

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

Michele Digiaro^{a,*}, Toufic Elbeaino^a, Giovanni Paolo Martelli^b

^a *Istituto Agronomico Mediterraneo di Bari (IAMB), Via Ceglie 9, 70010 Valenzano-Bari, Italy*

^b *Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale del CNR, Sezione di Bari, Italy*

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Abstract

Based on the nucleotide sequence homology of RNA-1 and RNA-2 of nepoviruses isolated from grapevines, three sets of degenerate primers, one for each of the three subgroups of the genus (A, B and C), were designed and proved effective for RT-PCR detection of subgroups in infected grapevines and herbaceous hosts. Primers designed specifically for detecting subgroup A species amplified a fragment of 255 bp from samples infected by *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Tobacco ringspot virus* (TRSV) and *Grapevine deformation virus* (GDefV), but not from samples infected by other nepovirus species. Similarly, primers for detection of subgroup B nepoviruses amplified a 390 bp product from samples infected by *Grapevine chrome mosaic virus* (GCMV), *Tomato black ring virus* (TBRV), *Grapevine Anatolian ringspot virus* (GARSV) and *Artichoke Italian latent virus* (AILV). The third set of primers amplified a 640 bp fragment, only from samples infected by subgroup C nepoviruses, i.e. *Tomato ringspot virus* (ToRSV), *Grapevine Bulgarian latent virus* (GBLV), and *Grapevine Tunisian ringspot virus* (GTRSV). These primers were able to detect simultaneously all viral species belonging to the same subgroup and to discriminate species of different subgroups. Multiplex-PCR detection of subgroup A and B nepoviruses was obtained using a specific primer (*sense* for subgroup A and *antisense* for subgroup B) for each of the species of the same subgroup in combination with the degenerate subgroup-specific primers. In this way it was possible to detect four different viral species in single samples containing mixtures of viruses of the same subgroup. In particular, for viruses of subgroup A (TRSV, GFLV, ArMV and GDefV) amplicons of 190, 259, 301 and 371 bp were obtained, whereas amplicons of 190, 278, 425 and 485 bp, respectively, were obtained from samples infected with viruses of subgroup B (GCMV, AILV, GARSV and TBRV).

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1. Introduction

Grapevine (*Vitis vinifera*) hosts 15 different species of the genus *Nepovirus* and a single species of the genus *Sadwavirus* (Table 1) among which there are the alleged agents of “infectious degeneration” and “grapevine decline” (Martelli, 1993). With the exception of *Grapevine fanleaf virus* (GFLV), which is ubiquitous, all viruses have a restricted geographical distribution, based on which they are distinguished in European nepovirus species, i.e. GFLV, *Arabis mosaic virus* (ArMV), *Tomato black*

ring virus (TBRV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine Bulgarian latent virus* (GBLV), *Raspberry ringspot virus* (RpRSV) and *Cherry leaf roll virus* (CLRV), and American species, i.e. *Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus* (TRSV), *Blueberry leaf mottle virus* (BBLMV) and *Peach rosette mosaic virus* (PRMV) (Martelli and Taylor, 1990). Additional nepoviruses were recorded from North Africa, i.e. *Grapevine Tunisian ringspot virus* (GTRSV) in Tunisia (Ouertani et al., 1991), and Asia, i.e. *Grapevine deformation virus* (GDefV) and *Grapevine Anatolian ringspot virus* (GARSV) in Turkey (Digiaro et al., 2003).

Nepoviruses contain two species of single stranded positive-sense RNAs (RNA-1 and RNA-2) with molecular weight of $(2.4\text{--}2.8) \times 10^6$ Da and $(1.3\text{--}2.4) \times 10^6$ Da, respectively (Mayo

* Corresponding author. Tel.: +39 080 4606255; fax: +39 080 4606275.
E-mail address: digiaro@iamb.it (M. Digiaro).

