Degenerate Primers Facilitate the Detection and Identification of Potyviruses From the Northwest Region of Iran

Nemat Sokhandan-Bashir [*,1], Aisan Ghasemzadeh [1], Nahid Masoudi [1], Reza Khakvar [1], Davoud Farajzadeh [2]

[1] Department of Plant Protection, Faculty of Agriculture, University of Tabriz, Tabriz, IR Iran, Tabriz, IR Iran

[2] Department of Cellular and Molecular Biology, Madani Tarbiat Moallem University, Tabriz, IR Iran

*Corresponding author: Nemat Sokhandan-Bashir , Department of Plant Protection, Faculty of Agriculture, University of Tabriz, Tabriz, IR Iran. Tel.: +98-4113392036, Fax: +98-4113356006, E-mail: sokhandan@tabrizu.ac.ir.

ABSTRACT

Background: Potyviruses are accounted for 40% of viral diseases of various crops including vegetables and legumes. Potyviruses can be transmitted through plant sap, seeds and many aphid species. Due to ease of spread of these viruses detection of such viruses are crucial as to the control of the incited diseases.

Objectives: This study compared the efficiencies of two couples of primers in their detection of potyviruses infecting vegetables in North-West of Iran.

Materials and Methods: To identify potyviruses infecting vegetables, total RNA preparations from leaves of diseased plants were subjected to reverse transcription (RT) with oligo d(T)15, M4T or a potyvirus-specific primer, followed by polymerase chain reactions (PCR) with two pairs of degenerate primers including Sprimer/M4 or NIb2F/NIb3R that would yield ~ 1700 or 350 bp fragments, respectively. Amplification was achieved from 7 samples when Sprimer and M4 were used, but non- specific fragments were also amplified. However, specific amplifications from 31 samples were achieved with NIb2F2 and NIb3R. PCR products resulting from the use of NIb2F/NIb3R were subjected for cloning and sequencing.

Results: BLAST analysis of the sequenced data revealed that the PCR-amplified fragments belonged to *Bean common mosaic virus* (green bean), *Bean yellow mosaic virus* (broad bean), *Potato virus Y* (potato), *Watermelon mosaic virus* (squash, muskmelon or melon), *Zucchini yellow mosaic virus* (squash), and *Soybean mosaic virus* (bean).

Conclusions: This study demonstrated the efficiency of NIb2F2/NIb3R in detection of potyviruses and revealed the extent of infections with diverse potyviruses species in the northwest part of Iran.

Keywords: Bean common mosaic virus; *Bean yellow mosaic virus*; Phylogenetic; *Potato virus* Y; *Watermelon mosaic virus*

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▸Implication for health policy/practice/research/medical education:

This study has implication for researchers as well as virologists.

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1. Background

The genus Potyvirus in the family *Potyviridae* is one of the largest plant virus genera (1) that are accounted for 40% of losses caused by all plant viruses (2). It includes 111 confirmed species and 86 tentative species (3-5). Potyviruses are transmitted by aphids, especially species of *Aphidinae* (6). Potyvirus particles are flexuous and rod-shaped, 11 - 15 nm in diameter and 680-900 nm in length and are made up of a single structural protein surrounding an RNA molecule of approximately 10000 nucleotides (nts) (7, 8). The only open reading frame (ORF) of the virus genome codes for a single large polyprotein that is co- and post-translationally processed into 10 functional proteins (9). Polymerase chain reaction (PCR) with degenerate primers has facilitated the detection of many potyviruses and enabled partial genomic sequencing. Attempts have been made to design universal primers to detect all potyviruses since early 1990s (7, 10-16). An analysis of the seventeen most conserved sites within potyvirus genomes has revealed a decay in their consensus as newly generated sequences resulted in an apparent loss of stability and less efficiencies of previously designed degenerate primers (17). However, NIb2F and NIb3R have been reported as the more efficient primer pair compared to other primers (5).

2. Objectives

This study was carried out to compare the efficiencies of two couples of primers in their detection of potyviruses infecting vegetables in North-West of Iran.

