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Development of RT-PCR degenerate primers for the detection of two mandariviruses infecting citrus cultivars in India

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ABSTRACT

Indian citrus ringspot virus (ICRSV) and *Citrus yellow vein clearing virus* (CYVCV) are the mandariviruses infecting various citrus cultivars in India and around the world. In the fields, it was observed that citrus plants infected by both the viruses and frequently expressed only ringspot symptoms. The ICRSV-specific polyclonal-antibody used in immuno-sorbent electron microscopy (ISEM) and enzyme linked immuno-sorbent assay (ELISA) could detect only ICRSV in mixed infections. Therefore, the conserved sequences of the RNA dependent RNA polymerase (RdRP) gene of the alphaflexiviruses were exploited for developing a RT-PCR based assay for detection of both the mandariviruses simultaneously, if present. A degenerate primer pair was designed to amplify a ~435bp fragment by multiple alignments of the RdRP gene sequences of the members of genera *Mandarivirus*, *Potexvirus* and *Allexivirus*. The developed RT-PCR assay was validated for detecting both, CYVCV and ICRSV in mixed infections as well as in single virus-infected citrus plants. The presence of ICRSV or CYVCV or both of them together in such plants were confirmed by using primer pair specific to each of these viruses. Further, the identity of the amplicons was confirmed by sequencing and the virus species were determined with BLAST*N* analysis. The degenerate primers also amplified the corresponding target sequences of an allexivirus and a potexvirus from the respective infected garlic/ onion and tobacco plants. The use of the degenerate primers for the detection of these virus species of the genus *Mandarivirus* will be useful in citrus certification programmes.

1. Introduction

Citrus is one of the most important fruit crops globally grown in more than 142 countries under different climatic conditions. *Indian citrus ringspot virus* and *Citrus yellow vein clearing virus* are two species of the genus *Mandarivirus.* These two viruses, ICRSV and CYVCV are associated with ringspots and yellow vein clearing disease of citrus, respectively. The ringspot, vein flecking and chlorosis are the typical symptoms on Kinnow mandarin associated with ICRSV (Ahlawatand Pant, 2003; Prabha and Baranwal, 2011). CYVCV produces typical water socking of veins on ventral side, vein clearing and leaf distortion symptoms on Etrog citron, could be used as indicator host (Alshami et al., 2003; Loconsole et al., 2012). In India, yellow vein clearing disease was reported in Abohar, Punjab on citrus cultivar Etrog citron. The symptomatic leaf samples showed a mixture of ICRSV decorated with ICRSV specific polyclonal antibodies and undecorated flexuous virus particles in ISEM study (Alshami et al., 2003). That was first indication about the presence of a new virus and was tentatively named as citrus yellow vein clearing virus. The flexuous filamentous virions were 13–15 nm in diameter with a length of 650 and 685 nm (Ahlawat and Pant, 2003; Alshami et al., 2003). In another study CYVCV had been found to occur along with ICRSV in mixed infections on citrus plants showing ringspots, vein flecking and chlorosis symptoms (Meena and Baranwal, 2016). ELISA with ICRSV specific antibodies and RT-PCR assay by using ICRSV specific primers failed to detect the presence of associated virus, even though the samples were exhibiting typical ringspot symptoms (Prabha and Baranwal, 2011).

The capability of degenerate primers to detect more than one virus within a genus has been successfully demonstrated by many researchers (Saldarelli et al., 1998). *Alphaflexiviridae,* is the recently established family characterized by potexvirus like replication protein and possesses triple gene block (TGB) like movement protein. The recognized genera of major plant viruses under *Alphaflexiviridae* family are *Potexvirus, Allexivirus* and *Mandarivirus* (Martelli et al., 2007; ICTV, 2017). These members of plant infecting virus genera of the family possess positive single strand RNA genome and because of the low fidelity and