Table 1
List of nepovirus and sadwavirus species infecting grapevines

Nepovirus	First report
<i>Grapevine fanleaf virus</i> (GFLV)	Cadman et al. (1960) and Vuittenez (1960)
<i>Tomato ringspot virus</i> (ToRSV)	Gooding and Hewitt (1962)
<i>Arabidopsis mosaic virus</i> (ArMV)	Panjan and Saric (1963)
<i>Tomato black ring virus</i> (TBRV)	Stellmach and Bercks (1963)
<i>Grapevine chrome mosaic virus</i> (GCMV)	Martelli et al. (1965)
<i>Raspberry ringspot virus</i> (RpRSV)	Bercks and Stellmach (1966)
<i>Peach rosette mosaic virus</i> (PRMV)	Dias (1968)
<i>Tobacco ringspot virus</i> (TRSV)	Gilmer et al. (1970)
<i>Grapevine Bulgarian latent virus</i> (GBLV)	Ferreira and De Sequeira (1972) and Martelli et al. (1977)
<i>Artichoke Italian latent virus</i> (AILV)	Savino et al. (1976)
<i>Blueberry leaf mottle virus</i> (BBLMV)	Uyemoto et al. (1977) and Ramsdell and Stace-Smith (1980)
<i>Grapevine Tunisian ringspot virus</i> (GTRSV)	Ouertani et al. (1991)
<i>Grapevine deformation virus</i> (GDefV)	Cigsar et al. (2003)
<i>Grapevine Anatolian ringspot virus</i> (GARSV)	Gokalp et al. (2003)
<i>Cherry leaf roll virus</i> (CLRV)	Ipach et al. (2003)
Sadwavirus	
<i>Strawberry latent ringspot virus</i> (SLRSV)	Bercks et al. (1977)

and Robinson, 1996). Both RNA molecules have a poly(A) tail at the 3' end, and a VPg (Mr (3–6) × 10³ Da) covalently linked to the 5' end (Pinck et al., 1990).

Based on the size of RNA-2, sequence similarity and serological relationship, the genus *Nepovirus* is subdivided in three subgroups: (i) subgroup A has an RNA-2 with Mr ± (1.3–1.5) × 10⁶ present in both M and B components; (ii) subgroup B has an RNA-2 with Mr ± (1.4–1.6) × 10⁶, present only in M component; (iii) subgroup C has a RNA-2 with Mr ± (1.9–2.2) × 10⁶, present only in M component (Le Gall et al., 2005).

Subgroup A species infecting grapevines include GFLV, ArMV, GDefV, RpRSV and TRSV; subgroup B, AILV, GCMV, GARSV and TBRV; subgroup C, BBLMV, CLRV, GBLV, GTRSV, PRMV and ToRSV (Le Gall et al., 2005).

Although polyclonal antisera to all the above viruses have been raised, only some are commercially available and molecular detection is possible only when partial or complete genome sequences are available. On the other hand, the high number of grapevine-infecting nepoviruses makes their identification for practical purposes (certification programs, quarantine, surveys, etc.) a rather heavy task. The availability of a reliable, broad-range and quick detection method would therefore be highly desirable.

The wide sequence divergence existing among grapevine nepovirus genomes makes it difficult to design a single set of degenerate primers for the aspecific detection of all species (Le Gall et al., 1995). More promising seems the situation when members of single subgroups are taken into consideration, since several conserved sequence stretches can be identified in their genome. Sets of degenerate primers for each of the three subgroups were therefore designed, whose performance was tested in this study on the isolates listed in Table 2. Limited to the subgroups A and B members, the same degenerate primers

