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Use of primers with 5′ non-complementary sequences in RT-PCR for the detection of nepovirus subgroups A and B

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Two generic PCR protocols were developed to detect nepoviruses in subgroups A and B using degenerate primers designed to amplify part of the RNA-dependent RNA polymerase (RdRp) gene. It was observed that detection sensitivity and specificity could be improved by adding a 12-bp non-complementary sequence to the 5′ termini of the forward, but not the reverse, primers. The optimized PCR protocols amplified a specific product (~340 bp and ~250 bp with subgroups A and B, respectively) from all 17 isolates of the 5 virus species in subgroup A and 3 species in subgroup B tested. The primers detect conserved protein motifs in the RdRp gene and it is anticipated that they have the potential to detect unreported or uncharacterised nepoviruses in subgroups A and B.

1. Introduction

The genus *Nepovirus* is classified in the family *Comoviridae*, together with the genera *Comovirus* and *Fabavirus*. Genomes of nepoviruses consist of two RNA species, both of which are transcribed as single polyproteins. RNA-1 encodes the helicase, polymerase and proteinase, and RNA-2 encodes for the capsid and movement proteins. Nepoviruses are categorised into three subgroups, A (8 species), B (9 species) and C (15 species), according to their serological relationships, sequence similarities, and the length and arrangement of RNA-2 (Fauquet et al., 2005). A number of species, e.g., *Cherry leaf roll virus* (CLRV), *Tomato black ring virus* (TBRV) and *Tomato ringspot virus* (ToRSV), have well characterised strains with particular biological characteristics.

At least 12 nepoviruses (e.g. *Arabis mosaic virus* [ArMV], *Tobacco ringspot virus* [TRSV] and TBRV) are transmitted by nematodes of the family Longidoridae, one (*Blackcurrant reversion virus*, [BRV]) is transmitted by a mite (*Phytoptus ribis*), but some have no known biological vector (e.g., *Grapevine Bulgarian latent virus* and *Myrobalan latent ringspot virus*) (Harrison and Murant, 1977). At least 9 members of the genus are also seed- and/or pollen-transmitted, e.g. *Blueberry leaf mottle virus*, *Raspberry ringspot virus* (RpRSV) and TRSV (Card et al., 2007). Many nepoviruses have become widely distributed geographically through movement of infected seed, pollen and planting material; these characteristics together with nematode transmission make these viruses particularly hard to eradicate or control (Harrison and Murant, 1977; Fauquet et al., 2005).

A few nepoviruses have a narrow host range (e.g. *Cocoa necrosis virus* [CoNV] infects only *Theobroma cacao* naturally) but most have a wide host range, e.g., ArMV infects many wild and cultivated monocotyledonous and dicotyledonous species naturally and at least 93 species in 28 dicotyledonous families experimentally (Murant, 1970). Many species are of considerable economic importance, such as ArMV and *Grapevine fanleaf virus* (GFLV) in grapevine (Digiaro et al., 2007).

Prevention of infection, either by quarantine or by selecting healthy seed/planting material, is the most effective way to avoid the damage caused by nepovirus infection. Therefore, reliable and sensitive diagnostic methods are required to ensure that infected material is identified. There are published serological and molecular methods for some nepoviruses, e.g., commercial sources of antisera and there are published PCR protocols (e.g., Digiaro et al., 2007). However for others, such as *Cassava American latent virus* and *Mulberry ringspot virus*, there is no diagnostic method. Moreover, because of the wide host range of many nepoviruses and the lack of sequence information for many members of the genus (Fauquet et al., 2005), the availability of a general detection method for all species in the genus would be highly desirable.

A primer flap is a nucleotide segment which is non-complementary to the target and is linked to the 5′ terminus of a primer. Mackenzie et al. (1998) used bacteriophage promoter sequences (SP6 and T7) as flaps on plant potyvirus universal primers and found that they not only made sequencing of the PCR products more convenient but also increased amplification

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specificity. Binladen et al. (2007) also reported that primers with a 5′ flap improved the sequencing quality of PCR products. It has recently been recognized that a 5′ AT-rich non-complementary flap can improve significantly the efficiency of Real Time PCR (Afonina et al., 2007). The aim of this study was to develop a rapid, reliable and sensitive RT-PCR method for the detection of different species in each of the nepovirus subgroups. Therefore, the addition of an AT-rich flap to the newly designed universal nepovirus primers was investigated. It was hypothesized that this approach would increase PCR efficiency in conventional PCR.

