarantine (PEQ) stations across Australia and the accidental or intentional importation of these viruses has the potential to cause significant losses for the Australian grains industry. A number of commercial tests using enzyme-linked immunosorbent assay (ELISA) and/or immunocapture polymerase chain reaction (IC-PCR) are available for the detection of viruses such as BSMV and RGMV (Ay et al., 2008). Virus-specific reverse transcription polymerase chain reaction (RT-PCR) assays are also available for some members of these genera but not all (Chen et al., 1996; Salm et al., 1996; Webster et al., 1996; Diao et al., 1999; Clover et al., 2001; Koenig et al., 2002; Budge et al., 2008; Valianopoulos et al., 2009).

RT-PCR assays utilising broad-range primers have been demonstrated to provide a faster tool for the detection of multiple viruses, and broad detection is also more likely to detect unreported species (Ha et al., 2008; Zheng et al., 2010). To date, there are no genus-specific primers for the detection of furoviruses, hordeiviruses and rymoviruses. In this study, RT-PCR assays for the detection of these

| Virus species | Acronym | Genus | Natural host/s | Geographical distribution | Full-length sequence used for primer design (GenBank accession number) |
|---------------|--------|-------|----------------|--------------------------|---------------------------------------------------------------|
| Chinese wheat mosaic virus | CWMV | Furovirus | Triticum spp. | China (Diao et al., 1999), Japan (Fukuta et al., 2013), Japan (Saito et al., 1964; Shirako and Ebara, 1986), possibly France (Hariri and Meyer, 2007) | AJ012005; AJ271838; AB299271; AB033689 |
| Japanese soil-borne wheat mosaic virus | JSBWMV | Furovirus | Hordeum vulgare, Triticum spp., and Secale cereale | Germany, Poland, Denmark, UK, Italy, France, possibly North America and Africa (Lapiere and Signoret, 2004) | AJ132578 |
| Oat golden stripe virus | OGSV | Furovirus | Avena sativa | UK, Ireland, France and the south-east USA (Lapiere and Signoret, 2004; Plumb et al., 1977) | AJ132576; AJ252151; AF146278; AF146279; AF146280 |
| Soil-borne cereal mosaic virus | SBCMV | Furovirus | Secale cereale, Triticum species (T. aestivum, T. durum, T. turgidum) and triticale | Central USA, China, Japan, France, Italy, Argentina, Brazil and New Zealand (Lapiere and Signoret, 2004) | L07937; AJ298068 |
| Sorghum chlorotic spot virus | SgCSV | Furovirus | Sorghum bicolor | USA (Kendall et al., 1988) and England (Catherall and Chamberlain, 1980) | AB033691 |
| Anthoxanthum latent blanching virus | ALBV | Hordeiviruses | Anthoxanthum odoratum | None available | |
| Barley stripe mosaic virus | BSMV | Hordeiviruses | Hordeum vulgare and occasionally Triticum spp. and Avena sterilis | World-wide (Lapiere and Signoret, 2004) | U35769; U35770; U35771; U35772; AV79694; X03854; JF080284; Z46351 |
| Lycnhs ringspot virus | LRSV | Hordeiviruses | Lycnhs divaricata, Lycnhs chalcedonica, Lycnhs coronaria, Lycnhs haageana | Hungary (Brunt et al., 1996) | |
| Poo semilatent virus | PSLV | Hordeiviruses | Elynus trachycalcaus and Pinus palustris | Canada (Brunt et al., 1996) | M81486 |
| Agropyron mosaic virus | AgMV | Rymovirus | Agropyron or Triticum species, Aristida spp., Bothriochloa laguroides, Elynus smithii and Sorghastrum nutans | USA, southern Canada, Finland, Germany, Great Britain and Hungary (Lapiere and Signoret, 2004) | AY623626 |
| Hordeum mosaic virus | HoMV | Rymovirus | Hordeum vulgare, Agropyron trachycaulum, Agrohordeum (Elynus) macouni and Hordeum jubatum | Canada (Brunt et al., 1996) | AY623627 |
| Ryegrass mosaic virus | RGMV | Rymovirus | Festucoid species within the family Poaceae: Lolium perenne, L. multiflorum and Dactylis glomerata | UK, Netherlands, USA, Canada, Italy, Australia, New Zealand and South Africa (Lapiere and Signoret, 2004) | Y09854; AF035818 |
three virus genera were developed, aiming to provide a more effective strategy in the routine diagnosis and surveillance of these viruses. These genus-specific primers were designed using a bioinformatic approach, similar to that used previously for potyvirus genus-specific primers (Zheng et al., 2010) and all conserved sites identified in this study were assessed in silico for their suitability as target sites for genus-specific primers prior to in vitro testing. The validated assays were then used to screen wheat and barley samples collected during a three-year survey in western Victoria, Australia for the presence of furoviruses, hordeiviruses and rymoviruses.