Table 2
List of nepovirus and sadwavirus isolates used in this study

Virus	Isolates	Origin
GFLV	C-1	Turkey
	D-63	Turkey
	UR-11	Russia
	E-3	Spain
	Pr-22	Italy
ArMV	NA-14	Italy
	Ni-52	France
	6–29	France
	Fr-2	Germany
RpRSV	KK-TY	Tchek Rep.
	Ch-Ag	Italy
	T-83	France
GCMV	Sy-1	Germany
	H-6	Austria
	H-15	Hungary
TBRV	H-27	Hungary
	Mirs	Hungary
ToRSV	USA8	USA
GDefV	N-66	Turkey
GARSV	A-34	Turkey
AILV ^a		Italy
GTRSV ^a		Tunisia
GBLV ^a		Bulgaria
TRSV ^a		USA
SLRSV	T-29	France

^a Isolates available only in herbaceous hosts.

were used in a protocol for detection of each single virus with Multiplex-PCR tests.

2. Materials and methods

2.1. Alignment of RNA-1 and RNA-2 of subgroup A, B and C of grapevine nepoviruses

Available nucleotide sequences of RNA-1 and RNA-2 of grapevine-infecting nepoviruses were retrieved from GenBank and assembled using the Strider 1.1 Program (Marck, 1988). The alignment for homology using the nucleotide and the amino acid sequences of RNA-1 and RNA-2 of species belonging in the same subgroup was done with the Clustal X Program. Viral species and accession numbers of RNA-1 and RNA-2 considered in this study are: (i) subgroup A: GFLV (NC-003615, AY371032), ArMV (NC-006057, X55460), GDefV (AY291208), TRSV (NC-007095, L09205) and RpRSV (NC-005267, NC-00526); (ii) subgroup B: GCMV (NC-003662, X15163), AILV (X87254), TBRV (NC-004439, AY157994) and GARSV (AY291207); (iii) subgroup C: BBLMV (U20621, U20622), PRMV (AF016626) and ToRSV (NC-003839, NC-003840).

2.2. Design of degenerate primers for subgroup-selective detection

Clustal alignment of RNA sequences of subgroup members revealed the presence of several conserved nucleotide tracts. Dif-

Table 3
List of nepovirus isolates and accession numbers used in the Clustal alignment

Virus isolate	Accession number	Reference
Subgroup A		
GFLV (NW)	NC-003623	Serghini et al. (1990)
GFLV (F13)	AY017338	Wetzel et al. (2001)
GFLV (A17)	AY780901	Vigne et al. (2005)
GFLV (B19)	AY780903	Vigne et al. (2005)
ArMV (NW)	AY017339	Wetzel et al. (2001)
ArMV (U)	X81814	Loude et al. (1995)
ArMV (S)	X81815	Loude et al. (1995)
GDefV	AY291208	Abou Ghanem-Sabanadzovic et al. (2005)
TRSV	NC-005096	Moon et al. (2003)
TRSV	AY363727	Moon et al. (2003)
RpRSV (Ch)	NC-005267	Ebel et al. (2003)
RpRSV (RAC815)	AY310445	Wetzel et al. (2006)
RpRSV	AY303788	Ebel et al. (2003)
Subgroup B		
GCMV	NC-003621	Brault et al. (1989)
TBRV	X80831	Le Gall et al. (1995)
TBRV	AY157994	Jonczyk et al. (2004)
TBRV	NC-004440	Jonczyk et al. (2004)
AILV	X87254	Grieco (1996)
GARSV	AY291207	Abou Ghanem-Sabanadzovic et al. (2005)

ferent sets of primers were therefore designed in these areas and tested with all isolates under study for assessing the: (i) capacity of detecting the highest possible number of species in the same subgroup; (ii) selectivity in detecting virus species within the same but not in other subgroups; (iii) sensitivity for virus detection in infected grapevine tissues; (iv) absence of aspecific reactions.

2.3. Amino acid sequence homology at primers sites

The level of amino acid conservation of all selected primers sites was analyzed by aligning amino acid sequences of all iso-

lates of the same species of subgroups A and B available in GenBank (Table 3).