2. Materials and methods

2.1. Nepovirus isolates

The nepoviruses used in this study were obtained as fresh or freeze-dried infected leaves from commercial sources or relevant researchers (Table 1). Except those viruses purchased from commercial sources, the identity of each isolate was confirmed by sequencing (data not shown).

2.2. Analysis of nepoviruses sequences and primer design

All available nepovirus sequences (including mulitple sequences for individual viruses where available) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov) in May 2007. The sequences were first organized into different subgroups (A, B and C), species, RNA types (RNA1 and RNA2) and/or genes (e.g., coat protein [CP] gene, RNA-dependent RNA polymerase [RdRp] gene etc.). The sequences were then aligned using BioEdit version 7.0.4 (Hall, 1999) and conserved regions were identified visually. Primers for conserved regions were designed using Oligo Explorer 1.1.0 (Teemu Kuulasmaa, University of Kuopio, Finland [http://molbiol-tools.ca/molecular_biology_freeware.htm]). Primers were synthesized (Invitrogen, Auckland, New Zealand) either with or without the addition of a 12-bp primer non-complementary sequence or "flap", "AAT AAA TCA TAA", at the 5′ terminus (Afonina et al., 2007).

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from infected leaves using an RNeasy® Plant Mini Kit (Qiagen, Doncaster, Australia) following the manufacturer's protocol and stored at −80 °C. cDNA was synthesized by reverse transcription (RT) using SuperScriptII Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. The RT was performed in a total volume of 20 μl. In the first RT step, 4 μl total RNA, 0.1 μl (40 U/μl) RNasin® Plus RNase Inhibitor, 4 μl 5 × first strand buffer, 0.5 μl (0.5 ng/μl) random hexamer primers, 2 μl (10 μg/μl) bovine serum albumin (BSA) (Sigma–Aldrich, Auckland, New Zealand) and 5.4 μl nuclease-free water were mixed and denatured at 70 °C for 10 min and then kept at room temperature for 15 min. To this mixture, 0.5 μl (200 U/μl) SuperScriptII Reverse Transcriptase, 0.1 μL (40 U/μl) RNasin® Plus RNase Inhibitor, 2 μl 0.1M DTT, 1.0 μl 10 mM dNTPs and 0.4 μl nuclease-free water were added before incubating at 42 °C for 1 h. The synthesized cDNA was stored at −20 °C.

2.4. Optimization of the RT-PCR conditions

Four different primer pair combinations were investigated in one- and two-step PCR: (1) both primers without flaps; (2) both primers with flaps; (3) reverse primer only with flap; and (4) forward primer only with flap. The components of the different PCR reaction mixes in one- and two-step PCR are described in Table 2. The following conditions were optimized for two-step RT-PCR: (1) primer concentrations (final concentrations at 0.5, 1.5 or 3.0 μM); (2) annealing temperature (50 to 60 °C) and time (30 s to 3 min); and (3) extension temperature (60 to 72 °C) and time (30 s to 3 min). One- and two-step RT-PCR assays were done using either Platinum® *Taq* (Invitrogen) or Go*Taq*® DNA polymerase (Promega, Auckland, New Zealand). Gradient PCR was performed using a Palm-Cycler™ machine (Corbett Research, Sydney, Australia) to determine the optimal annealing and extension temperatures for each primer pair. Annealing temperatures for each of the four combinations of primers were optimized at an extension temperature of 72 °C. Optimization of extension temperatures was then done at the optimal annealing temperature. Optimal PCR conditions were used for all subsequent PCR assays. The use of one-step RT-PCR was