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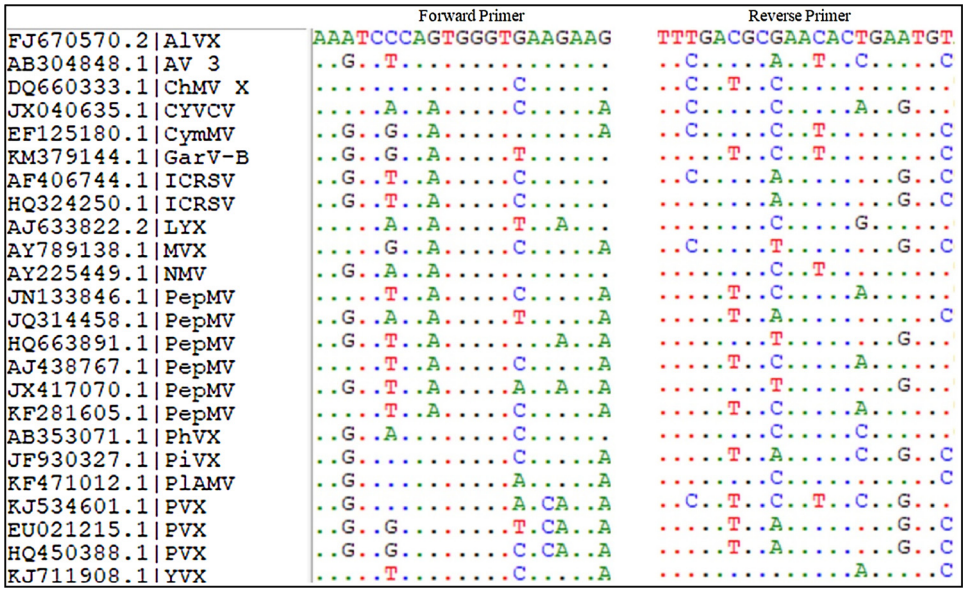

Fig. 1. Multiple sequences alignment of the genomes of the virus species in the Alphaflexiviridae family used for designing the degenerate primers for a RT-PCR assay: Allium virus X (AlV X), Asparagus virus 3 (AV 3), Citrus yellow vein clearing virus (CYVCV), Chenopodium mosaic virus X (ChMV X), Cymbidium mosaic virus (CymMV), Garlic virus B (GarV B), Indian citrus ringspot virus (ICRSV), Lily virus X (LVX), Mint virus X (MVX), Narcissus mosaic virus (NMV), Pepino mosaic virus (PepMV), Phaius virus X (PhVX), Pitaya virus X (PiVX), Plantago asiatica mosaic virus (PlAMV), Potato virus X(PVX) and Yam virus X (YVX). Based on the sequence alignment, the primers identified were: forward primer, Alflex F- AARTCDCAATGGGTYAAG-AAR; reverse primer, Alflex R- GCAYTCWGT-RTTNGCRTCRAA. The degenerate bases were: R = A or G; D = A or G or T; Y = C or T; W = A or T; N = any of the four bases) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
Nucleotide sequences, annealing temperatures (Ta), amplified region and amplicon size of each of the primer pairs used in RT-PCR assays.

Primer Name	Primer sequencing (5'——3')	Tm (°C)	Amplified region	Amplicon size	Reference
ICM101-F	AGCGTAACCATTCTTACGCCC	60 °C	Partial RdRp & CPG	758bp	Meena and Baranwal (2016)
ICM102-R	GGTTCAGGAGCCTTCGTCTG				
CYVdt-F	CCCTAGAGAGACACCCTAC	60 °C	Partial NABP	496bp	This study
CYVdt-R	GGCACTAACAGGATTTTCGGTTAG				
Alflexi- F	AARTCDCAATGGGTYAAGAAR	45°C	Partial RdRp	~435bp	This study
Alflexi -R	GCAYTCWGTRTTNGCRTCRAA				

Note: (a) Wobble base positions of the degenerate nucleotides are indicated by the following codes: M = A or C; Y = C or T; R = A or G; D = A or G or T; W = A or T; N = A or T or G or C and (b) CPG: coat protein gene, (c)NABP: Nucleic acid binding protein, RdRp: RNA-dependent RNA polymerase.

variability, are also known to exist as quasispecies with some conserved region in viral RNA-dependent RNA polymerase (RdRP) (Zanotto et al., 1996). In the present study, attempts were made to design novel degenerate primers, based on a conserved domain in the RdRP genes of the viruses in the family *Alphaflexiviridae*, for the detection of mandariviruses.