2. Materials and methods

2.1. Genus-specific primer design

2.1.1. Plant virus nucleotide sequence datasets

Nucleotide sequences from each virus species within their respective genera were retrieved from the public nucleotide sequence database GenBank. All sequences were ordered by their year of deposition in GenBank and separated into subsets of sequences representing their respective sub-RNA components, i.e. “RNA1” and “RNA2” for furovirus sequences; “RNAα”, “RNAβ” and “RNAγ” for hordeivirus sequences. In all datasets, only complete sequences were considered for the purpose of broad detection primer design. The number of distinct virus species represented by sequences in each dataset was determined, and the dataset with the most representative sequences was used for primer design.

A total of 13 full-length sequences from furovirus RNA1 were available in GenBank as of August 2013, representing all six confirmed species from the genus, whilst the RNA2 sequence dataset also consisted of 13 full-length sequences, the sequences represented only five out of the six confirmed furovirus species (data not shown). Consequently, sequences from the RNA1 dataset were used for primer design (Table 1).

The “hordeivirus” dataset contained three subsets, each contained full-length sequences from the RNAα, RNAβ and RNAγ of the hordeivirus genomes respectively. The RNAβ dataset was used for primer design as it contained nine full-length sequences representing three out of the four confirmed species from the genus Hordeivirus (Table 1) whilst the RNAα and RNAγ subsets contained only sequences from BSMV (data not shown). The full length sequence of Anthoxanthum latent blanching virus (ALBV) (Catherall and Chamberlain, 1980; Edwards et al., 1989) is not yet available.

For rymoviruses, a total of four full-length sequences were available (as of August 2013), representing all three confirmed species in the group (Table 1).

2.1.2. Identification and assessment of conserved sites for primer design

Alignment was generated using the multiple sequence alignment programme MUSCLE (Edgar, 2004). Conserved sites within the alignment were identified by the nucleotide conserved site finder (NCSF) programme using five different measures: average nucleotide variants, entropy, minimum redundancy, minimum variants and maximum count with the site length parameter set to 20 (Zheng et al., 2008). Conserved sites were also identified by eye, where the site must be equal or greater than 17 base pairs (bp) in length, and a maximum of only one non-consecutive variable position in every three bases. The top five sites chosen by each measure, along with sites that were identified by eye were analysed for their rate of consensus decay using methods previously published (Zheng et al., 2008). The consensus decay of a site is measured by its “average nucleotide score” (N), representing the apparent stability of consensus sequences at sites over time. When used to rank the sites, the lower the N score, the more conserved a site is. The best ranked sites were further assessed for their suitability as primer targets based on their sequence information (e.g. how variable the sequences are as represented by their N scores), the possibility of self-hybridisation (i.e. internal loops) and primer dimer formation using the programme OligoAnalyzer 1.2 (Gene-Link software) and OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html).

Sites were also selected for their proximity to each other (<1 kb apart) to allow for efficient amplification of target sequences; sites that were located less than 200 bp and more than 1 kb apart were not considered for genus-specific primer design.

To decrease the high degeneracy of broad detection primers, deoxyinosine (di; DNA analogue) bases were incorporated into positions of greater than 3-fold degeneracy within the degenerate primers following the recommendation by Zheng et al. (2008). Consequently, no more than 4 dls were incorporated into any primer and all dls were placed more than five bases away from the 3’ end of the primer to minimise the chance of mis–priming. The degeneracy of all resultant primers designed and developed in this study was kept at or below 12-fold (Table 2).

2.2. Positive and negative virus controls

Twenty-three positive virus controls from the genera Furovirus, Hordeivirus and Rymovirus, and 14 virus isolates from the genera

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Table 2

| Primer names | Nucleotide positions | Primer sequence (5’→3’) | Sense of primer | Target site rank | N score* in 2009 |
|-------------|----------------------|------------------------|----------------|-----------------|-----------------|
| Furo1F       | 4060–4078*           | AAACGTTTTCGGCCGCGAA   | Sense          | 1               | 0.00            |
| Furo2F       | 4197–4213*           | TTTCCGCGGTAGTGTAC    | Sense          | 2               | 0.12            |
| Furo2R       | 4699–4708*           | GCTTCTTTTCTTCTTCCCG  | Anti-sense     | 16              | 0.55            |
| Furo3R       | 4725–4744*           | GACATTTTACCTCTCCTCAT | Anti-sense     | 5               | 0.25            |
| Horde1F      | 1794–1816*           | TAAACATTCCAGAGCGCGA   | Sense          | 5               | 0.25            |
| Horde1R      | 2383–2407*           | GAACACTGGCAATATCTTNGG | Anti-sense     | 2               | 0.17            |
| Rymo1F       | 7370–7385*           | AAGCTGCTACGATGAAGAAC  | Sense          | 2               | 0.15            |
| Rymo1R       | 7722–7731*           | GACATCTGGGATGTTGCTAT | Anti-sense     | 4               | 0.20            |
| Rymo2F       | 8207–8226*           | CTTGAGCAAGCCTATACCAA  | Sense          | 1               | 0.10            |
| Rymo3R       | 9179–9189            | GAWGTTATTTCTAGAGTCT   | Anti-sense     | 1               | 0.10            |

* Average nucleotide (N) score generated by the software NCSF.