Table 1
Nepovirus isolates used during the study

Subgroup A	Host	Country of origin (supplier)
1. ArMV	*Chenopodium quinoa* (ex *Daphne* sp.)	New Zealand (MAF, Auckland, New Zealand)
2. GFLV	*Chenopodium quinoa* (ex *Vitis* sp.)	New Zealand (MAF, Auckland, New Zealand)
3. GFLV	Unknown	Unknown (20707, Bio-Rad, Auckland, New Zealand)
4. GFLV	Unknown	Unknown (LPC27701, Agdia, Elkhart, IN, USA)
5. GFLV	Unknown	USA (Foundation Plant Service, California)
6. *Potato black ringspot virus* (PBRSV)	*Nicotiana bigelovii* (ex *Solanum tuberosum*)	Scotland (Scottish Agricultural Science Agency Edinburgh, UK)
7. TRSV	*N. sylvestris*	Canada (Agriculture & Agri-Food, Summerland, Canada)
8. TRSV	*Rubus* sp.	USA (PV-0234, DSMZ, Braunschweig, Germany)
9. TRSV	Unknown	Unknown (Agdia, Elkhart, IN, USA)
10. RpRSV	Unknown	Scotland (Scottish Crop Research Institute, Dundee, UK)
Subgroup B	Host	Country of origin
1. CoNV	Unknown	Ghana (ATCC, Manassas, VA, USA; PV-283)
2. CNSV	*Cycas* sp.	Japan (MAFF, Japan)
3. CNSV	*Nicotiana clevelandii* (ex *Paeonia lactiflora*)	New Zealand (MAF, Auckland, New Zealand)
4. CNSV	*Chenopodium amaranticolor* (ex *Paeonia lactiflora*)	New Zealand (MAF, Auckland, New Zealand)
5. TBRV	Unknown	Unknown (Bio-Rad, Auckland, New Zealand)
6. TBRV	Unknown	Unknown (Neogen, Auchincruive, UK)
7. TBRV	Unknown	Unknown (PV554, ATCC, Manassas, VA, USA)
Subgroup C	Host	Country of origin
BRV	*Ribes nigrum* var. Baldwin	Canada (Canadian Food Inspection Agency, Saanichton, BC, Canada)
CLRV	*Prunus* sp.	USA (Prosser, WA, USA)
Peach rosette mosaic virus	*Prunus* sp.	USA (Prosser, WA, USA)
ToRSV	Unknown	Unknown (LPC87700, Agdia, Elkhart, Indiana, USA)

Table 2
Components of the one- and two-step PCR assays

Two-step RT-PCR (10 μl total volume)			
Platinum® *Taq* DNA polymerase (invitrogen)		Go*Taq*® (promega)	
10 × PCR buffer	1.0 μl	2 × Go*Taq* Master Mix	5.0 μl
50 mM $MgCl_2$	0.4 μl	Forward primer	~ μl
10 mM dNTP	0.2 μl	Reverse primer	~ μl
Forward primer	~ μl	cDNA	1.0 μl
Reverse primer	~ μl	Nuclease-free water	1.0 μl
5U/μl platinum *Taq* polymerase	0.1 μl		
cDNA	1.0 μl		
Nuclease-free water	~ μl		
One-step RT-PCR (10 μl total volume)			
Platinum® *Taq* DNA polymerase (Invitrogen)		Go*Taq*® (Promega)	
10 × PCR buffer	1.0 μl	2 × Go*Taq* Master Mix	5.0 μl
50 mM $MgCl_2$	0.4 μl	Forward primer	~ μl
10 mM dNTP	0.2 μl	Reverse primer	~ μl
Forward primer	~ μl	RNA	1.0 μl
Reverse primer	~ μl	0.1M DTT	0.5 μl
5 U/μl platinum *Taq* polymerase	0.1 μl	10 μg/μl BSA	1.0 μl
RNA	1.0 μl	RNasin® plus RNase inhibitor (Promega)	0.1 μl
0.1M DTT	0.5 μl	Superscript™ II Reverse Transcriptase (20U/μl, Invitrogen)	0.1 μl
10 μg/μl BSA	1.0 μl	Nuclease-free water	~ μl
RNasin® plus RNase inhibitor (Promega)	0.1 μl		
Superscript™ III Reverse Transcriptase (20U/μl, Invitrogen)	0.1 μl		
Nuclease-free water	~ μl		

Table 3
Healthy plants used to test the specificity of the newly designed primers

Number	Plant name (species)
1	Carnation (*Dianthus caryophyllus*)
2	Chestnut (*Castanea sativa*)
3	Hop (*Humulus lupulus*)
4	Walnut (*Juglans regia*)
5	Sweet potato (*Ipomoea batatas*)
6	Apple (*Malus* × *domestica*)
7	Pepino (*Solanum muricatum*)
8	Potato (*Solanum tuberosum*)
9	Cherry (*Prunus avium*)
10	Blackcurrant (*Ribes nigrum*)
11	Strawberry (*Fragaria vesca*)
12	Tomato (*Solanum lycopersicum*)
13	Nasturtium (*Tropaeolum majus*)
14	Grapevine (*Vitis riparia*)
15	Grapevine (*Vitis vinifera*)

investigated using the optimal PCR conditions. PCR products were analysed by agarose gel electrophoresis.