3. Materials and Methods

Leaves showing characteristic symptoms of virus infection including mosaic, mottling, leaf and fruit malformation and green blistering, and ring patterns on fruits were collected from vegetables during the years 2009 and 2010. Samples were preserved on calcium chloride granules at 4 °C and then stored at -20 °C until processing. Inoculations were done on diagnostic and propagation hosts including *Chenopodium amaranticolor*, *Cucurbita pepo*, *Phaseolus vulgaris* and *Nicotiana tabacum*. Total RNA was extracted from the original (field samples) or the infected experimental hosts using 'Method 4' of Rowhani et al. (18) with some modifications as reported elsewhere (19). Two primer pairs including Sprimer/M4 (11) or NIb2F/NIb3R (17) were used in the PCR reactions. Reverse transcription (RT) was done with Oligo d(T)15, M4T (5'GTTTTCCCAGTCACGAC(T)153') or NIb3R (5'TCIACIACIGTIGAIGGYTGNCC3'). Sprimer (5'GGNAAYAAYAGYGGNCAZCC3'), NIb2F (5'GTITGYGTIGAYGAYTTYAAYAA3') and NIb3R corresponded to NIb (nuclear inclusion protein b) coding region in the potyvirus genome. M4 (5'GTTTTCCCAGTCACGAC3') is an adaptor primer designed to anneal to the very 3' end of the potyvirus genome right before the poly (A) tail. Moloney Murine Leukemia virus (M-MuLV) reverse transcriptase (Fermentas, Lithuania) was used in the synthesis as described elsewhere (20). PCR reactions were done in 25 µL or 12.5 µL containing 20 pmol of each primer, 1 unit of *Taq* DNA polymerase (Fermentas, Lithuania), 0.2 mM each dNTP, 2 mM MgCl2 and 2 µL of the synthesized cDNA. PCR with Sprimer/M4 was performed with an initial denaturation at 94 °C for 2 min; followed by 35 cycles at 94 °C for 30 sec, 47 °C for 1 min and 72 °C for 2 min; then a final polymerization at 72 °C for 10 min. PCR with NIb2F/NIb3R was performed similarly, but annealing was at 45 °C and duration of the denaturation, annealing and extension was 45 sec for each. Some optimizations were done to improve efficiencies of the PCR reactions as follows; different quantities of the primers (1, 1.5, 2 or 3 pmol final concentrations) and cDNA (1, 1.5, 2, 2.5 or 3 µL per 25 µL reaction mix) were used with 'Hot-start' (20, 21) or 'Touch down' (22) PCR methods when Sprimer and M4 were applied. In the 'Touch down' PCR, the annealing temperature was gradually decreased from 60 °C to 47 °C during the first 13 cycles, then other 22 cycles with annealing at 47 °C were appllied. In PCR with NIb2F/NIb3R, initially a 0.5 pmol (final concentration) of each primer was used, but in the next runs this was increased 10 times to 5 pmol of each primer.

4. Results

PCR with Sprimer/M4 on subsamples of 15 samples resulted in amplification of the expected ~1700 bp fragment from 7 samples (data not shown). However, three other fragments with smaller sizes were also produced. There were samples from which no targeted fragment was produced, but instead smaller non targeted fragments were generated. Even the optimizations did not improve efficiency and specificity of the PCR. However, PCR with NIb2F/NIb3R on 61 symptomatic samples resulted in amplification of the expected 350 bp fragment from 31 samples (*Table 1*). The amplification was faint when the primers were used at 0.5 pmol, whereas an intense band was amplified with 5 pmol of the primers. Among the tested samples, there were 4 samples that gave the expected fragments with either of the two primer sets (*Table 1*). PCR products amplified by Sprimer/M4 were not subjected to cloning and subsequent sequencing since they were faint. However, sixteen out of the thirty-one PCR products resulting from amplification with NIb2F/NIb3R were selected for cloning and sequencing. These included isolates A74, A185, A186, A187, A206, A213, T41, T67, T172, T180, T192, T198, T205, T209, T210 and U167 which were from different hosts and geographical locations. PCR products with the expected size from these isolates were purified from agarose gel by the use of Genomic DNA Purification Kit (Fermentas, Lihuania) and then ligated into pTZ57R/T (Fermentas, Lithuania) following the manufacturers' instructions. The ligated mixture was transformed into *Escherichia coli* strain DH5α cells which had been made competent according to Chung et al. (23).