** Positions of primers are given in reference to the genomic RNA 2 of Soil-borne wheat mosaic virus (NC_002041).

† Positions of primers are given in reference to the genomic beta RNA of Barley stripe mosaic virus (X03854).

‡ Positions of primers are given in reference to the genome of Ryegrass mosaic virus (Y09854).

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1 The software Nucleotide Conserved Site Finder is being relocated at the time of writing. While in transit, this software is offline. To obtain more information on this software, please contact the author.
Table 3
Reference isolates of viruses and healthy species used to validate the RT-PCR detection methods for furoviruses, hordeiviruses and rymoviruses.

| Positive virus controls | Source | Type of material |
|-------------------------|--------|-----------------|
| **Furovirus**           |        |                 |
| 1 Chinese wheat mosaic virus | Y Shirako – Japan | Fresh tissue |
| 2 Chinese wheat mosaic virus | J-P Chen – China | Dry powder, nucleic acid |
| 3 Soil-borne cereal mosaic virus | DSMZ (PV-0552) | Dry powder, nucleic acid |
| 4 Soil-borne cereal mosaic virus | Loewe (07173-PC) | Dry powder |
| 5 Soil-borne cereal mosaic virus | Loewe (07127-PC) | Dry powder |
| 6 Soil-borne cereal mosaic virus | BioRad (3551984) | Dry powder, nucleic acid |
| 7 Soil-borne wheat mosaic virus | DSMZ (PV-0748) | Dry powder, nucleic acid |
| 8 Soil-borne wheat mosaic virus | Agdia (LPC-42001) | Dry powder |
| 9 Soil-borne wheat mosaic virus | Loewe (07174-PC) | Dry powder |
| 10 Soil-borne wheat mosaic virus | INRA, France | Dry tissue |
| 11 Soil-borne wheat mosaic virus | PHEL-MPI (03-2003-2886) | Dry tissue |
| **Hordeivirus**          |        |                 |
| 12 Barley stripe mosaic virus | DSMZ (PV-0330) | Dry tissue, nucleic acid |
| 13 Barley stripe mosaic virus | Agdia (LPC-19500) | Dry powder, nucleic acid |
| 14 Barley stripe mosaic virus | Loewe (07074-PC) | Dry powder |
| 15 Barley stripe mosaic virus | BioRad (3551870) | Dry powder |
| 16 Lychnis ringspot virus | ATCC (PV-82) | Dry tissue |
| 17 Poa semilatent virus | ATCC (PV-162) | Dry tissue |
| **Rymovirus**            |        |                 |
| 18 Agropyron mosaic virus | DSMZ (PV-0729) | Dry tissue, nucleic acid |
| 19 Agropyron mosaic virus | DSMZ (PV-0934) | Dry tissue, nucleic acid |
| 20 Hordeum mosaic virus | ATCC (PV-81) | Dry tissue |
| 21 Ryegrass mosaic virus | DSMZ (PV-0558) | Dry tissue, nucleic acid |
| 22 Ryegrass mosaic virus | PHEL-MPI | Fresh tissue |
| 23 Ryegrass mosaic virus | UK | Plant sap on FTA cards |
| **Bymovirus**            |        |                 |
| 24 Barley mild mosaic virus | DSMZ (PV-0329) | Dry tissue |
| 25 Barley mild mosaic virus | BioRad (3551865) | Dry powder |
| 26 Barley yellow mosaic virus | DSMZ (PV-0634) | Dry tissue |
| 27 Wheat yellow mosaic virus | BioRad (3551985) | Dry powder |
| **Potyvirus**            |        |                 |
| 28 Maize dwarf mosaic virus | BioRad (3551904) | Dry powder |
| 29 Sugar cane mosaic virus | Agdia (LPC-18100) | Dry powder |
| **Tritimovirus**         |        |                 |
| 30 Brome streak mosaic virus | DSMZ (PV-0932) | Dry tissue |
| 31 Barley streak mosaic virus | Loewe (07123-PC) | Dry powder |
| 32 Wheat streak mosaic virus | DSMZ (PV-0356) | Dry tissue |
| 33 Wheat streak mosaic virus | DSMZ (PV-0889) | Dry tissue |
| 34 Wheat streak mosaic virus | DSMZ (PV-0935) | Dry tissue |
| 35 Wheat streak mosaic virus | Agdia (LPC-17801) | Dry powder |
| 36 Wheat streak mosaic virus | Loewe (07048-PC) | Dry powder |
| 37 Wheat streak mosaic virus | INRA, France | Dry tissue |
| **Healthy graminaceous species** |        |                 |
| 38 Hordeum vulgare | PHEL-MPI | Fresh tissue |
| 39 Zea mays | PHEL-MPI | Fresh tissue |
| 40 Setaria italicca | PHEL-MPI | Fresh tissue |
| 41 Avena sativa | PHEL-MPI | Fresh tissue |
| 42 Lolium multiflorum | PHEL-MPI | Fresh tissue |
| 43 Sorghum bicolor | PHEL-MPI | Fresh tissue |
| 44 Triticum aestivum | PHEL-MPI | Fresh tissue |