2.5. Specificity and sensitivity of the developed detection assays

The specificities of the new PCR assays were investigated by sequencing amplicons of the expected sizes to confirm their identity. In addition, healthy plants of 15 different species (Table 3) which are susceptible to nepoviruses, were tested using the assays to investigate the specificity of the new primers.

The sensitivities of the new PCR assays and that of Digiaro et al. (2007) were assessed and compared using serially diluted templates of five isolates representing three species (ArMV, GFLV and TRSV).

3. Results

3.1. Sequence analysis and primer design

A total of 447 nucleotide (nt) sequences and 480 amino acid (aa) nepovirus sequences were analysed. The sequences were organized into subgroup (A, B and C), species, RNA type (RNA1 and RNA2) and/or gene (e.g., coat protein [CP] gene, RNA-dependent RNA polymerase [RdRp] gene etc.). Two short conserved motifs ("TSEGYP" and "LPCQVGI") with the desired separation of ~100 aa were identified in the RdRp gene of subgroup A nepoviruses (Fig. 1). For subgroup B, multiple short conserved protein motifs were also found in the RdRp gene and two regions were selected for primer design: "ELDFLKRKF" and "LMELYLH" (Fig. 1). No other conserved sequences suitable for primer design were found in subgroups A and B, nor any part of the genome of subgroup C (data not shown). Based on the conserved protein regions, a pair of primers was designed for each subgroups A and B (Table 4).

3.2. Optimization of two-step RT-PCR and comparison with one-step RT-PCR

The primers designed in this study amplified a specific product of ~340 bp with the 10 isolates of 5 species in subgroup A, and a specific product of ~250 bp with the seven isolates of 3 species in subgroup B. The subgroup A primers did not amplify the specific product with viruses in subgroup B or C, nor did subgroup B primers amplify the specific product with viruses in either subgroup A or C (data not shown)

Optimal amplification efficiency of all viruses from subgroups A and B was obtained using a forward primer with a 5′ flap and a conventional reverse primer at a final concentration of 1.5 μM using the PCR cycling conditions described in Table 4 (Fig. 2). Both primer pairs shared the same cycling conditions except that the optimal extension temperatures was 69 °C and 65 °C for the subgroup A and subgroup B primer pairs, respectively. For the subgroup A primer pair, addition of a 5′ flap to both primers reduced sensitivity slightly compared to the best primer combination (Fig. 2). For subgroup B, addition of a 5′ flap to both primers not only reduced sensitivity but also caused non-specific amplification (Fig. 2). All other primer combinations had similarly reduced specificity and sensitivity (Fig. 2). In two-step RT-PCR, Platinum® *Taq* DNA polymerase had greater specificity and sensitivity than Go*Taq*® DNA polymerase for viruses in both subgroup A and B primers.