2. Materials and methods

2.1. Plant materials, viruses and transmission electron microscopy

Surveys were conducted during the years 2012-14 and bud sticks and symptomatic leaf samples were collected from different citrus orchards in Punjab, Maharashtra, Manipur, West Bengal and Rajasthan. The fresh leaf samples collected were assayed for ICRSV and CYVCV using specific primers and then stored at -80 °C for future testing and validation of degenerate primers described in this paper. The collected bud sticks were grafted onto healthy sweet orange and Kinnow mandarin seedlings and maintained in a glasshouse of the Division of Plant Pathology, IARI, New delhi for further experimental studies, including the validation of degenerate primers. Symptomatic leaves from the grafted plants as well as those from the fresh field samples were examined by transmission electron microscopy (TEM) following the leaf dip standard protocol (Milne and Luisoni, 1977). Kinnow mandarin plants grown from seeds were used as healthy control. Garlic and onion leaves, collected from the research farm of IARI, New Delhi, and confirmed to be positive for garlic virus X and shallot virus X infections using the allexivirus-specific primers, were used as positive controls for testing and validating the degenerate primers. Tobacco (*Nicotiana benthamiana*) plants, infected with potato virus X and maintained in the glasshouse, were also used as positive controls.

2.2. Designing of degenerate RT-PCR primers

To design the degenerate primer pair, the reference sequences of mandariviruses (CYVCV and ICRSV) and those of the closely related virus genera, *viz., Potexvirus* (*Allium virus X; Asparagus virus 3; Chenopodium mosaic virus X; Cymbidium mosaic virus; Lily virus X; Mint virus X; Narcissus mosaic virus; Pepino mosaic virus; Phaius virus X; Pitaya virus X; Plantago asiatica mosaic virus; Potato virus X and Yam virus X*) and *Allexivirus* (*Garlic virus B*) were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/) and aligned using the Clustal W package of BioEdit 7.2.5.0. The genomic region of RNA-dependent RNA polymerase (RdRP) gene, which contained the most conserved sequences in the alignment, was exploited for designing a primer pair that amplified a 435 nt long genomic fragment (Fig. 1).

2.3. RNA isolation and RT-PCR assays

Total RNA was extracted from a 100 mg leaf sample of each of the respective symptomatic and healthy leaves of citrus plants, by macerating in liquid nitrogen and using the commercial RNeasy Plant Mini Kit (QIAGEN, Germany) according to manufacturer's instructions. The RNA (1 µg) was reverse-transcribed into cDNA using the *Alflex-R*(antisense) primer and the M-MuLV reverse transcriptase kit (NEB, Germany) by following the manufacturer's instructions. PCR using the degenerate primers was carried out in a total volume of 20 µl reaction mixture containing 2.5 µl of template cDNA, 0.25 µl (5 u/µl) *Taq* DNA polymerase (NEB), 0.5 µl of 10 mM dNTP, 1 µl of 25 mM $Mgcl_2$, 0.5 µl of each of the 10 mM *Alflex-F* and *Alflex-R* primers and the remaining

Table 2
Validation of degenerate primers using unknown samples: sample code, associated plant hosts, symptoms and results obtained using the *Alflex-F* and *Alflex-R* (degenerate primers RT-PCR), *CYVdt-F* and *CYVdt-R* (CYVCV RT-PCR) and the *ICM101-F* and *ICM102-R* primers (ICRSV RT-PCR), sequence identities of the amplicons with the CYVCV and ICRSV reference sequences.