2.4. Design of species-specific primers for multiplex PCR detection

Of a number of anti-sense or sense species-specific primers designed on conserved sequences of members of subgroups A and B, those shown in Table 4 proved the most reliable in several preliminary tests. These primers were used for single or Multiplex-PCR assays in conjunction with the group-specific degenerate primers Nepo-A (s/a), Nepo-B (s/a), and Nepo-C (s/a) (Table 4).

2.5. Plant material and virus sources

About 25 isolates of 12 grapevine nepoviruses and of the sad-wavirus *Strawberry latent ringspot virus* (SLRSV) in infected grapevine accessions coming from different countries and maintained under screen at the Mediterranean Agronomic Institute of Valenzano-Bari (Italy) (Table 2) were used for the validation of designed primers.

2.6. Total nucleic acid extraction

Total nucleic acids (TNA) were extracted from about 100 mg of cortical scrapings of infected grapevine canes ground in 1 ml grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc and 2.5% (w/v) PVP-40) and silica-purified according to Foissac et al. (2001).

2.7. RT and Multiplex-PCR

Eight to 10 µl of TNA extracts were mixed with 1 µl random hexamer primers, (Boehringer Mannheim, GbmH) (0.5 µg/µl), denatured at 95 °C for 5 min and quickly chilled on ice. Reverse

Table 4
Sequence of degenerate primers for subgroup detection in RT-PCR, sense and antisense species-specific primers used in Multiplex-PCR and their nucleotide positions in the genome

Primers code	Sequence	Subgroup/virus	Location/position	Amplified product (bp)
Nepo-A (s/a)	5'-GGHDTBCAKTMYSARRARTGG-3' 5'-TGDCCASWVARYTCYCCATA-3'	Subgroup A	CP-RNA2	255
Nepo-B (s/a)	5'-ATGTGYGCHACYACWGGHATGCA-3' 5'-TTCTCTDHAAGAAATGCCTAAGA-3'	Subgroup B	CP-RNA2	390
Nepo-C (s/a)	5'-TTRKDYTGKYKAAMYYCCA-3' 5'-TMATCSWASCRHGTGSKKGCCA-3'	Subgroup C	UTR-RNA1	640
GFLV-sp-s	5'-TCAGATTTTAAGGGCGTCCA-3'	GFLV	RNA2-88	259
ArMV-sp-s	5'-GCGGGAATATATCTGAAA	ArMV	RNA2-47	301
GDefV-sp-s	5'-GCCAGGATGAGCTCTACTAC-3'	GDefV	RNA2-18	371
TRSV-sp-s	5'-CAGTGAGGATGCACGCC-3'	TRSV	RNA2-184	190
GCMV-sp-a	5'-CTGCAAGGGAACCTTGATAAGGG-3'	GCMV	RNA2-3518	190
AILV-sp-a	5'-AGAGGTGGATTGAACGCGT-3'	AILV	RNA2-1160	278
TBRV-sp-a	5'-ATGACACTCTAGAAGAAAGTTG-3'	TBRV	RNA2-3636	485
GARSV-sp-a	5'-AGGCACCTAAAAGCCCCACA-3'	GARSV	RNA2-3732	425

sp: specific; s: sense; a: antisense; B (C + G + T); D (A + G + T); H (A + C + T); M (A + C); R (A + G); S (C + G); W (A + T); Y (C + T); K (G + T).

transcription was done for 1 h at 39 °C by adding 4 µl M-MLV buffer 5x (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂), 2 µl of 10 mM DTT, 0.5 µl of 10 mM dNTPs, and 200 units Moloney Murine Leukaemia virus (M-MLV) reverse transcriptase (Bethesda Research Laboratories, USA) in a final volume of 20 µl.