Plasmid DNA was extracted from overnight culture of the resulting white colonies using the small-scale alkaline lysis method (24) or Qiagene Miniprep Kit. Integration of the PCR products into the vector was confirmed by restriction analysis. The cloned fragments were then subjected to dideoxy terminator cycle sequencing in Macrogen Inc. (Seoul, South Korea). The sequencing data were submitted to BLASTN (Basic Local Total Alignment Search Tool- Nucleotide) (25) facility in the NCBI website (www.ncbi.nlm.nih.gov) to identify the best matching sequences recorded in GenBank. The cloned isolates were considered to be potyvirus provided that they matched a potyvirus sequence in GenBank in the expected NIb region. According to the demarcation criteria established for potyvirus by the ICTV if there is < 85% nucleotide identity between the two isolates they are considered as distinct potyviruses. At the level of amino acid sequences, < 80% identity means that the isolates are distinct potyviruses (4). The BLASTN revealed A74, A185 and Adb-pot187 as the most similar sequences to *Potato virus Y* (PVY); A186, A206, A213, T41, cT180, T192, T205 and U167 to *Watermelon mosaic virus* (WMV); T172 to *Bean common mosaic virus* (BCMV); T67 to *Bean yellow mosaic virus* (BYMV); T209 to *Soybean mosaic virus* (SMV); and T210 to *Zucchini yellow mosaic virus* (ZYMV). Overall, these similarities were above 97%. These newly identified isolates were named as PVY isolates Adb-Glr-pot74 (JX683528), Hir-pot185 (JX683529) and pot187 (JF707767); WMV isolates Nia-pot186 (JX683531), Aga-Sq206 (not submitted to GenBank due to its short length), Glr-pot213 (JX683534), Gow-Sq41 (JX124710), Kha-Msk180 (JX124711), Gmk-Msk192 (JX683530), Ahr-sq205 (JF707768) and Zei-Sq167 (JX124709); BCMV isolate Mgh-Bn172 (JF707770); BYMV isolate Ghk-Brn67 (JF707769), SMV isolate Ahr-bn209 (JX843813); and ZYMV isolate Gmk-sq210 (JX843814) (*Figure 1*). The closest previously reported accessions to the isolates reported in this study are presented in *Table 2*. However, the sequence from sample T198 seemed to have a host plant origin because it matched the *Vitis vinifera* (grapevine) hypothetical protein gene.

Table 1. Characteristics of Samples and Results From the RT-PCR Assays

Isolate	Geographical Location	Original Host	Symptom[a]	PCR[b](Sprimer/M4)	PCR (NIb2F/NIb3R)
A187[c], A188, A189	Ardebil	Potato	M, RL, WL[a]	ND[b]	+
A80, A185[c], A186[c], A206[c]	Ardebil	Potato	MO[a]	ND[b]	+
A110, A184	Ardebil	Potato	MO,YM[a]	ND[b]	+
A183	Ardebil	Potato	YM[a]	ND[b]	-
A207	Ardebil	Potato	YM[a]	ND[b]	+
A73	Ardebil	Potato	MO[a]	-	-
A14, A31	Ardebil	Potato	YM[a]	+	+
A1, A30, A74[c]	Ardebil	Potato	MO,RL[a]	-	+
A213[c]	Ardebil	Potato	YM,CS[a]	-	+
T180[c], T198[c]	Tabriz	Muskmelon	YM[a]	ND[b]	+
T190	Tabriz	Muskmelon	GB[a]	ND	+
T26	Tabriz	Muskmelon	VB[a]	ND	-
T192[c]	Tabriz	Muskmelon	GV[a]	ND	+
T37, T196	Tabriz	Muskmelon	GB[a]	+	-
T211	Tabriz	Muskmelon	GV[a]	-	+
T210[c]	Tabriz	Muskmelon	GB,FP[a]	-	+
T126	Tabriz	Tomato	MO[a]	ND	-
T181	Tabriz	Tomato	RS[a]	ND	-
T168	Tabriz	Pepper	RS[a]	ND	-
T132	Tabriz	Spinach	CS[a]	ND	+
T17	Tabriz	Lettuce	VC[a]	-	-
T67[c]	Tabriz	Broad bean	MO, FP[a]	+	+
T97	Sarab	Cabbage	VC[a]	ND	-
T36	Sarab	Potato	CS,Y[a]	-	-
T208	Khodafarin	Squash	YM, FB[a]	ND	-