No.	Sample Code	Host	Symptoms	DP -assay	CYVCV-assay	ICRSV-assay	Seq. identity (%)		Genus
							CYVCV	ICRSV	
1.	KGH	@Kinnow	Asym	-ve	-ve	-ve	X	X	*Mandarivirus*
2.	KMg	#Kinnow	VF, RS	+ve	-ve	+ve	79	79	
3.	KRFa	#Kinnow	RS, M	+ve	+ve	-ve	81	79	
4.	KVfa	#Kinnow	CRS, Mo	+ve	X	+ve	80	80	
5.	AKh	#Kinnow	CRS, M	+ve	+ve	-ve	79	79	
6.	KPune	@Kinnow	RS, VF	+ve	+ve	-ve	95	77	
7.	RPK1	#Kinnow	RS, M	+ve	+ve	-ve	81	79	
8.	KVfp	#Kinnow	CRS, M	+ve	+ve	-ve	X	X	
9.	KMlu	@Kinnow	VF, RS	+ve	+ve	-ve	96	76	
10.	MMkf	@Mandrin	RS, M	+ve	+ve	-ve	80	79	
11.	CTiru	@Sweet lime	M	-ve	-ve	-ve	X	X	
12.	MUk	@Mandrin	MO	-ve	-ve	+ve	X	X	
13.	MAnd	#Mandrin	RS	+ve	X	+ve	79	79	
14.	PASOa	@Sweet orange	VF, RS	+ve	+ve	-ve	97	76	
15.	MNgp	#Sweet orange	RS	+ve	X	X	X	X	
16.	CDnd	#Lemon	M	+ve	X	X	X	X	
17.	CRFa	#Lemon	RS	+ve	+ve	-ve	81	79	
18.	CPdv	@Lemon	M	-ve	-ve	-ve	X	X	
19.	CLab	#Lemon	RS, YS	+ve	-ve	+ve	80	80	
20.	CSOnd	#Sweet lime	CRS	+ve	X	X	X	X	
21.	CTL	#Lemon	RS	-ve	X	X	X	X	
22.	CECa	@Lemon	VF	+ve	+ve	-ve	96	76	
23.	GFahd	#Grape fruit	RS, M	+ve	X	X	X	X	
24.	GFfP	#Grape fruit	Asym	-ve	X	X	X	X	
25.	Nagri	@Sweet orange	VF	+ve	X	X	X	X	
26.	PUMne	#Pumello	MO	-ve	X	X	X	X	
27.	SOnd	#Sweet orange	CRS	+ve	X	X	X	X	
28.	CCIFT	#Lemon	MO	-ve	X	X	X	X	
29.	JRRl	#Orange	RS	+ve	X	X	X	X	
30.	JMa-1	@Orange	RS	+ve	-ve	+ve	80	80	
31.	JMa-2	@Orange	RS	+ve	+ve	-ve	82	79	
32.	MARSg	#Orange	Asym	-ve	X	X	X	X	
33.	KPAU-1	$Kinnow	RS	+ve	X	X	X	X	
34.	KPAU-2	$Kinnow	RS	+ve	X	X	X	X	
35.	PVc	$Lemon	M	-ve	X	X	X	X	
36.	RMg-1	@Malta	RS	+ve	+ve	+ve	81	79	
37.	RMg-2	@Malta	CRS, N	+ve	+ve	-ve	95	77	
38.	CG-112	#Garlic	Asym	-ve	X	X	X		*Allexivirus*
39.	G-282	#Garlic	M, C	+ve	X	X	X		
40.	BGSD-1225	#Garlic	M, C	+ve	X	X	X		
41.	SCL-383	#Onion	M& Stunting	+ve	X	X	Garlic virus C		
42.	NB-1	@ Tobacco	MO	+ve	X	X	potato virus X		*Potexvirus*

Note: "Asym = No visible symptoms; C = Chlorosis; CRS = Conspicuous Ringspot; M = Mosaic; Mo = Mottling; N = Necrosis; RS = Ringspot; VF = Vein Flecking.".
*X- "Not done".
*Samples used in this study indicated as: @ = samples from glasshouse; # = fresh samples from field and $ = Samples stored at -80.

volume was made up with nuclease-free water. The PCR program consisted of one cycle at 94 ^{0}C for 4 min, 35 cycles at 94 ^{0}C for 30 s, 45 ^{0}C for 45 s, and 72 ^{0}C for 1 min each, followed by a final extension cycle at 72 ^{0}C for 10 min. For the specific detection of ICRSV and CYVCV, the PCR was performed at 60 ^{0}C annealing temperature with the *ICM101-F* & *ICM102-R*, and *CYVdt-F* & *CYVdt-R* primers pairs, respectively (Table 1), while the other parameters remained the same as those described for the degenerate primers. The PCR products were electrophoresed in 1.0% agarose gels containing ethidium bromide and visualized under a gel documentation system (Bio-Rad, USA).