4 Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).
5 Loewe Plant Diagnostics (Loewe Biochemica GmbH, Sauerlach, Germany).
6 Bio-Rad (Berkeley, United States of America).
7 Agdia (Elkhart, United States of America).
8 National Institute for Agriculture Research, France.
9 Plant Health & Environment Laboratory, Ministry for Primary Industries, New Zealand.
10 American Type Culture Collection (Manassas, United States of America).

Bymovirus, Potyvirus and Tritimovirus were obtained from various sources as nucleic acid, dry powder, or plant tissue (Table 3). The 23 positive virus controls were: 11 furovirus isolates representing three distinct furovirus species Chinese wheat mosaic virus (CWMV), Soil-borne cereal mosaic virus (SBCMV) and Soil-borne wheat mosaic virus (SBWMV); six hordeivirus isolates representing three hordeivirus species Barley stripe mosaic virus (BSMV), Lychnis ringspot virus (LSRV) and Poa semilatent virus (PSLV) and six rymovirus isolates, representing all three members of the genus Rymovirus: Agropyron mosaic virus (AgMV), Hordeum mosaic virus (HoMV) and Ryegrass mosaic virus (RGMV) (Table 3). Virus isolates were either imported as nucleic acid extracts suspended in ethanol or as plant sap spotted onto FTA (Flinders Technology Associates) cards (Whatman, Maidstone, UK). Healthy samples from seven graminaceous species were also included in the evaluation (Table 3).
2.3. Survey samples

Crops in the wheat growing region of western Victoria, Australia, were surveyed in spring (September–November) when cereal plants were in the vegetative growth and early heading stages for three consecutive years (2010, 2011 and 2012) (Fig. 2). Crops were selected randomly while ensuring a minimum of 10 km distance between sample sites. Green leaf tissue was collected from an unbiased selection of 100 plants at 10 m intervals following a “W” pattern from the centre of the crop. A single leaf from the base of a plant that was still actively green was sampled from each of the plants. Due to the large numbers of individual plant samples that are needed for the detection of virus presence in broad acre field crops, surveyed samples are often pooled and virus incidence can be estimated (Fletcher, 1993; Freeman et al., 2013). Consequently, 100 leaves were grouped into 10 separate groups of 10 leaves and approximately 2 cm length of the leave tissue was subsampled from the base of each bundled group for nucleic acid extraction.

In 2010, 2300 plants were sampled from 13 wheat and ten barley crops resulting in a total of 230 pooled samples. In 2011, 17 wheat and 15 barley crops were sampled, resulting in 320 pooled samples. In 2012, 3400 plants from 24 wheat and ten barley crops were surveyed resulting in a total of 340 pooled samples (Table 4). All samples were subjected to nucleic acid extraction and assayed for fuviruses, hordeiviruses and rymoviruses in the Australian laboratory using the genus–specific primers developed in this study.

2.4. RNA extractions

Imported virus isolates in the form of nucleic acid suspended in ethanol were precipitated via ethanol precipitation (Sambrook et al., 1989a) and re-suspended in 50 μL of RNase/Dnase-free water and stored at −20 °C until use.

Viral RNA was imported as the plant sap spotted on FTA cards was isolated following the manufacturer’s protocol. Briefly, four 6 mm discs were excised from the sample spot using a Harris Uni-Core puncher and placed in a sterile 1.5 mL tube. One-hundred μL of RNA processing buffer (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 800 U/mL RNaseOut (Life technologies, Carlsbad, USA), 2 mM DTT) was mixed with the FTA discs and incubated at room temperature for 15 min. The discs were then removed and the buffer containing the eluted RNA was further concentrated via ethanol precipitation (Sambrook et al., 1989a). The pellet was then suspended in 50 μL of TE−1 (10 mM Tris, 0.1 mM EDTA, pH 8.0) buffer and stored at −20 °C.

Virus isolates positive controls from plant tissue were extracted using either the InvigMag® Plant DNA mini Kit (Invimag, STRATEC, Berlin, Germany) on a Kingfisher mL instrument (Thermo Scientific, Waltham, MA, USA) or an RNasy Plant Mini Kit (Qiagen, Doncaster, Australia) following the manufacturer’s instructions. Nucleic acid extracted using the InvigMag Plant DNA mini kit was eluted in 100 μL of elution buffer whilst nucleic acid extracted using the RNasy Plant Mini Kit was eluted in 50 μL of nuclease-free water, and both were stored at −80 °C until use.