T48	Khosroshahr	Squash	GV[a]	-	-
T212	Khosroshahr	Squash	GB,VB[a]	ND	-
T133	Shabestar	Eggplant	RS, N[a]	ND	-
T128	Shabestar	Squash	GB, M[a]	ND	+
T123, T140	Shabestar	Squash	M,VB[a]	ND	-
T122	Shabestar	Pepper	MO, LM[a]	ND	-
T137	Shabestar	Bean	GB, LM[a]	ND	-
T173, T174	Bonab	Squash	GB,FM[a]	ND	-
T171, T175	Bonab	Squash	VB, FP[a]	ND	-
T205[c], T209[c]	Ahar	Squash	VB[a]	ND	+
T45	Ajab shir	Squash	GV[a]	-	-
T177	Jolfa	melon	GB[a]	+	-
T213	Jolfa	melon	YM[a]	-	+
T41[c]	Gogan	Squash	YM[a]	+	+
T172[c]	Maragheh	Bean	YM, GB, LM[a]	ND	+
U167[c]	Urmia	Squash	YM, FM[a]	ND	+

[a] CS, chlorotic spot; FB, fruit blistering; FL, fan leaf; FM, fruit malformation; FP, fruit pattern; GB, green blistering; GV, green vein; LM, leaf malformation; M, mosaic; Mo, mottling; RL, ragged leaf; RS, ring spot; VB, vein banding; VC, vein clearing; WL, wavy leaf; YM, yellow mosaic
[b] Not determined.
[c] Cloned and sequenced.

Table 2. List of the Most Similar Previously Reported Potyviruses to Isolates From Iran Based on the Sequenced NIb Region

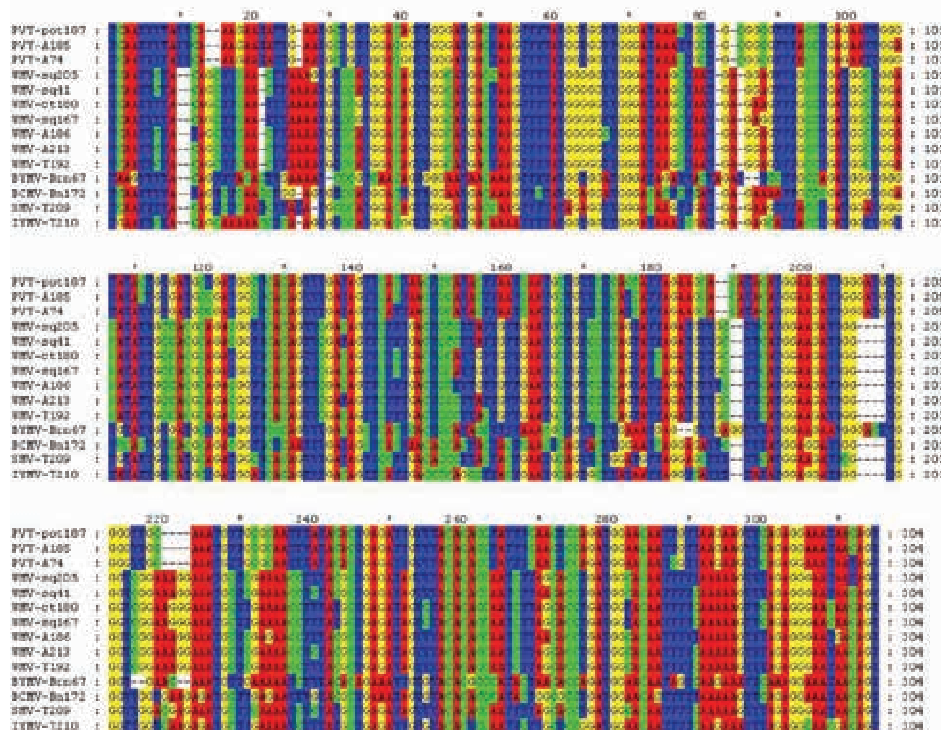
New Isolate (s)	Best Matching Previously Reported Isolates	Identity, %
PVY- Adb-pot187, - Hir-pot185	PVY isolates Wilga, nwilga and LW (Poland); pB209, OR-1, ID-1, Alt and pN10A (USA), 156 var (Germany); and Frkv15 (France)	98
PVY- Glr-pot74	PVY strain N-W, serotype O, isolate 09-3a	98
WMV-Sq205, -T192	IR02-54 (Iran)	98
WMV-A213, -T41	WMV CO5-463, CO5-464, CO5-337 and CH102-481 (France)	99
WMV-ct180, -sq167	WMV isolate USA	98
SMV-T209	SMV isolate WS151 (Korea)	98
ZYMV-T210	ZYMV AG (Israel)	99
BCMV-T172	MS1 and NWA1 (Australia)	97
BYMV- T67	BYMV-s (Australia)	98