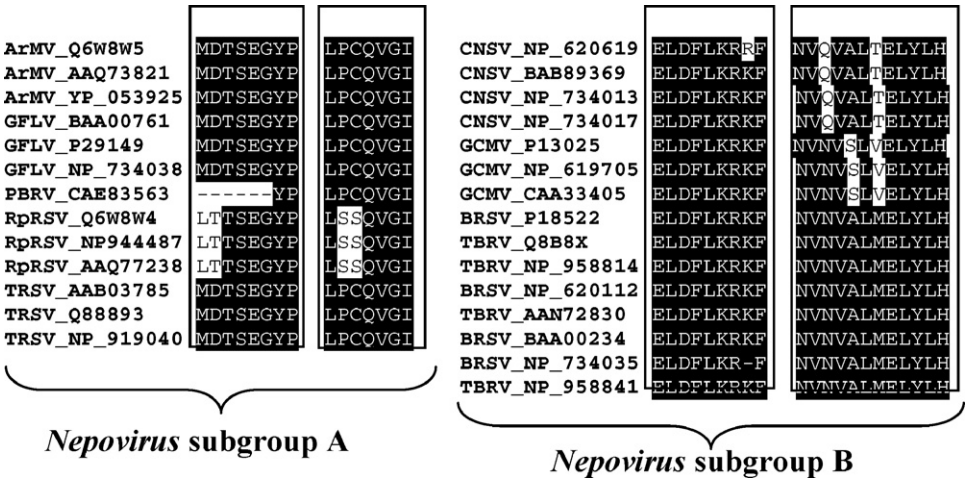

Fig. 1. Conserved protein regions in the RNA-dependent RNA polymerase gene of *Nepovirus* subgroups A and B used for primer design. ArMV, *Arabis mosaic virus*; BRSV, *Beet ringspot virus*; CNSV, *Cycas necrotic stunt virus*; GCMV, *Grapevine chrome mosaic virus*; GFLV, *Grapevine fanleaf virus*; PBRV, *Potato black ringspot virus*; RpRSV, *Raspberry ringspot virus*; TRSV, *Tobacco ringspot virus*; TBRV, *Tomato black ring virus*; ——, in subgroup A alignment means that there is no sequence available for PBRV in this part of the genome.

Table 4
Information on primer and PCR parameters

Primer name and sequences (5′-3′)	Location	Size	PCR cycling conditions	
NepoA-F (forward): ACDTCWGARGGITAYCC	RdRp gene	~340 bp	94 °C, 5 min	
			94 °C, 30 s	35 cycles
			50 °C, 30 s	
			69 °C, 2 min	
NepoA-R (reverse): RATDCCYACYTGRCWIGGCA			72 °C, 5 min	
NepoB-F (forward): TCTGGITTTGCYTTRACRGT	RdRp gene	~250 bp	94 °C, 5 min	
			94 °C, 30 s	35 cycles
			50 °C, 30 s	
			65 °C, 2 min	
NepoB-R (reverse): CTTRTCACTVCCATCRGTAA			72 °C, 5 min	

3.3. Specificity and sensitivity of the developed RT-PCR assays

The primers designed in this study were able to detect all isolates of the viruses in subgroups A and B tested. Sequencing of the PCR products amplified from ten isolates (representing all 8 species tested) confirmed the identity of the products, except CoNV for which there is no publicly available sequence (Table 5). This work also demonstrated that the newly designed primers can be used directly for sequencing. By comparison, the assays published by Digiaro et al. (2007) did not detect at least some isolates of PBRSV, RpRSV, CNSV and TBRV. The primers in this study not only detected all viruses but with greater sensitivity (except CoNV) than those of

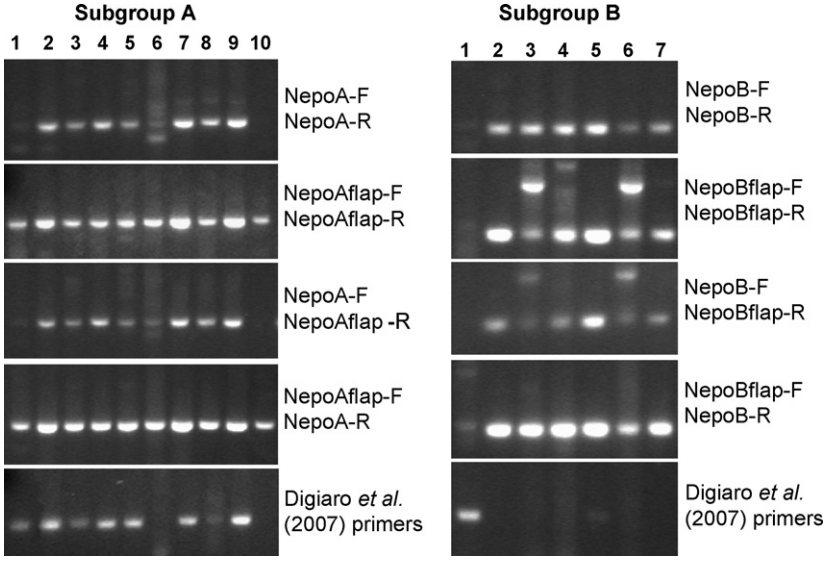

Fig. 2. Comparison of different primer combinations, with or without flaps, for the detection of nepoviruses belonging to subgroups A and B. Final primer concentration was 1.5 μM and PCR cycling conditions were as described in Table 3. The viruses in lanes 1–10 (subgroup A) and lanes 1–7 (subgroup B) are as described in Table 1.