For RT-PCR with degenerate primers for subgroup detection, 2.5 µl cDNA mixture were submitted to amplification with the addition of 2.5 µl of 10× Taq polymerase buffer (Promega Corporation, USA), a final concentration of 1.5 mM MgCl₂ for subgroup A, or 2 mM MgCl₂ for subgroups B and C, 0.5 µl of 10 mM dNTPs, 0.5 µl of 20 µM sense and antisense of each degenerate primers (Table 4), and 0.2 µl of Taq polymerase (5 unit/µl) in a final volume of 25 µl. Different combinations of denaturing and annealing conditions (temperature and time of treatment) were used with each set of primers for determining the optimal operative conditions for PCR amplification. For Multiplex-PCR, 2.5 µl of 10×Taq polymerase buffer (Promega Corporation, USA) were added to 2.5 µl cDNA mixture, 0.5 µl of 10 mM dNTPs and 0.2 µl Taq polymerase (5 u/ml). Final concentration of MgCl₂ was 1.5 mM for subgroup A and 1 mM for subgroup B nepoviruses. Antisense and sense degenerate primers (0.5 µl of 20 µM) were combined with sense and antisense species-specific primers (0.5 µl of 10 µM) for subgroups A and B nepoviruses, respectively (Table 4). Identical denaturation, annealing and elongation temperature conditions, and the same number of cycles were used. Amplification products were run in 6% polyacrylamide gel and visualized by silver staining.

3. Results

3.1. Sequence homology analysis of RNA-1 and RNA-2 of nepovirus subgroups

Nucleotide and amino acid sequences of subgroups A and B viruses showed more highly conserved regions on RNA-2

rather than RNA-1, especially in the coat protein gene (Fig. 1). Conserved sequences for subgroup C were selected on the 3' untranslated region (UTR) of RNA-1 (Fig. 1) since RNA-2 sequences of only a few viruses are available.

RpRSV RNA-2 showed a low sequence homology with that of all other members of subgroup A and lack of useful conserved regions. Thus, the sequence of this virus was not taken into account for primer design.

3.2. Amino acid conservation level at the primer sites

Clustal alignment of amino acid sequences selected for primer design of different nepovirus species and isolates showed a very high level of conservation, especially for subgroup A and B nepoviruses (data not shown).

3.3. PCR primers testing for the simultaneous and differential detection of nepoviruses

Potential subgroup-specific primers were initially tested and screened against different nepoviruses in infected plant tissues. After optimization of the testing conditions, three sets of primers proved particularly efficient for RT-PCR detection.

The primer set “Nepo-A-s/a” generated an amplicon of 255 bp from vines infected by GFLV, ArMV, GDefV and TRSV, but not from samples infected by RpRSV and viruses of subgroups B and C (Fig. 2a). Primers “Nepo-B-s/a”, designed on subgroup B virus sequences, detected efficiently all members of this subgroup (GCMV, TBRV, GARSV and AILV), yielding an amplicon of 390 bp, but gave no amplification from grapevine accessions infected with viruses of other subgroups (Fig. 2b). Primers “Nepo-C-s/a” amplified selectively only ToRSV, GBLV and GTRSV, yielding a 640 bp product (Fig. 2c).

None of the three sets of primers was able to detect SLRSV in infected grapevine tissues, thus confirming the molecular distance of this virus from nepovirus species.

Subgroup A viruses		Site of primer Sense	Site of primer Antisense
ArMV	RNA-2	GGTATTCA GTACGAAAAGTGC	TATGGAGA ACTCTGTGGTCA
GDefV	RNA-2	GGAATCCAGTATGAGAAAGTGC	TATGGGGAATTGTGTGGCCA
GFLV	RNA-2	GGCGTCCA GTATGAAAATGG	TATGGAGAATTGTGTGGTCA
TRSV	RNA-2	GGTTTGCA TTCCAAAGAATGG	TATGGGAGCTTACTGGACA
Subgroup B viruses			
GCMV	RNA-2	ATGTGTGCCACTTACTGGCATGCA	TCTTAGGCATTTCTTGAAGAGAA
TBRV	RNA-2	ATGTGTGCCACCCTTGGTATGCA	TCTTAGGCATTTCTTACAGAGAA
GARSV	RNA-2	ATGTGCGCAACTTACTGGCATGCA	TCTTAGGCATTTCTTTAGAGAA
AILV	RNA-2	ATGTGCGCTACTTACAGGAATGCA	TCTTAGGCATTTCTTATAGAGAA
Subgroup C viruses			
BBLMV	RNA-1	TTATTCTGGTGA AATTCCA	TGGCACCCACTTGC TTGGATGA
PRMV	RNA-1	TTGTACTGGTGA AATTCCA	TGGCACCCACG TGC TTGGATGA
ToRSV	RNA-1	TTGGGTTGGCTAA CCCCCA	TGGCCAGCACACGGTACGATTA