2.4. Validation of the degenerate primers for virus detection

The degenerate primer set designed for the RT-PCR detection of mandariviruses was validated using the total RNA of 37 citrus samples which comprised the frozen field samples collected during 2012 to 2014 and fresh samples from the grafted plants mentioned above and as indicated in Table 2. The allexiviruses infected garlic and onion plants and glasshouse potato virus X infected tobacco plants described above were used as positive controls in the validation experiments (Table 2).

2.5. Cloning and sequencing of RT-PCR products

The amplified DNA fragments of the expected sizes were excised from the gels and purified with Qiagen Gel Extraction Kit (Hilden, Germany). The purified product ligated by using the T&A Cloning vector kit, (RBC, USA) following manufacturer's instructions and transformed into the DH5α strain *E. coli* (Stratagene) competent cells using standard protocol (Sambrook and Russell, 2001). Two positive clones were selected for plasmids isolation using Thermo scientific GeneJET Plasmid Miniprep kit (Lithuania) and sequenced. Sequencing was performed with an automated sequencer ABI 3130 Genetic Analyzer at Xcelris Genomics Labs Ltd, Ahmedabad, India.

Fig. 2. Electron micrograph showing the flexuous virion particles (Right panel) in the sap extract of a symptomatic citrus (Malta) leaf sample (Left panel) from scion collected from the field.

2.6. Sequence analysis and phylogenetic tree construction

The DNA sequences of the cloned RT-PCR fragments from 17 citrus samples were initially processed with BLAST*N* analysis. These nucleotide sequences were aligned using Clustal W algorithm of BioEdit 7.1.3. Further, a phylogenetic tree was constructed using the aligned nucleotide sequences and DNA sequences of the reference isolates of CYVCV-Y1 (JX040635.1), ICRSV-Pu (HQ324250), *Potato virus X* (HQ450388.1) and *Allium virus X* (FJ670570.1) of the *Alphaflexiviridae* retrieved from GenBank. The phylogenetic inference was accomplished with 1000 bootstrap replicates following the maximum likelihood phylogeny of the MEGA6 software (Tamura et al., 2013).

3. Results

3.1. Electron microscopy of symptomatic samples of different citrus species

Symptomatic leaf samples of different citrus species *viz*., Etrog citron (2), Malta (2), sweet **orange** (1) and Kinnow mandarin (2) collected from different citrus orchards during surveys were used either directly or from grafted scions for leaf dip preparation. Presence of the flexuous virion particles was observed under the TEM, (JeolJEM1011, Fig. 2). Diameter of virions ranged from 13 to14 nm with a length of 580 to 785 nm under electron microscopy. All these samples were confirmed positive for mandariviruses with RT-PCR using the degenerate primers (Table 2).

3.2. Testing the degenerate primers for mandarivirus/allexivirus/potexvirus detection using positive control samples

With the RT-PCR, by using the synthesized degenerate primers, the expected size of 435bp amplicons were recovered from ICRSV and CYVCV infected citrus samples, maintained as positive control in the glass house. Further, infection of ICRSV and CYVCV was confirmed using specific primers (Table 1). Gel electrophoresis profile revealed that the degenerate primers successfully amplified the RNA isolated from the ICRSV and CYVCV infected plants samples (Fig. 3A). The degenerate primer could also detect garlic virus X and shallot virus X in onion and garlic samples, respectively, previously tested positive for these viruses (Fig. 3B). Presence of potato virus X was also tested in tobacco (Fig. 3C). In all positive samples, amplicons of expected size of 435bp were recovered. Sequencing results of amplicons from citrus plants confirmed the presence of ICRSV and CYVCV in respective samples. Sequencing results of allexiviruses and potato virus X confirmed that the degenerate primer could also detect these viruses.