Table 5
Comparison of the sequence of the PCR products amplified by newly designed primers with those in GenBank

Number	Viruses	Product size	GenBank Accession no.	Sequence identity with previous accessions in GenBank
1	ArMV	341 bp	EU741688	No match
		113 aa		96% to ArMV-AAQ73821
2	GFLV-New Zealand isolate	345 bp	EU741689	87% to GFLV-D00915
		115 aa		96% to GFLV-NP_619689
3	GFLV-Agdia isolate	345 bp	EU741690	No match
		115 aa		96% to GFLV-NP_619689
4	PBRSV	353 bp	EU741691	93.5% to PBRSV-AJ616715
		117 aa		95.7% to PBRSV-CAE83563
5	TRSV-Canada isolate	354 bp	EU741692	93% to TRSV-U50869
		118 aa		95% to TRSV-NP_919040
6	TRSV-DSMZ isolate	344 bp	EU741693	97% to TRSV-AJ698718
		114 aa		98% to TRSV-NP_919040
7	RpRSV	353 bp	EU769555	77% to RpRSV-AY310444
		117 aa		86% to RpRSV-NP_944487
8	CoNV	243 bp	EU741694	No match
		81 aa		No match
9	CNSV-New Zealand isolate	220 bp	EU741695	98% to CNSV-AB073147
		73 aa		98% to CNSV-NP_734017
10	TBRV-ATCC isolate	221 bp	EU741696	90% to TBRV-AY157993
		73 aa		97.2% to TBRV-NP_958841

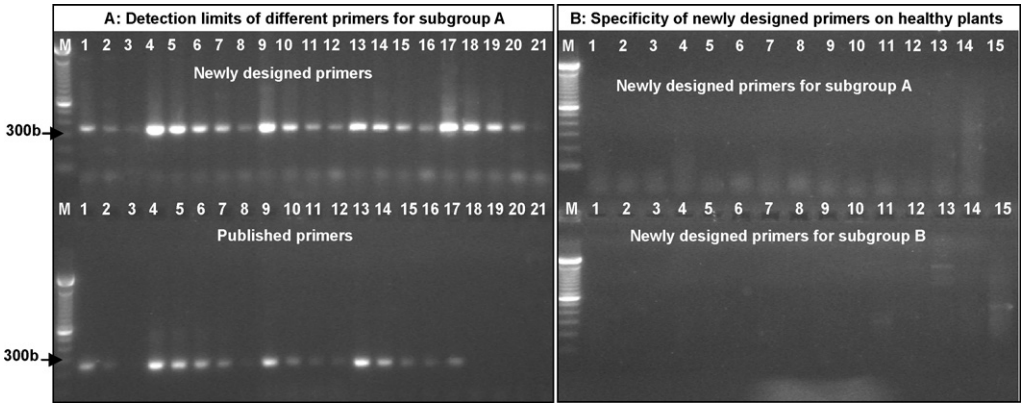

Fig. 3. (A) Comparison of the detection limits between newly designed primers and published primers for *Nepovirus* subgroup A. Lanes 1–3, 4–8, 9–12, 12–16 and 17–21 represented 10 times serial dilution of ArMV, GFLV-New Zealand isolate, GRLV-Agdia isolate, TRSV-Canada isolate and TRSV-DSMZ isolate (Table 1). Lane M: 100 bp DNA ladder (Invitrogen). (B) Specificity of the newly designed primers for subgroups A and B were tested using 15 different healthy plants. Lanes 1–15 correspond to the number described in Table 3. Lane M: 100 bp DNA ladder (invitrogen).

Digiaro et al. (2007) (Fig. 2). In addition, the newly designed primers did not amplify a specific product from healthy plants of any of the 15 species tested (Fig. 3B).

The detection limits of the newly developed assays were up to 1000 times greater (around 10 times greater for ArMV, GFLV and TRSV-Canada isolate, and around 1000 times greater for TRSV-DSMZ isolate) than the protocol published by Digiaro et al. (2007) (Fig. 3A).

In contrast with two-step RT-PCR, the primers designed in this study were unable to detect viruses in subgroup A in one-step RT-PCR, and only some viruses in subgroup B primers, albeit with less sensitivity (Fig. 4).