Total RNA from fresh field samples collected in Victoria, Australia were extracted using a modified lysis buffer (Mackenzie et al., 1997) and the QIAxtractor (Qiagen) in 96-well plates (Constable et al., 2012). RNA was eluted in 200 μL of RNase and Dnase free water into a 96-well elution plate (Qiagen) and stored at −20 °C until use.

2.5. RT-PCR and cycling conditions

All nucleic acid extracts were tested by RT-PCR for the presence of amplifiable host RNA using primers targeting either the plant gene NAD with primers NAD2.1a and NAD2.2b (Victoria laboratory) (Thompson et al., 2003) or with primers nads-5 and nads-5 as (New Zealand laboratory) (Menzel et al., 2002). For the initial validation of the five primer pairs, one-step RT-PCR tests were done using two enzyme reagent systems SuperScript® III Platinum Taq One-step RT-PCR kit (Life Technologies) and VersoTM 1-Step RT-PCR ReddyMixTM (Thermo Fisher Scientific, Epsom, UK). Reactions were done in a 20 μL volume as per the manufacturer’s instructions. One μL of total RNA isolated from the reference virus positive control was used. PCR cycling conditions, extension time and expected amplicons size are described in Table 5. For survey samples, one-step RT-PCR was done using the SuperScript® III Platinum Taq One-step RT-PCR kit in a 20 μL volume reaction as per the manufacturer’s instructions and 4 μL of total RNA. All PCR products were separated by agarose gel electrophoresis and visualised using either ethidium bromide or SYBR® safe DNA gel stain (Life Technologies). When the assay produced amplicons of the expected sizes, the assay was assumed to have successfully detected the virus isolate.

2.6. Cloning and sequencing

To confirm positive PCR results, selected amplicons were purified using the QiAquick PCR Purification Kit (Qiagen) and then cloned into the pGEM Easy Vector 2 (Promega, Madison, USA) following the manufacturers’ instructions. Plasmid DNA was extracted from white colonies using the small scale alkaline lysis method (Sambrook et al., 1989b) and inserts were detected by restriction enzyme analysis. Three clones were sequenced from each cloned amplicon in both directions using the SP6 and T7 promoter primers and an ABI BigDye Terminator Version 3.1 kit on an ABI3730xl sequencing machine (Applied Biosystems; Australian Genome Research Facility, Melbourne, Australia). A consensus sequence for each amplicon was compiled using Clustal X (Thompson et al., 1997) and the GenBank non-redundant nucleotide database was searched using BLASTN to identify the sequences that matched most closely the consensus sequences (Altschul et al., 1997). A positive detection is considered to be successful if the consensus sequence matched sequences (from their perspective genera) in GenBank in the expected region of the genome.

2.7. Specificity tests

To validate the specificity of the fuviruses-specific primers, RNA from 11 fuvirus isolates, representing three distinct fuvirus species (samples 1–11) were used as positive virus controls whilst RNA from four hordeivirus isolates representing BSMV (samples

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**Table 4** Number of barley and wheat plants surveyed in the Victorian survey.

| Year | Number of plants sampled from each crop |
|------|----------------------------------------|
|      | Wheat | Barley | Total  |
| 2010 | 1300  | 1000   | 2300   |
| 2011 | 1700  | 1500   | 3200   |
| 2012 | 2400  | 1000   | 3400   |
| Total| 5400  | 3500   | 8900   |

**Table 5** Cycling condition and expected amplicon size of each group-specific RT-PCR assays.

| Primer combinations | Annealing temperature (°C) | Extension time (s) | Expected amplicon size (bp) |
|---------------------|-----------------------------|-------------------|-----------------------------|
| Furo2F-2R           | 48                          | 40                | 511                         |
| Furo1F-1R           | 54                          | 45                | 685                         |
| Hordei1F-1R         | 50                          | 40                | 614                         |
| Rymo1F-1R           | 50                          | 30                | 362                         |
| Rymo2F-3R           | 50                          | 90                | 983                         |
12–15) and seven healthy graminaceous species (samples 38–44; Table 3) were used as negative controls.

To confirm specificity of the furovirus primers to the three furovirus species not available for testing in this study, namely Japanese soil-borne wheat mosaic virus (JSBWWMV), Oat golden stripe virus (OGSV) and Sorghum chlorotic spot virus (SGCSV), the primer sequences were aligned against available furovirus sequences in the public sequence database GenBank using the function primer-BLAST. At positions of degeneracy, the most dominant nucleotide base was used for the BLAST search, and the search was done against the nucleotide database with the organism limited to “Furovirus” and the primer-pair specificity check parameters set to default except for one modification, which is to ignore targets that have 7 or more mismatches to the primers, instead of the default number of 6 or more mismatches. This modification was made to allow for the degeneracy of genus-specific primers, which would commonly have several positions of degeneracy.

The specificity of the hordeivirus-specific primers was tested against six hordeivirus isolates (samples 12–17), representing three distinct virus species within the genus as positive controls, and 11 furovirus isolates (samples 1–11) and seven healthy graminaceous species (samples 38–44; Table 3) as negative controls.