Fig. 1. CLUSTAL X (1.81) multiple alignment of nucleotide sequences at the two sites where the degenerate primers (sense and antisense) were designed.

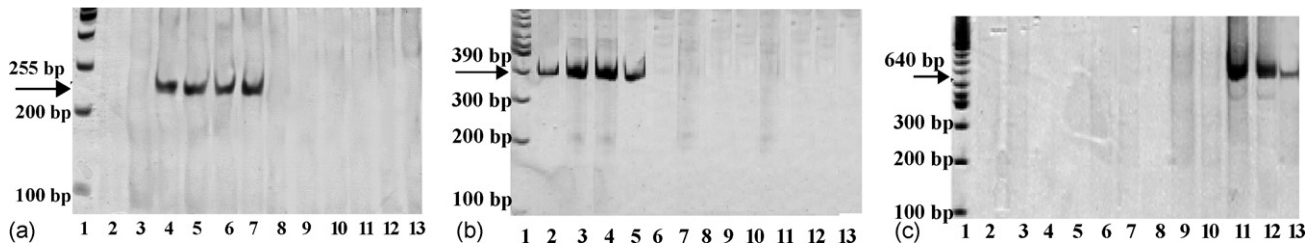


Fig. 2. (a) Electropherogram showing the 255 bp product amplified by degenerate primers Nepo-A (s/a) only from samples infected by nepoviruses of subgroup A. Lane 1: DNA ladder marker; lane 2: GTRSV; lane 3: GBLV; lane 4: GFLV; lane 5: ArMV; lane 6: GDefV; lane 7: TRSV; lane 8: ToRSV; lane 9: RpRSV; lane 10: GCMV; lane 11: GARSV; lane 12: TBRV; lane 13: negative control. (b) Electropherogram showing the 390 bp product amplified by degenerate primers Nepo-B (s/a) only from samples infected by nepoviruses of subgroup B. Lane 1: DNA ladder marker; lane 2: GCMV; lane 3: TBRV; lane 4: AILV; lane 5: GARSV; lane 6: GFLV; lane 7: ArMV; lane 8: GDefV; lane 9: TRSV; lane 10: GBLV; lane 11: GTRSV; lane 12: ToRSV; lane 13: negative control. (c) Electropherogram showing a 640 bp, product amplified by degenerate primers Nepo-C (s/a) only from samples infected by nepoviruses of subgroup C. Lane 1: DNA ladder marker; lane 2: GFLV; lane 3: ArMV; lane 4: GDefV; lane 5: TRSV; lane 6: GARSV; lane 7: AILV; lane 8: TBRV; lane 9: GCMV; lane 10: negative control; lane 11: GBLV; lane 12: GTRSV; lane 13: ToRSV.

The best PCR protocol encompassed 35 cycles after an initial denaturation at 94 °C for 5 min. Each cycle consisted of denaturation at 94 °C for 35 s, annealing temperature of 50 °C for 45 s for all subgroups and an extension temperature of 72 °C for 35, 45, 50 s for subgroups A, B and C, respectively. In all cases, final extension was at 72 °C for 7 min.