Phylogenetic analysis was performed to reconfirm the potyvirus affiliation of the newly identified isolates. First, by the use of GeneDoc software (26), sequences of the new isolates were aligned with counterpart regions of the previously characterized potyvirus species and Ryegrass mosaic virus (RGMV) was set as the outgroup (*Table 3*). Then, a phylogeneic tree with 1000 bootstrap replicates was generated by the use of Treecon (27) on the basis of Jukes-Cantor distance method (28). As a result, each new isolate was clustered with the same most similar virus as that determined by the BLASTN analysis (*Figure 2*). This provided further proof for the potyvirus affiliation of the isolates.

Branches with $<$ 500 bootstrap values were collapsed. Branch lengths are proportional to genetic distances. *Ryegrass mosaic virus* (RGMV) is designated as the root. The newly detected potyvirus species/isolates from Iran are bolded.

Here, infections with potyviruses were detected in 31 of 60 samples. By selection of appropriate representative isolates according to the origin and host, distinct species were intercepted. We showed that when testing for potyviruses even in new locations, the primer pair NIb2F/NIb3R was the most efficient and specific. Consistent amplification of a 350 bp fragment for majority of the isolates was in agreement with Zheng et al. (5). Because these primers target highly conserved sites in the genome, they resulted in successful detection of potyviruses from Iran. However, amplification with Sprimer/M4 was not satisfactory because it was achieved from a relatively smaller number of samples and was always associated with non-specific bands. This primer pair was tested for 15 samples, which resulted in amplification of the expected 1700 bp as well as non specific fragments from 7 samples.

Figure 1. An Alignment of 304- Nucleotide Sequences From 14 Potyvirus Isolates (6 Species) From Iran Amplified by NIbF2/ NIbR3 Primers



The primer sequences were removed.