3.3. Validation of the degenerate primers for RT-PCR assay using unknown samples

A total of 37 citrus samples collected during the period 2012–2014, were tested either directly as fresh samples/stored samples or from scions of graft inoculated plants (Table 2) for validating the designed degenerate primer pair. Those leaf samples included from different citrus species exhibited various symptoms such as vein flecking, mosaic, chlorosis to ringspot (Table 2). Out of 37 samples tested, in 27 amplicons of the expected sizes were documented. Gel image of amplified products from nine citrus field samples (KMg; KRFa; KPune; CLab; KVFa; MMkf; PASOa; JMa-1 and RMg-1, respectively) have been given in Fig. 3(D). Out of three garlic samples from field, in two amplicons of 435bp was observed (Fig. 3E). Moreover, no amplification was observed in healthy samples with the use of degenerate primers.

Further, the results for ICRSV and CYVCV were confirmed using specific primers in RT-PCR assays. Among the samples, tested positive with degenerate primers, one sample of each *viz*., Kinnow, lemon and orange showing ringspot symptoms, however, no amplification was observed by using CYVCV specific primers on gel profile. Whereas, out of the 27 tested positive with degenerate primers, 12 were found positive for CYVCV using specific primers and negative for ICRSV. The amplified products of viruses from 17 citrus samples were purified and cloned, and two positive clones of each sample were sequenced. The sequencing results revealed that all 17 amplicons showed similarity with the sequences of members of mandarivirus (Table 2).

3.4. Comparison and phylogenetic analysis of the cloned virus sequences

Sequence analysis of seventeen cloned amplicons showed 80–97% sequence similarity with CYVCV as compared to 76–81% similarity with ICRSV. A comparison in nucleotide sequences of sequenced cloned from this study with the reference sequences of CYVCV and ICRSV showed interesting results. The 435bp partial sequences of RdRP gene from the five samples, *viz*., RMg-2, CECa, PASOa, KMlu and KPune shared 95–97% sequence similarity with the CYVCV (JX040635.1) and 76–77% with ICRSV (HQ324250.1). Six samples *viz*., CLab, JMa-1, MAnd, AKh, KMg and KVfp shared nucleotides sequence identity equally with both the viruses, while the remaining contigs of six samples *viz*., RMg-1, RPK-1, CRFa, JMa-2, MMKf and KRFa shared 79–81% sequence identities with the ICRSV and CYVCV reference sequences (Table 2). All the seventeen sequence contigs showed 62–67% sequence identity with potexvirus (potato virus X). The sequences of amplicons of the allexiviruses from garlic and onion, and potato virus X from tobacco shared 100 % identity with the corresponding reference sequences of