4. Discussion

Due to the variety, complexity and wide host ranges of nepoviruses, accurate identification from putatively infected plants is difficult. Attempts have been made to develop universal primers for detecting multiple species in a single PCR assay; a strategy that has proved successful for other genera such as potyviruses (Langeveld et al., 1991), closteroviruses (Saldarelli et al., 1998), and foveaviruses and vitiviruses (Dovas and Katis, 2003). However, published nepovirus primers detect either a limited number of species (Wetzel et al., 2002) or species affecting specific plant hosts (Digiaro et al., 2007).

In this study, universal primers were designed to detect a conserved region of the RdRp gene in nepovirus subgroups A and B.

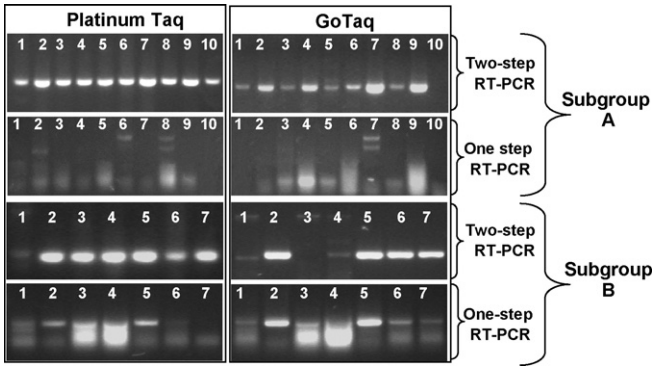

Fig. 4. Detection of nepoviruses using one- and two-step RT-PCR and two *Taq* DNA polymerases. Primer pairs for subgroups A and B are NepoAflap-F/NepoA-R and NepoBflap-F/NepoB-R, respectively. Final primer concentration was 1.5 μM and PCR cycling conditions were as described in Table 3. The viruses in lanes 1–10 (subgroup A) and lanes 1–7 (subgroup B) are as described in Table 1.

Addition of a 12-bp non-complementary sequence or "flap" at the 5′ terminus of both forward primers increased detection specificity and sensitivity. However, addition of a flap to both forward and reverse primers, or only reverse primers decreased sensitivity and/or specificity. Optimization of the PCR assay showed that primer concentration, PCR cycling conditions and the type of *Taq* DNA polymerase affected amplification efficiency. For example, increasing the extension period (but not the annealing period) to 2 min improved amplification efficiency. Platinum® *Taq* DNA polymerase also provided better amplification efficiency than Go*Taq*® DNA polymerase despite Go*Taq*® having shown better performance in other assays used routinely in our laboratory.

Using the optimized PCR assays, the newly designed primers were able to detect all 17 isolates of the eight virus species in subgroups A and B tested. Sequencing confirmed the identity of the amplicons (except CoNV for which there is no sequence available). Two isolates, ArMV and GFLV-Agdia, shared a close amino acid sequence identity with previously sequenced isolates but little nucleotide identity (Table 5) indicating the variability of their nucleotide sequences. In contrast, the primers of Digiaro et al. (2007) did not detect two species in each subgroup (PBRSV and RpRSV in subgroup A, CNSV and TBRV in subgroup B) (Fig. 2), and in general were less sensitive than those developed in the current study. Digiaro et al. (2007) reported that their assay detected TBRV but this could not be repeated in the current study. This difference may result from variation in the sequences in the primers sites between the TBRV isolates used in the different studies. Alternatively, the TBRV templates may form secondary or tertiary structures under the conditions used in this study which prevent the primers binding. This has been observed in microarray assays in which perfectly matched targets and probes may fail to form duplexes resulting in a negative signal (Bodrossy et al., 2003; Wilson et al., 2002).

One-step RT-PCR assays are being increasingly adopted in pathogen diagnosis (e.g., Sánchez-Navarro et al., 2005). Compared to two-step assays such methods are faster to set up and complete, and cross-contamination is less likely. However, the sensitivity and specificity of one- and two-step RT-PCR is rarely compared. In the current study, one-step RT-PCR was shown to be unsuitable for nepovirus detection because of the large reduction in the specificity and sensitivity of detection compared to the two-step assay.