The specificity of the rymovirus-specific primers was tested against six rymovirus isolates, representing all confirmed species in the Rymovirus genus as positive controls. Negative controls included four virus isolates from the genus Bymovirus (samples 24–27), two isolates from the genus Potyvirus (samples 28–29), eight isolates from the genus Tribivovirus (samples 30–37) and RNA from seven healthy graminaceous samples (samples 38–44; Table 3).

2.8. Sensitivity test

Sensitivity assays were done using selected positive virus control of targeted viruses to determine the detection limit of the RT-PCR assays. Specifically, ten-fold serial dilutions of total RNA extracts from selected isolates were used as templates in one-step RT-PCR. The concentration of the neat total RNA extract was measured using a Nanodrop spectrophotometer (ND-1000; Thermo Fisher Scientific); then serially diluted in RNase/DNase-free water in a 10-fold serial dilution series up to 10−5 and 1 μL of the diluted RNA was used as template for each assay. The concentration of the neat total RNA extracts from the selected positive virus controls ranged from 10.36 ng/μl to 378.98 ng/μl with varied purity based on their ratio of absorbance at wavelengths 260 nm and 280 nm (Table 6). The neat total RNA concentrations were not adjusted, to mimic real life diagnostic samples which often have a wide range.

| Table 6 | Concentration and purity of the neat total RNA extracts used in the sensitivity test. |
|-----------------|-------------------------------------------------|
| Positive control virus | Concentration (ng/μL) | Purity (260/280 ratio) |
| SBCMV | 74.20 | 1.88 |
| SBWMV | 161.99 | 2.02 |
| CWMV | 378.98 | 2.05 |
| BSMV | 10.36 | 1.96 |
| LRSV | 151.60 | 2.14 |
| PSLV | 50.75 | 2.10 |
| AgMV | 20.08 | 2.04 |
| RGMV | 12.08 | 2.33 |
| HoMV | 45.03 | 2.11 |

* A ratio of ~2.0 is generally accepted as “pure” for RNA.

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of starting concentrations. For furovirus-specific primer pair, three furoviruses, CWMV (sample 1), SBCMV (samples 4) and SBWMV (sample 11; Table 3) were used to evaluate the sensitivity of detection. For hordeivirus-specific primer pair, three hordeiviruses, BSMV (sample 13), LRSV (sample 16) and PSLV (sample 17; Table 3) were used to evaluate the sensitivity of detection. For rymovirus-specific primer pair, three rymoviruses, AgMV (sample 19), HoMV (sample 20) and RGMV (sample 21) were used to evaluate the sensitivity of detection.

3. Results

3.1. Identification and assessment of genus-specific primer target sites

Four conserved sites were chosen from a total of 26 unique conserved sites identified in the furovirus RNA1 sequence alignment based on their final average N scores as targets for genus-specific primer design. These four sites are: Furo1F (top ranked), Furo2F (2nd ranked), Furo2R (16th ranked) and Furo3R (5th ranked; Table 2). Furo1F and Furo2F were selected as target sites for furovirus-specific degenerate primer design based on their low N scores (0.00 and 0.12 respectively; Table 2), their minimum potential to form internal loops and low probability of self-hybridisation. Furo2R (N score 0.55) and Furo3R (N score 0.25) were also chosen for primer design due to their proximity to the top 2 ranked sites (Fig. 1a, Table 2).

Hordei1R (3rd ranked) and Hordei1F (5th ranked) were chosen as target sites for hordeivirus-specific genus-specific primer design due to their low N scores (0.17 and 0.25 respectively; Table 2), their proximity to each other (Fig. 1b) and their low potential for self-hybridisation and primer dimmer formation from a total of 16 conserved sites identified from the hordeivirus RNA6 sequence alignment.

Four unique sites were chosen as targets for rymovirus detection from a total of 25 conserved sites identified in the rymovirus genome sequence alignment. These sites are: Rymo2F (top ranked) and Rymo3R (top ranked) each with an N score of 0.10, Rymo1F (2nd ranked; N score 0.15) and Rymo1R (4th ranked; N score 0.2) (Fig. 1c; Table 2).

3.2. Assessment of genus-specific primers

All nucleic acid extracts tested positive by RT-PCR using either the primers NAD2.1a and NAD2.2b or the primers nad5-s and nad5-as, confirming the presence of host amplifiable RNA in all control and survey samples.

3.2.1. Specificity tests

Two sets of genus-specific primers (Furo2F-2R and Furo1F-3R) were designed for furovirus detection and tested against 11 furovirus isolates (samples 1–11) and 11 negative controls (samples 12–15 and samples 38–44; Table 3) using two commercial available enzyme systems. The “Furo1F-3R” primer pair accurately detected all of the furovirus isolates tested with no false positives and was highly specific when used in both RT-PCR kits. The accuracy and specificity of the “Furo2F-2R” primer pair varied however when used with two different RT-PCR kits. Whilst the “Furo2F-2R” primer pair accurately detected all positive furovirus isolates tested with high specificity when used with the Superscript® III RT-PCR kit, both accuracy and specificity of the primer pair declined when used with the Verso™ RT-PCR kit, producing multiple amplicons in some positive virus controls and weak false positives from healthy barley and oat samples.