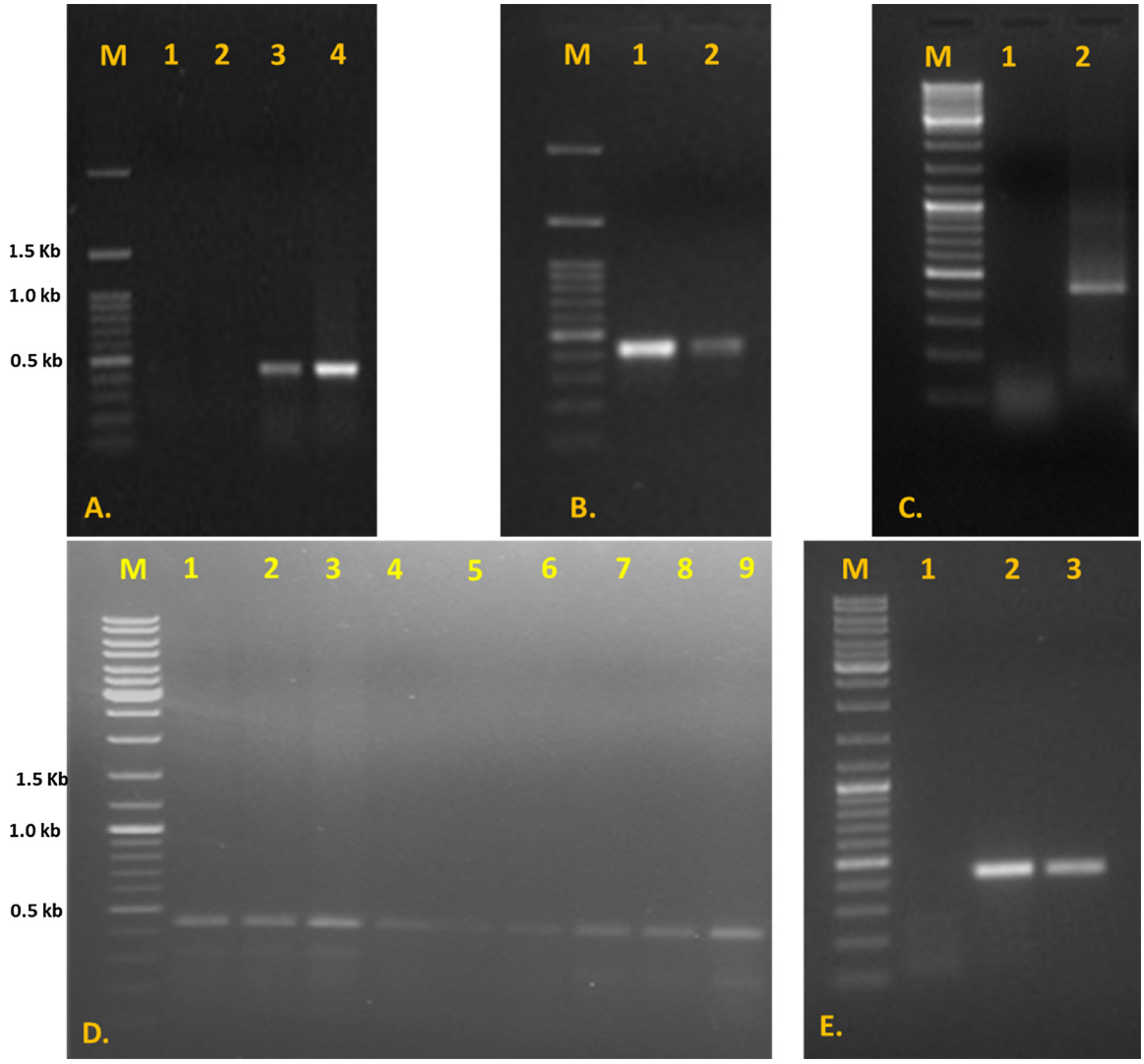

Fig. 3. Agarose gel profiles of the detection of Alphaflexiviridae members using degenerate primers in RT- PCR (A.) Detection of Mandarivirus using degenerate primers: Lanes: M, 100bp DNA ladder; 1. Water control; 2. Healthy sample (–ve); 3. Positive control (+ve) for ICRSV and 4. Positive control for CYVCV. (B.) Detection of Allexivirus using degenerate primers: Lanes: M, 100bp DNA ladder; 1. Garlic virus X positive sample in onion and 2. Shallot virus X positive sample in garlic. (C.) Detection of Potexvirus using degenerate primers: Lanes: M, mixed DNA ladder; 1. Healthy sample (–ve) and 2. Potato virus X in Nicotiana benthamiana. (D.) Detection of Mandarivirus virus from field samples of citrus using degenerate primers: Lanes: M, mixed DNA ladder; Lane 1–4 stored at -80 (KMg; KRFa; KPune; CLab) and Lane 5–9 maintained in glass house (KVFa; MMkf; PASOa; JMa-1 RMg-1). (E.) Detection of Allexivirus from unknown field samples using degenerate primers: Lanes: M, mixed DNA ladder; 1-3. Field samples of garlic for validation.

viruses (Data not shown).

To elucidate genetic relationship among the seventeen sequenced contigs, a phylogenetic tree was constructed. The results revealed that these sequences from the infected citrus species segregated into two separate groups, indicating the involvement of two virus species (Supplementary Fig. 1). The first group further segregated into two subunits; first cluster included five sequences grouped with the CYVCV sequence; whereas, the second group comprised twelve sequences which did not show any distinct grouping either with CYVCV or with ICRSV, however, showed close proximity with CYVCV. The ICRSV reference sequence segregated on a separate branch. The sequences and phylogenetic analysis of seventeen sequenced contigs showed that these isolates associated with two species of mandariviruses.