No conserved regions suitable for primer design could be identified amongst the sequences of nepoviruses in subgroup C. As more sequences become available it may become possible to design generic primers for this subgroup. However for the time being, the detection of viruses in this subgroup will continue to rely on specific methods such as ELISA and specific PCR assays, e.g., those described for BRV (Jones and McGavin, 2002) and ToRSV (Griesbach, 1995).

In summary, two new primer pairs and optimized PCR protocols have been developed for the detection of nepoviruses belonging to subgroups A and B. The primers have been designed to detect conserved protein motifs in the RdRp gene and it is anticipated that they have the potential to detect unreported or uncharacterised nepoviruses.

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References

Afonina, I., Ankoudinova, I., Mills, A., Lokhov, S., Huynh, P., Mahoney, W., 2007. Primers with 5′ flaps improve real-time PCR. BioTechniques 43, 770–772.
Binladen, J., Gilbert, M.T., Campos, P.F., Willerslev, E., 2007. 5′-tailed sequencing primers improve sequencing quality of PCR products. BioTechniques 42, 174–176.
Bodrossy, L., Stralis-Pavese, N., Murrell, J.C., Radajewski, S., Weilharter, A., Sessitsch, A., 2003. Development and validation of a diagnostic microbial microarray for methanotrophs. Environ. Microbiol. 5, 566–582.
Card, S.D., Pearson, M.N., Clover, G.R.G., 2007. Plant pathogens transmitted by pollen. Aust. Plant Path. 36, 455–461.
Digiaro, M., Elbeaino, T., Martelli, G.P., 2007. Development of degenerate and species-specific primers for the differential and simultaneous RT-PCR detection of grapevine-infecting nepoviruses of subgroups A, B and C. J. Virol. Meth. 141, 34–40.
Dovas, C.I., Katis, N.I., 2003. A spot nested RT-PCR method for the simultaneous detection of members of the *Vitivirus* and *Foveavirus* genera in grapevine. J. Virol. Meth. 107, 99–106.
Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., 2005. Virus Taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, California, USA.
Griesbach, J.A., 1995. Detection of tomato ringspot virus by polymerase chain reaction. Plant Dis. 79, 1054–1056.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41, 95–98.
Harrison, B.D.; Murant, A.F., 1977. Nepovirus group. CMI/AAB Descriptions of Plant Viruses No. 185.
Jones, A.T., McGavin, W.J., 2002. Improved PCR detection of *Blackcurrant reversion virus* in *Ribes* and further evidence that it is the causal agent of reversion disease. Plant Dis. 86, 1333–1338.
Langeveld, S.A., Dore, J.M., Memelink, J., Derks, A.F., van der Vlugt, C.I., Asjes, C.J., Bol, J.F., 1991. Identification of potyviruses using the polymerase chain reaction with degenerate primers. J. Gen. Virol. 72, 1531–1541.
Mackenzie, A.M., Nolan, M., Wei, K.J., Clements, M.A., Gowanlock, D., Wallace, B.J., Gibbs, A.J., 1998. Ceratobium mosaic potyirus; another virus from orchids. Arch. Virol. 143, 1–12.
Murant, A.F. 1970. Arabis mosaic virus. CMI/AAB Descriptions of Plant Viruses No. 16.
Saldarelli, P., Rowhani, A., Routh, G., Minafra, A., Digiaro, M., 1998. Use of degenerate primers in an RT-PCR assay for the identification and analysis of some filamentous viruses, with special reference to clostero- and vitiviruses of the grapevine. Eur. J. Plant Pathol. 104, 945–950.
Sánchez-Navarro, A., Aparicio, F., Herranz, M.C., Minafra, A., Myrta, A., Pallás, V., 2005. Simultaneous detection and identification of eight stone fruit viruses by one-step RT-PCR. Eur. J. Plant Pathol. 111, 77–84.
Wetzel, T., Jardak, R., Meunieer, L., Ghorbel, A., Reustle, G.M., Krczal, G., 2002. Simultaneous RT/PCR detection and differentiation of arabis mosaic and grapevine fanleaf nepoviruses in grapevines with a single pair of primers. J. Virol. Meth. 101, 63–69.
Wilson, W.J., Strout, C.L., DeSantis, T.Z., Stilwell, J.L., Carrano, A.V., Andersen, G.L., 2002. Sequence-specific identification of 18 pathogenic microorganisms using microarray technology. Mol. Cell. Probes 16, 119–127.