In silico assessment of the primer pair “Furo1F-3R” showed that it matched 13 out of 13 full length furovirus RNA1 sequences available in GenBank, including JSBWMV, OG5V and SgCSV, without any matches to non-furovirus species. Consequently, the primer pair “Furo1F-3R” was used to screen all survey samples for the presence of furoviruses.

One RT-PCR assay was assessed for the detection of hordeiviruses. Its accuracy and specificity were tested against six hordeivirus isolates (samples 12–17), representing three distinct virus species within the genus and 18 negative controls, including 11 furovirus isolates (samples 1–11) and seven healthy graminaceous species (samples 38–44; Table 3). The assay was able to detect all hordeivirus isolates accurately with no false positives from any of the negative controls and with high specificity (no non-target sequences were amplified) in both RT-PCR kits. In silico assessment of the specificity of hordeivirus-specific primers to ALBV could not be done due to a lack of sequence data for ALBV.

Two generic assays containing four genus-specific primers were developed for rymovirus detection (Table 2) and tested against six rymovirus isolates (samples 18–23) and 21 negative controls (samples 24–44) in the two different enzyme systems. The Rymo1F-1R primers were able to detect all six rymovirus isolates tested, representing all confirmed species in the Rymovirus genus, as well as a potyvirus, Maize dwarf mosaic virus (MDMV, sample 28) in both enzyme systems. Furthermore, when used with the Verso™ RT-PCR kit, the primers also detected Wheat streak mosaic virus (WSMV, sample 32), as well as producing multiple non-specific amplicons in multiple negative virus controls (Table 3). The cross-reaction with WSMV and non-specific annealing were not observed when the Rymo1F-1R primers were used with the Superscript III RT-PCR kit. RT-PCR assay containing Rymo2F-3R detected five out of six rymovirus isolates tested as it did not detect the RGMV isolate extracted from FTA card (sample 23). It had no cross-reactions with bymoviruses and potyviruses tested in this study using both enzyme systems. It did detect a tritmovirus, WSMV (sample 32; Table 3) and produced multiple non-specific annealing when used with the Superscript III RT-PCR kit. Conversely, when used with the Verso™ RT-PCR kit, primers Rymo2F-3R did not detect WSMV and no non-specific annealing was observed.

In silico assessment of the primer pair “Rymo2F-3R” showed that it matched 15 rymovirus sequences available in GenBank, representing all three confirmed species within the genus Rymovirus, without any matches to other non-rymovirus species. Consequently, the primer pair “Rymo2F-3R” was used to screen all survey samples for the presence of rymoviruses.

3.2.2. Sensitivity tests

The detection limit of the RT-PCR was determined at a point where the amplicons were visible by eye in a 10-fold serial dilution of viral total RNA extracts (Fig. 3). The primer set Furo1F-3R

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was able to detect dilution of total RNA extracts in water down to $1 \times 10^{-5}$ for all three furoviruses SBCMV (7.42 pg), SBWMV (16.20 pg) and CWMV (37.90 pg) (Fig. 3a). For primers Hordei1F-1R, the detection limit was $1 \times 10^{-4}$ for all three hordeiviruses BSMV (10.36 pg), LRSV (151.6 pg) and PSLV (50.8 pg) (Fig. 3b). And for primers Rymo2F-3R, the detection limit was $1 \times 10^{-3}$ for all three rymoviruses AgMV (2.00 pg), RGMV (1.21 pg) and HoMV (4.56 pg) (Fig. 3c).

### 3.3. Survey results

A total of 5400 wheat plants and 3500 barley plants from the western districts around Horsham of Victoria, Australia were sampled over a three-year period (Fig. 2; Table 4). The integrity of each of the 890 test samples (in the form of nucleic acid extract) was assessed by RT-PCR with the internal control gene, NADH dehydrogenase ND2 subunit (Thompson et al., 2003). PCR products of the expected size were amplified from all nucleic acid extracts, indicating the presence of amplifiable RNA in all samples. All survey samples were tested for the presence of furovirus, hordeivirus and rymovirus using the three RT-PCR assays Furo1F-3R, Hordei1F-1R and Rymo2F-3R respectively and all 890 samples were found to be negative in all three tests.

### 4. Discussion

Three novel RT-PCR assays were developed and validated for the broad-ranged detection of viral species within the genera Furovirus, Hordeivirus and Rymovirus. Each assay was assessed primarily based on its accuracy and specificity, and robustness to a lesser degree, resulting in three assays which were used in a field survey in the cereal growing regions of western Victoria, Australia for three consecutive years (2010–2012).