4. Discussion

CYVCV and ICRSV infect commercially grown citrus species and produce nearly identical symptoms such as vein flecking, mosaic and ringspots on citrus hosts. The symptoms are more prominent during the winter and based on the expressed symptoms the two species of viruses could not distinguished. Coexistence of both species of the mandariviruses in same citrus plants, had been observed with ISEM studies using the diseased tissues (Alshami et al., 2003). The particles of both virus species are alike in shape and size. The widely and routinely practiced ELISA for plant virus detection was developed for the detection of ICRSV (Ahlawat and Pant, 2003). The results of ELISA and ISEM revealed that CYVCV and ICRSV are serologically distinct species of genus *Mandarivirus* (Alshami et al., 2003; Loconsole et al., 2012), with some degree of cross reactivity. As serological assays are not preferable for detection of closely related mandariviruses (Alshami et al., 2003), use of degenerate oligonucleotide primers from conserved amino acid sequences in RNA-dependent RNA polymerase gene is a better option to amplify closely related virus species as has been shown for two approved virus species: European mountain ash ringspot-associated virus (EMARaV) and Fig mosaic virus (FMV) in RT-PCR assay (Elbeaino et al., 2013). The RdRP genomic region of flexiviruses is highly variable with some conserved sequence fragments which could be used for synthesis of degenerate oligonucleotide primers. The developed RT-PCR assay

using the degenerate primers *AFlex-F* and *AFlex-R* promises the detection of both, ICRSV and CYVCV species of genus *Mandarivirus*. Further, the degenerate primer set could also detect two allexiviruses and a potexvirus and may be tested for detection of other viruses in different genera of *Alphaflexiviridae*. RdRP based degenerate primers have been also used for detection of other members of *Alphaflexiviridae viz., Trichovirus, Capillovirus* and *Foveavirus* (Foissac et al., 2005). The power of degenerate primers to detect and diagnose, have been proven for other genera such as *Ilarvirus, Potyvirus, Cucumovirus, Tobamovirus* (Chu et al., 2001; Maliogka et al., 2007; Zheng et al., 2010; Seo et al., 2014; Li et al., 2018). Chu and associates employed degenerate primers based on conserved region of RdRP for the detection of five different serogroups of tospoviruses (Chu et al., 2001). The primers based on replicase used to detect about nine viruses in the genus *Tobamovirus* (Li et al., 2018). These studies indicate that degenerate oligonucleotide primers can be very useful for detection of related and unrelated viruses at genus level in a family.

In the present study many citrus samples were co-infected with both ICRSV and CYVCV, but only CYVCV could be confirmed after sequencing of amplicon. This could be due to a lower concentration of ICRSV than the CYVCV in the co-infected tissues and lower RNA replicase preference for ICRSV than that of CYVCV, thus resulting in amplification of only CYVCV. Similar results had been observed when only Olive mild mosaic virus (OMMV) was amplified in a mixed infection with Tobacco necrosis virus D (TNV-D) in olive tree, where OMMV was at a higher level compared to TNV-D (Varanda et al., 2010). Another possibility could be that CYVCV better be fitted to the host and thus become dominant in co-infected plants. All these possibilities need further investigations and corroboration in the light of the observation of more detection of CYVCV in co-infected samples.

Sequencing and phylogenetic analysis of sequenced contigs of RdRP region from symptomatic citrus samples, consistently positioned CYVCV and ICRSV on the same branch in phylogenetic tree. RNA viruses are prone to genetic variability, evolution and adaption to new environmental conditions more faster due to the high rates of mutation (at least 10^5 times higher than those of their hosts) as a consequence of the lack of proof-reading activity of the RNA polymerases (García-Arenal and Fraile, 2011) and the members of *Alphaflexiviridae* are not exception. The partial RdRP sequences retrieved in the present study, indicated genomic variability in mandariviruses. The degenerate primers can be used for exploration of novel virus species as have been done for several new DNA virus species in *Badnavirus* (Baranwal and Sharma, 2017) The main objective of the present study was to devise a universal primer pair for detection of mandarivirus for citrus certification programmes and it has been achieved successfully.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113753.

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