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Phylogenetic Relationships between Iranian *Poaceae* Potyviruses

Sasan Ghasemi

Plant Protection Dept, College of Agriculture, Islamic Azad University, Arsanjan Branch.
ghasemi@iaua.ac.ir

Abstract. Phylogenetic analysis of DNA or protein sequences has become an important tool for studying the evolutionary history of organisms from viruses to humans. It has been shown that the highly variable 3' untranslated regions of potyviral genomes may be used as strain- and virus-specific probes for sensitive virus detection and differentiation, while the sequences can also be used for estimating close phylogenetic relationships. The study was carried out to determine if the Iranian Poaceae potyviruses could be differentiated by comparison of their 3′-terminal sequences and comparison of these 3′-terminal sequences with other Poaceae potyvirus sequences available from the GenBank to compose a phylogenetic relationship. RNA extracts from virus preparation or infected plant tissues were conducted to amplify the approximately 700 bp nucleotide sequences, using Oligo1n/OligodT primers in RT-PCR. Each of the nucleotide sequences contained a 462 bp fragment corresponding to the C-terminal portion of the CP followed by a non-coding region of 231bp and a polyadenylated terminus. Maximum Likelihood (ML) phylogenetic analysis of the 691bp region (Capsid protein C-terminal plus 3'-UTR) of 35 potyvirus isolates was carried out using the PHYLIP DNAML program. Two Iranian sugarcane isolates (SC-Q86 and SC-CP68) together with 10 other SCMV isolates formed a major SCMV clade (84% support), with the other isolates split into four minor clades, an 8-isolate MDMV clade (100% support), a 7-isolate ZeMV clade (95% support), a 4-isolate SrMV clade (98% support), and a 2-isolate JGMV clade (100 % support), with the SrMV-CHNXi appearing to be in a clade of its own. These results showed that Iranian poaceous potyviruses could be divided into three distinct potyvirus groups: (1) SCMV-group, including SC-Q86 and SC-CP68, that are closely related to US sugarcane isolates; (2) MDMV-group, including SC-L66, JG-Sar, JG-Isf, and Mz-Isf isolates, that are closely related to European MDMV isolates, although, JG-Isf and Mz-Isf formed a significantly separate subclade; (3) ZeMV-group, including JG-Shi, JG-And, JG-Krj, JG-Ham, Sr-Khz, and Mz-Khz. This group has been previously called Iranian Johnsongrass mosaic virus group, which is more closely related to SrMV and MDMV than SCMV clade.

1. Introduction

Phylogenetic analysis of DNA or protein sequences has become an important tool for studying the evolutionary history of organisms from viruses to humans. In recent years, a great number of potyvirus sequences have been reported. This has allowed nucleotide and amino acid sequence comparisons and clarified some conserved motifs in the potyviral genome (Marie-Jeanne *et al*., 2000). Knowledge of conserved viral gene sequences has been useful in the construction of oligonucleotide primers for use in group-specific polymerase chain reaction (PCR) amplification (Saiki *et al*., 1985) and characterization of novel potyviruses (Langeveld *et al*., 1991). Degenerate oligonucleotide primers complementary to conserved genomic sequences shared by all known members of a virus group have been shown to enable the identification of new related members of the group (Rybicki and Hughes, 1990). It has been shown that the highly variable 3' untranslated regions of potyviral genomes may be used as strain- and virus-specific probes for sensitive virus detection and differentiation, while the sequences can also be used for estimating close phylogenetic relationships (Frenkel *et al*., 1989; 1992; McKern *et al*., 1992). The study was carried out to determine if the Iranian Poaceae potyviruses could be differentiated by comparison of their 3′-terminal sequences and comparison of these 3′-terminal sequences with other Poaceae potyvirus sequences available from the GenBank to compose a phylogenetic relationship.

2. Material and Methods

An mRNA capture kit (Roche) was used for RNA extraction from virus preparation or infected plant tissues. Virus isolate used in this study and their origins are listed in Table 1. The following Oligonucleotides were used to amplify the 3' region of these viruses. Oligo dTs (5'-TTTTTTTTTTTTTTTTTTTTY-3')/ Oligo n1 (5'-ATGGTHTGGTGYATHGARAAYGG-3').To prepare first strand cDNA, the following mixture was added to virus RNA captured in mRNA capture kit (Roche) tubes: 10 µl of 5x First Strand buffer (provided by Fermentas with Moloney-murine Leukemia Virus (M-MLV) Reverse Transcriptase), 1µl of Dithiotheritol (DDT) 0.1 M, 1ul of dNTPs (10 mM each), 1ul of RNase-inhibitor (40 u/µl, Boehringer), 2 µl of reverse primer (10 µM), 1 µl of M-MLV reverse transcriptase (RT, Fermentas) and sterile distilled water to a final volume of 50 µl. The RT reaction mixture was incubated for 1 h at 42 ^{o}C. These cDNAs were amplified under following condition: one cycle 94 ^{o}C for 5 min, and then 35 cycles of 94 ^{o}C for 1 min, 55 ^{o}C for 45 sec, and 72 ^{o}C for 1 min, followed by 30 min at 72 ^{o}C. The PCR products were visualized in 1% agarose gel, after ethidium bromide staining and UV illumination. To obtain ultrapure PCR fragments for use in DNA cloning, the PCR fragments were extracted from agarose gel using the Zymoclean Gel DNA Recovery Kit.