For furovirus detection, the primer pair Furo1F-3R was shown to be superior to the primer pair Furo2F-2R, in its range of detection, its specificity to the target virus group and its robustness to be used with two different RT-PCR kits. The better specificity of the Furo1F-3R primer pair could be due to a number of reasons, including primer design. Broad detection primer designs are generally based on highly conserved sites to minimise degeneracy in the primer sequences which in turn, can increase the specificity of the assay. As the conserved sites identified in this study were ranked based on their N scores and the Furo1F-3R targeted sites ranked with a lower combined N score (total N score of 0.25) than the N scores of the sites targeted by Furo2F-2R (total N score of 0.67), the Furo1F-3R primer pair had less degeneracy by design, which may have in turn, contributed to its higher accuracy. Less degeneracy can also contribute to better reaction efficiency and sensitivity due to the higher concentration of the correct primer sequences being available. When more primers anneal to the template with high specificity, this leads to higher yields of specific PCR products and increases the efficiency of the amplification reaction, which results in increased sensitivity of the assay as smaller quantity of the starting DNA template is required for successful detection. The N scores of conserved sites can therefore be used as a tool to guide degenerate primer design whereby the primer sites can be ranked in terms of their variability. This method has been used in the past to help facilitate the process of designing genus-specific primers for potyvirus detection (Zheng et al., 2010).

Based on the same principle, the primer pair Hordei1F-1R was designed for the detection of hordeiviruses. The N scores of both primer sites were equal to or lower than 0.25 and they were among the top 5 most conserved sites within the beta RNA of hordeiviruses. Validation showed that the primer pair Hordei1F-1R was highly accurate, specific and robust, as it detected all positive hordeivirus isolates tested without any false positive results when tested against selected virus species from the genera Tritimovirus, Bystrovirus and Rymovirus, as well as seven healthy samples.

For rymovirus-specific primers, both primer sets cross-reacted with non-intentional targets: Rymo2F-3R cross-reacted with WSMV, a tritimovirus and Rymo1F-1R cross-reacted with WSMV and MDMV, a potyvirus. Given that the genus Rymovirus is closely related to both Potyvirus and Tritimovirus, it is likely that the most conserved sites within the rymovirus genomes will also be highly conserved in the potyvirus and tritimovirus genomes. This could explain why broad detection primer designs based on the most conserved sites within the rymovirus genomes were able to detect isolates from the other two closely related genera. Despite not being able to detect RGMV in sample 23, the primer pair Rymo2F-3R was ultimately chosen to screen the survey samples due to its better specificity for rymovirus detection. Its inability to detect sample 23 could be due to the sub-optimal quality of the RNA extract isolated from FTA cards, since the pair was able to detect the other two RGMV isolates tested (samples 21 and 22). In silico assessment also indicates that the primers Rymo2F-3R were able to detect all confirmed species within the genus.

The use of these primer sets with two different enzyme systems also highlighted the importance of the reaction conditions in a PCR test. With the exception of the hordeivirus-specific generic primers, all other primer sets produced different results when used with different PCR reagents. Similar findings have been reported by Lu et al. in 2010, in which they found that different real-time PCR systems (both the instrument and reagents) yielded different gene expression values using the same templates (Lu et al., 2010), indicating that the reliability and consistency of PCR reagents could have a profound impact on the accuracy of a test result. It is therefore necessary to evaluate diagnostic tests under different buffer and enzyme systems to ensure the robustness of the assay.

A survey of wheat and barley crops from the cereal growing regions of western Victoria, Australia was done, with the three generic RT-PCR assays containing the primer pairs Furo1F-3R, Hordei1F-1R and Rymo2F-3R used on samples collected over a three year period. These three assays are capable of detecting at least nine distinct virus species from the genera Hordeivirus, Furovirus and Rymovirus, and all samples surveyed in the study...
were free of these viruses, with no false positive detected in any samples. This is the first time a broad-spectrum molecular test has been used in a survey for exotic grain viruses in Australia allowing: (1) to assess the three generic assays using field samples; testing both the accuracy of the assays (no false positive reactions were recorded) and the preparation of the samples, which is essential to the adaptions of these tests for routine diagnosis; and (2) results from the survey provided important evidence of absence of these viruses in the regions surveyed, addressing the World Trade Organisations mandate to demonstrate freedom of a pest/disease as being “known not to occur” within a country or region (http://www.wto.org/english/tratop_e/sps_e/sps_agreement_cbt_e/c2s10p1_e.htm), thereby supporting the phyto-sanitary status of the Victorian grains industry.

The development of these three generic tests is also timely, given the recent discovery of the protozoan vector for furoviruses, Polymyxa graminis in Queensland (Thompson et al., 2011) and Western Australia (Cox et al., 2012). Whilst this survey did not test for the presence of P. graminis, none of the furoviruses that can be transmitted by this vector were detected in the regions surveyed.

In summary, the three generic assays developed in this study are capable of detecting a wide range of virus species within the genera Hordeivirus, Furovirus and Rymovirus. The application of these tests can greatly reduce the amount of time, effort and cost required for routine testing as well as an opportunity to discover previously unreported virus species.

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