3.4. Multiplex-PCR

Degenerate and species-specific primers used in combination were able to detect single infections by nepoviruses of subgroup A and B. Primers specifically designed for subgroup A species amplified a fragment of 190, 259, 301 and 371 bp from samples infected by TRSV, GFLV, ArMV and GDefV, respectively, but not from samples infected by other nepovirus species (Fig. 3a). Similarly, primers for subgroup B virus detection amplified efficiently and selectively a 190, 278, 425 and 485 bp product from samples infected by GCMV, AILV, GARSV and TBRV, respectively (Fig. 3b).

Degenerate primers designed for the aspecific detection of subgroup C species proved efficient only when used in RT-PCR, but not in Multiplex-PCR in combination with species-specific primers. For this subgroup, the high level of primer degeneracy required the use of high MgCl₂ concentration and low annealing

temperature, factors that may have interfered with the activity of specific primers.

The same primers successfully detect mixed natural infections in grapevine samples (Fig. 4a). However, given the unavailability of sources naturally infected by more than three viruses of subgroups A and B, mixed infections were artificially built by mixing cDNAs of the four species of each subgroup and successfully tested in Multiplex-PCR (Figs. 4 and 5).

4. Discussion

Polymerase chain reaction with polyvalent primers is a simple strategy that has been widely applied for the aspecific and simultaneous detection of members of several genera and families of plant viruses (James et al., 2006). In the case of grapevine viruses, degenerate primers were successfully used for the simultaneous detection of closteroviruses (Saldarelli et al., 1998), foveaviruses and vitiviruses (Dovas and Katis, 2003), and fleck-like viruses (Sabanadzovic et al., 2000). For nepoviruses the application of polyvalent primers was limited to GFLV and ArMV, using primers designed on a conserved portion of the movement protein gene (Wetzel et al., 2002).

In the present study, the comparative analysis of genomic sequences of grapevine-infecting nepoviruses belonging in the

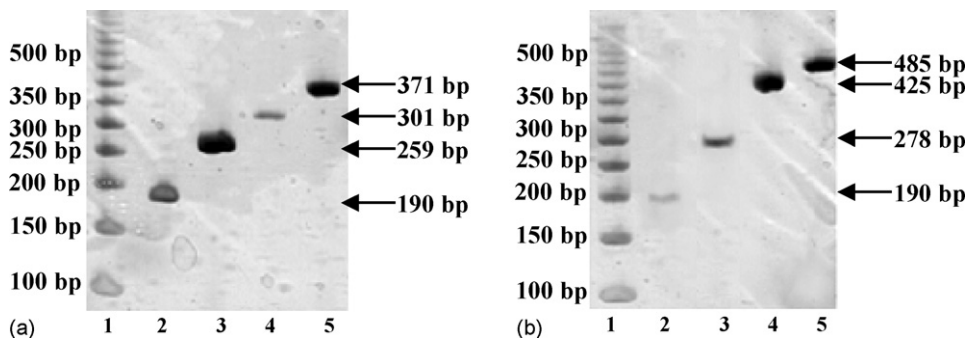


Fig. 3. (a) Electropherogram of amplification products from sources infected by subgroup A nepoviruses. RT-PCR reactions were done with the antisense degenerate subgroup A primers with the appropriate subgroup A species-specific sense primers. Lane 1: DNA ladder marker; lane 2: TRSV; lane 3: GFLV; lane 4: ArMV; lane 5: GDefV. (b) Electropherogram of amplification products from sources infected by subgroup B nepoviruses. RT-PCR reactions were done with the sense degenerate subgroup B primer with the appropriate subgroup B species-specific antisense primers. Lane 1: DNA ladder marker; lane 2: GCMV; lane 3: AILV; lane 4: GARSV; lane 5: TBRV.