Table1- Specification of virus isolates used in phylogenetic analysis

Isolate designation	Source plant	Collection area
JG-Shi	Johnsongrass	Shiraz
JG-Kaj	Johnsongrass	Karaj
JG-Isf	Johnsongrass	Isfahan
JG-Sar	Johnsongrass	Sari
JG-And	Johnsongrass	Andimeshk
JG-Ham	Johnsongrass	Hamadan
Mz-Isf	Maize	Isfahan
Mz-khz	Maize	Khuzestan
Sr-Khz	Sorghum	Khuzestan
SC-Q86	Sugarcane	Khuzestan
SC-L66	Sugarcane	Khuzestan
SC-CP68	Sugarcane	Khuzestan

The PCR products cloning were carried out using pGEMT-Easy vector system (Promega). Transformed colonies were selected and high purity plasmid DNA minipreps were used for DNA sequencing. Sequence data were subjected to process in BioEdit Sequence Alignment Editor (version 5.0.9) and Sequence Navigator (version 2.2) for initial editing and preparation of consensus sequence for each isolate. All sequences were subjected to database search using BLAST® program for the comparison against the nucleotide databases. Sequence data were aligned automatically using the Clustal X program with default settings. The output file in PHYLIP (extention .phy) format was created and used in PHYLIP (version 3.6) program (http://evolution.genetics. washington.edu/phylip.html, Felsenstein, 2002).

3. Results and Discussion

Using Oligo1n/OligodT primers in RT-PCR, the expected amplification product of approximately 700 bp was obtained from all virus isolates, but not from healthy plant preparations. The pattern for sugarcane isolates is shown in Fig. 1. The PCR products were cloned and sequenced. Each of the nucleotide sequences

contained a 462 bp fragment corresponding to the C-terminal portion of the CP. The CP sequence was followed by a non-coding region of 231bp and a polyadenylated terminus. The downstream primer was crucial for our method as potyviruses have a long poly-A tail of about 200-250 adenosine residues. An oligo-dT primer is not useful since there is the possibility to anneal to overlapping poly-A residues in different (or the same) template molecules thereby resulting in a population of molecules with varying lengths of poly-A tails. Therefore, the PCR reverse primer consisted of a mixture of three oligonucleotides each comprising 21 T residues but with a different terminal residue either A, C or G (Pappu *et al*., 1993). This method combines the advantages of the group-specific PCR detection method of Langeveld *et al*. (1991) with the strain-specific 3′-UTR hybridization/sequencing approach of Frenkel *et al*. (1992).

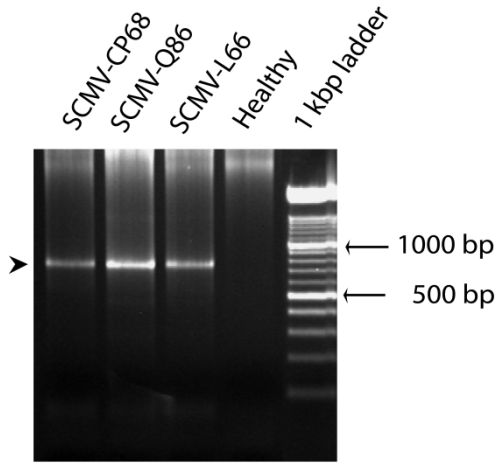

Fig. 1: RT-PCR amplification of 700 bp 3′ end of sugarcane isolates (The same pattern was obtained for other isolates)

Maximum Likelihood (ML) phylogenetic analysis of the 691bp region (Capsid protein C-terminal plus 3'-UTR) of 35 potyvirus isolates was carried out using the PHYLIP DNAML program. A F84 plus Gamma nucleotide substitution model was used and parameters were estimated (expected transition/tranversion ratio of 1.84; Alpha parameter of 0.61). The log likelihood of the best tree was –7805.04. Bootstrap analysis (100 replicates) was carried out using the PHYLIP SEQBOOT and CONSENSE programs.

The ML tree was reasonably well-resolved, based on the interpretation of bootstrap support values of greater than or equal to 70% (Fig 2). Two Iranian sugarcane isolates (SC-Q86 and SC-CP68) together with 10 other SCMV isolates formed a major SCMV clade (84% support), with the other isolates split into four minor clades, an 8-isolate MDMV clade (100% support), a 7-isolate ZeMV clade (95% support), a 4-isolate SrMV clade (98% support), and a 2-isolate JGMV clade (100 % support), with the SrMV-CHNXi appearing to be in a clade of its own.