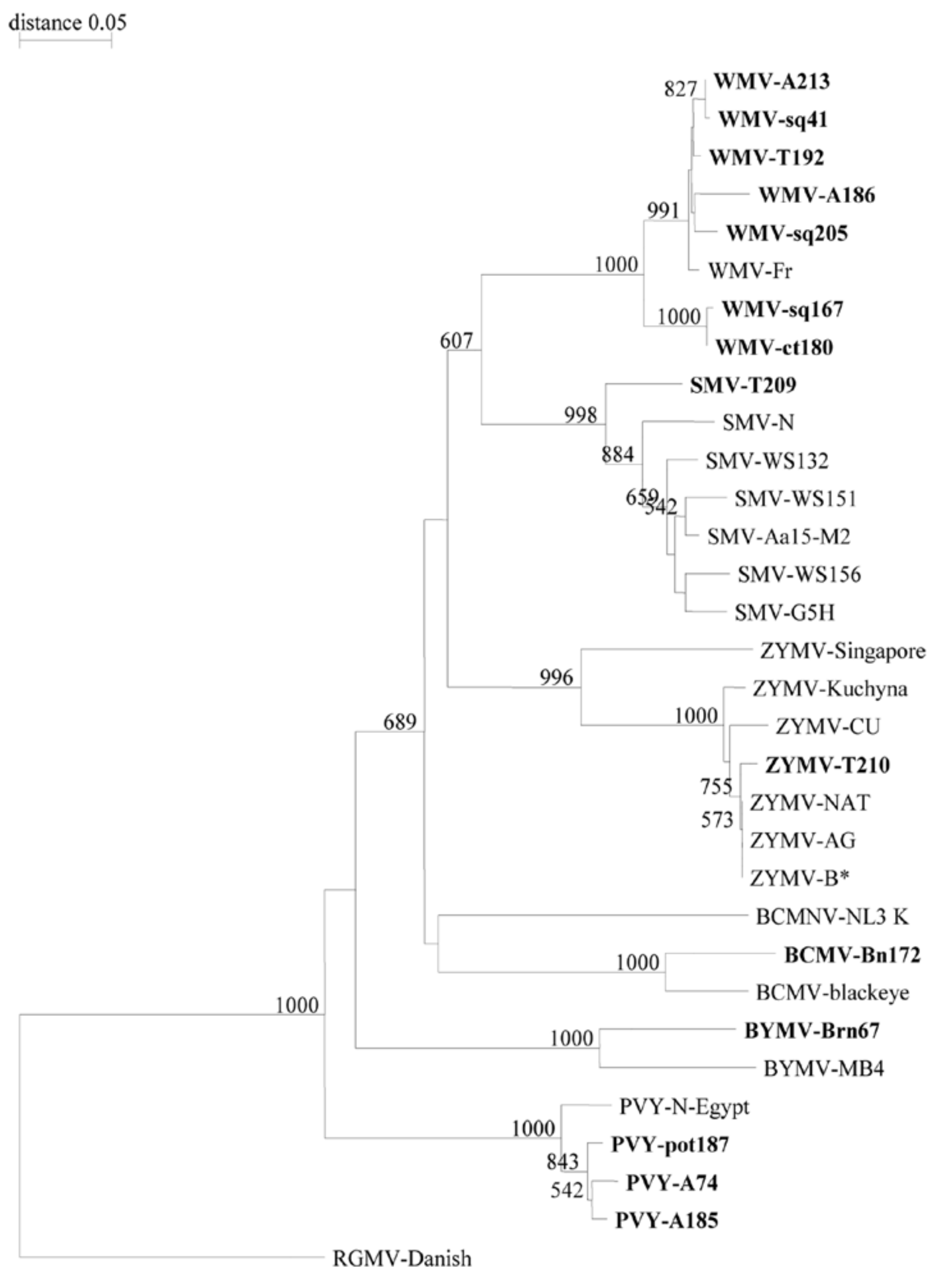
Table 3. Accession Numbers and Characteristics of previously Reported Potyviruses Used in the Phylogenetic Analysis

Accession Number	Isolate Name/Strain[a]	Country[b]	Host[c]
AY864314	BCMNV strain NL-3 K	USA	*Phaseolus vulgaris*
AY575773	BCMV4 strain Blackeye	Taiwan	
D83749	BYMV strain MB4	Japan	
AJ243766	PVV-DV 42	United Kingdom: Scotland	
AF522296	PVY Strain N-Egypt	Egypt	
Y09854	RGMV isolate Danish		
AB100443	SMV-Aa15-M2	Japan	
FJ376388	SMV-G5H	South Korea	*Glycine max*
FJ640964	SMV-WS132	South Korea	Wild soybean
FJ640969	SMV-WS151	South Korea	Wild soybean
FJ640971	SMV-WS156	South Korea	Wild soybean
D00507	SMV strain N	USA	
AY437609	WMV- FR	France	
AJ307036	ZYMV-CU	China	cucumber
AY188994	ZYMV-B*		
DQ124239	ZYMV-Kuchyna	Slovakia	*Cucurbitapepo*
EF062582	ZYMV-NAT	Israel	