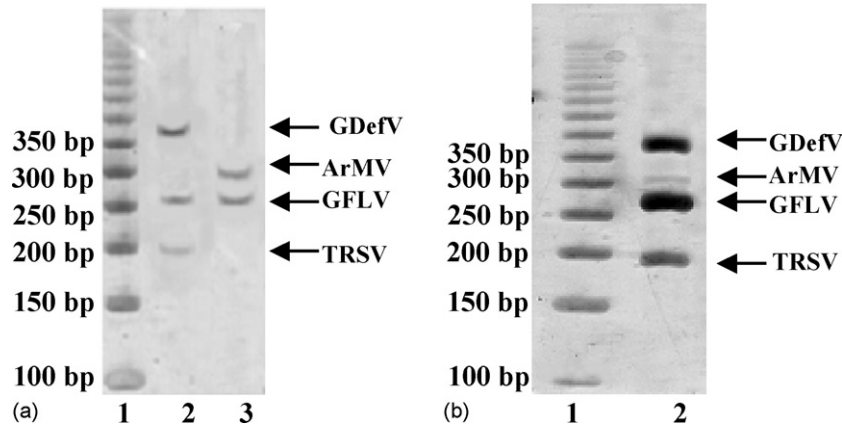


Fig. 4. Electropherogram of amplification products from sources with multiple infections by subgroup A nepoviruses. The cocktail of species-specific primers used in Multiplex-PCR generated amplicons of different size, allowing the identification of single viral species. (a) Lane 1: DNA ladder marker; lane 2: artificial triple infection by TRSV, GFLV and GDefV; lane 3: natural mixed infection of GFLV and ArMV in grapevine. (b) Lane 1: DNA ladder marker; lane 2: quadruple infection by subgroup A nepoviruses TRSV, GFLV, GDefV and ArMV.

three known subgroups of the genus allowed the identification of several highly conserved regions in the RNA-2 (coat protein gene) of subgroup A and B, and in the UTR of subgroup C. Hence, several sets of degenerate primers for the aspecific detection of all members of the same subgroup were designed. However, because none of the identified regions was common to all members of the genus no universal degenerate primers could be developed.

Although several molecular and serological techniques are currently available for nepovirus detection in grapevines, their use is not deprived of shortcomings when large number of samples are to be tested for a large number of viruses, as in the case of grapevines. Furthermore, serological reagents are not commercially available for all viruses and in field-grown vines virus concentration falls below the detection threshold of traditional diagnostic techniques (ELISA or mechanical transmission to herbaceous hosts) in summer months, especially in warm climates. For the above reasons, the development of the three sets of primers for multiple detection of nepoviruses belonging to

subgroups A, B and C represents an useful tool for improving the sensitivity of detection and reducing costs of large scale surveys.

The degenerate group-specific primers presently designed not only represent fast, efficient and low cost tools for large scale diagnosis, but they can also be used for detecting possible new nepovirus species in grapevines. This conclusion seems to be tenable even though three grapevine-infecting nepoviruses of subgroup C, i.e. BBLMV, PRMV and CLRV, were not included in our tests.

As shown in the present work, an extension of the potential use of group-specific primers for nepovirus detection is offered by their combination with species-specific primers in multiplex-PCR for quick virus identification at the species level.

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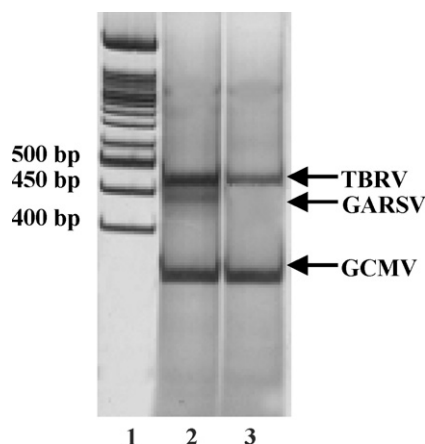


Fig. 5. Multiplex RT-PCR analysis of double and triple infection by subgroup B nepoviruses. All viral species are individually detected by a combination of sense degenerate group-specific primer and species-specific antisense primers. Lane 1: DNA ladder marker; lane 2: triple infection by GCMV, GARSV and TBRV; lane 3: double infection by TBRV and GCMV.

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