Some phylogenetic structure was observed within the SCMV clade. The four US isolates (SCMV-E/A/D/B) are grouped together and form part of a 6-isolate subclade with SCMV-Q86 and SCMV-CP68 (Two of the three Iranian isolates). Two of the US isolates (SCMV-D and -B) are closely related. Looking at the other 6 isolates out with the 6-isolate subclade, SCMV-Ger and SCMV-B in one side and SCMV-Mx and SCMV-CHN1 in the other side group together, but otherwise no other structure is apparent. Some phylogenic structures were observed within MDMV clade. Two Iranian isolates (JG-Isf and Mz-Isf) are grouped together and two other Iranian isolates (SC-L66 and JG-Sar) with two European isolates (MDMV-Hung and MDMV-Bulg) formed a 4-isolate subclade, which is closely related to MDVV-A and MDMV-Spin subclade. Within ZeMV clade two Iranian isolates (Mz-Khz and Sr-Khz) are grouped together and form part of a 6-isolate subclade with JG-Ham, JG-Krj, JG-And, and ZeMV-Isr isolates. This subclade is significantly separated from JG-Shi isolate. Some phylogenetic structures were observed within SrMV clade. Two Australian isolates (SrMV-I and SrMV-SCH) group together (81% support). There is some support (54%) for a 3-isolate subclade consisting of ((SrMV-I, SrMV-SCH), SrMV-M)) which is separated from SrMV-H.

These results showed that Iranian poaceous potyviruses could be divided into three distinct potyvirus groups: (1) SCMV-group, including SC-Q86 and SC-CP68, that are closely related to US sugarcane isolates; (2) MDMV-group, including SC-L66, JG-Sar, JG-Isf, and Mz-Isf isolates, that are closely related to European MDMV isolates, although, JG-Isf and Mz-Isf formed a significantly separate subclade; (3) ZeMV-group, including JG-Shi, JG-And, JG-Krj, JG-Ham, Sr-Khz, and Mz-Khz. This group has been previously called Iranian Johnsongrass mosaic virus group, which is more closely related to SrMV and MDMV than SCMV clade.

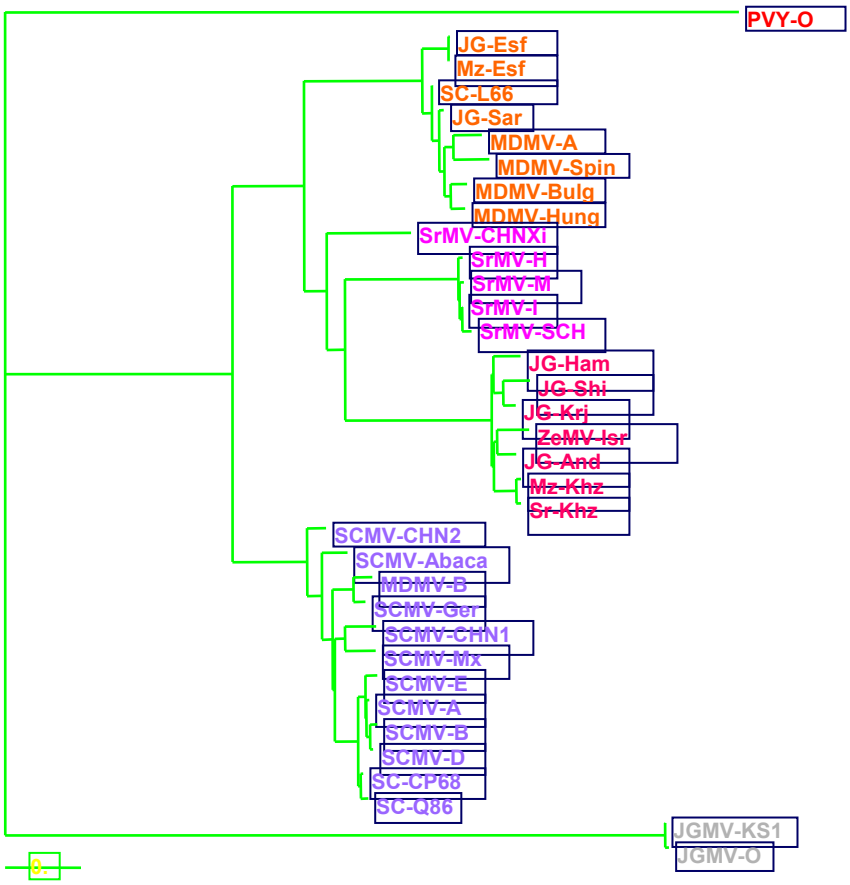

Fig. 2: Phylogenic statuses of Iranian Poaceae potyviruses

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