EF062583	ZYMV-AG	Israel
AF014811	ZYMV strain Singapore	Singapore

[a] Abbreviations: BCMV, *Bean common mosaic virus*; BCMNV, *Bean common mosaic necrosis virus*; BYMV, *Bean yellow mosaic virus*; PVY, *Potato virus Y*; RGMV, *Ryegrass mosaic virus*; SMV, *Soybean mosaic virus*; WMV, *Watermelon mosaic virus*; ZYMV, *Zucchini yellow mosaic virus*
[b] Origin is not written if not mentioned in the related accession.
[c] Host is not written if not mentioned in the related accession.

Figure 2. A Distance-Based Phylogenetic Tree Inferred From Nucleotide Sequences of the Potyvirus Genomic Region Flanked by the Primers NIb2F and NIb3R



Abbreviations: BCMV, Bean common mosaic virus; BCMNV, Bean common mosaic necrosis virus; BYMV, Bean yellow mosaic virus; PVY, Potato virus Y; RGMV, Ryegrass mosaic virus; SMV, Soybean mosaic virus; WMV, Watermelon mosaic virus; ZYMV, Zucchini yellow mosaic virus

5. Discussion

Results from this study justifies attempts that have been made to design potyvirus degenerate primers (7, 11, 12, 14-16). However, one major issue is that at any time the number of characterized (partiallly or completely sequenced) potyviruses is smaller than that which would be known in future thus as more and more new potyvirus sequences are accumulating it appears that the previously characterized conserved regions are not as much conserved as they were once thought. In other words, the consistency of conserved sites decays and, as a result, the previously designed degenerate primers fall short in detecting some potyvirus members. Accordingly, because Sprimer and M4 were designed about a decade ago when a relatively smaller number of potyvirus sequences were available they appear to be inefficient in detection. The other factor may be the geographical origins of the isolates especially from places where no detailed studies have been done before. Here, we studied samples from the northwest region of Iran where no potyvirus sequence has previously been recorded to consider in design of the primers. So, the newly characterized isolates may be well heterogeneous at the Sprimer site resulting in a non specific and inefficient amplification with Sprimer/M4. Compared to detection by serological methods, RT-PCR with broad spectrum primers offers cost-effectiveness, particularly in developing countries where antibodies to each virus are imported with considerable costs. Although a genus-specific antibody (antipoty) has become available there are yet some advantages for PCR with broad spectrum primers. With the genus specific antibody, one major problem can be cross reactivity between distinct potyvirus species (29), hindering species determination, whereas PCR with subsequent sequencing of the amplified fragment results in prudent identification of potyvirus species. There were also diseased samples that did not contain potyvirus as determined by RT-PCR suggesting that another virus (es) might be involved in these infections or distant genotypes of potyvirus with respect to the primer sites might be occurring in them. Because there has been no previous record of potyvirus sequence data from this geographical region it is possible that some distantly related potyviruses exist in the region that do not match the primers.Amplification from sample T198 appeared to have been derived from the host genome because the sequence data did not match any virus, but the host plant genome. Such a non targeted amplification has also been reported by Zheng et al (5) although without elaboration. Therefore, it seems that there are sites in the host genome complementary enough to pair with the primer sequences.The future direction would be the characterization of the newly identified species to understand the heterogeneity of their genotype with respect to the primers and design newer primers for improvement of detection.

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Authors' Contribution

Nemat Sokhandan-Bashir acted as the principle investigator (supervisor), Aisan Ghasemzadeh and N. Masoudi did the lab works, Reza Khakvar and D. Farajzadeh acted as associate supervisors.

Financial Disclosure

There is not any conflict of interest.

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