Short Communication

Phylogenetic analysis of four Iranian Cucurbit yellow stunting disorder virus isolates based on 3’ terminal region of RNA1

T. KESHAVARZ and K. BANANEJ
Assistant Professor and Professor, Respectively, Department of Plant Virus Research, Iranian Research Institute of Plant Protection, Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran
(Received: May 2017; Accepted: January 2018)

Abstract
To study phylogenetic analysis of some Iranian isolates of cucurbit yellow stunting disorder virus (CYSDV), 3’ terminal region of RNA1 (560 bp in length) of four CYSDV isolates, originated from four different locations of Iran including Boushehr, Kerman (Jiroft), Khuzestan (Dezfool) and Tehran provinces was amplified by RT-PCR using specific pair primers. The 3’ terminal region of RNA1 of four CYSDV was sequenced and compared with other available sequences in GenBank. Nucleotide sequences analysis of CYSDV isolates showed a high nucleotide sequence identity (98-99%) among all isolates and nucleotide sequences identity among Iranian CYSDV isolates was 99%. Phylogenetic tree constructed by neighbor-joining method using the nucleotide sequence of amplified region indicated that all CYSDV isolates were placed in one group however three of four Iranian isolates including Boushehr, Khuzestan and Tehran were clustered into a distinct subgroup with respect to other isolates.

Key words: Cucurbit yellow stunting disorder virus, Phylogeny, RT-PCR.

Corresponding author: ta_keshavarz@yahoo.com
Introduction

Whitefly-transmitted viruses (WTVs) are becoming important in recent years and causes severe economic yield losses in many cucurbit-growing areas of the world (Tzanetakis et al. 2013). *Cucurbit yellow stunting disorder virus* (CYSDV) is one of the most prevalent WTVs in cucurbits, worldwide (Tzanetakis et al. 2013) and can causes high yield losses (Abou-Jawdah et al., 2000). CYSDV is a member of the genus *Crinivirus* (family *Closteroviridae*) and viral particles are flexible rods with the length between 750 to 800 nm. (Martelli et al., 2002) CYSDV was first identified in the United Arab Emirates in 1982 (Hassan and Duffus, 1991), and then it has been reported from other countries, including, Mediterranean countries (Cyprus, Egypt, France, Greece, Israel, Lebanon, Morocco, Portugal, Spain, Tunisia, and Turkey) (Lecoq and Desbiez, 2012), North America (Kao et al., 2000; Kuo et al., 2007; Polston et al., 2008), South America (Brown et al., 2007), and Iran (Keshavarz and Izadpanah, 2005). CYSDV is transmitted from plant to plant by *Bemisia tabaci* in a semi-persistent manner and no replication within the vector (Celix et al., 1996). CYSDV symptoms started with interveinal chlorotic spots in mature leaves, then these spots enlarge and fuse together and finally yellowing of entire leaf except veins which remained green (Celix et al., 1996). CYSDV was previously believed to be restricted to cucurbits (Celix et al. 1996) but recently, it has been shown that CYSDV has a wide host range including common bean, *Phaseolus vulgaris*, lettuce *Lactuca sativa*, alkali mallow *Sida hederacea*, ground cherry *Physalis wrightii*, and the wild cucurbit, buffalo gourd *Cucurbita foetidissima* (Wintermantel et al. 2009). The CYSDV genome consists of two molecules of single-stranded RNA of positive polarity designated RNAs 1 and 2 (Celix et al., 1996). The complete nucleotide (nt) sequences of genomic RNAs 1 and 2 of CYSDV have been determined. RNA1 contains at least five open reading frames (ORFs). Computer-assisted analyses indicated papain-like protease, methyltransferase, RNA helicase and RNA-dependent RNA polymerase domains in the first two ORFs of RNA1. RNA2 contains four ORFs which encoding a heat shock protein 70 homologue, a 59 kDa protein, the major coat protein(CP) and a duplicate copy CP. Gene arrangement in the 3-terminal region of RNA1 is the most striking novel features in CYSDV compared to other criniviruses. This region encodes a protein which has no homologues in any database (Aguilar et al., 2003).

Evaluation of molecular variation of CYSDV isolates for part of heat shock protein 70 homologue (HSP70h) and the coat protein genes of RNA 2 from different geographical areas and isolates from different years showed genetic diversity of CYSDV isolates is unusually low compared to other members of the family *Closteroviridae*. Despite of this finding, CYSDV isolates could be differentiated into Eastern and Western groups (Rubio et al., 1999, 2001).

It has also been shown that the coat protein gene of CYSDV has more diversity compared to ORF2, ORF3, ORF4, and HSP70h coding regions (Marco and Aranda, 2005).

CYSDV was first reported in 2005 from Iran (Keshavarz and Izadpanah, 2005) and molecular variability of Iranian isolates according to entire CP gene was determined (Keshavarz et al., 2013). In this study we determined molecular properties of some Iranian isolate of CYSDV according to sequence of the 3’ terminal region of CYSDV RNA 1.

Materials and Methods

Four CYSDV isolates, originated from Bushehr, Tehran, Kerman and Khuzestan provinces, which were used in phylogenetic analysis of CYSDV isolates based on complete coat protein region of genome (Keshavarz et al., 2013), were used in this study.

Total RNA was extracted from the ELISA-positive samples using TriPure isolation reagent (Roche, St Louis, MO, USA) according to the manufacturer’s instructions, the pellet was resuspended in 20μl nuclease-free water. CYSDV specific primers (MA156 forward (5’- GAAGAATTCCAGGCAAGG -3') and MA129 reverse (5’- TCACATCATCAATCCAAAAG -3’) (Marco and Aranda, 2005), corresponding to nt 8191-8753 of CYSDV RNA 1) were used in RT-PCR. 4 μL of extracted total RNA was added to 16 μL of the reverse transcription (RT) mix (9 μL water, 2 μL 5 × RT buffer, 1 μL 10 mm dNTPs, 0.5 μL (100 U) M-MuLV reverse transcriptase (Fermentas,
Lithuania), 0.5 µL Rnasin (Fermentas, Lithuania) and 1 µL reverse primer (20 pmol)) and the reaction was incubated at 42°C for 1 h.

PCR was performed in a 25 µl reaction mixture containing PCR buffer (50 mM KCl; 10 mM Tris-HCl, pH 9.0; and 1.0% Triton X-100), 1.5 µl of MgCl₂ (50 mM), 1μl of dNTPs (10 mM), 2.5 units of Taq DNA polymerase (Fermentas, Lithuania), and 20 pmol of each primer. Amplification was performed in an automated thermal cycler (Eppendorf Mastercycler® 5330, Germany) programmed for the following thermo-cycling conditions: initial denaturation step at 94°C for 4 min, then PCR was performed for 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C, and final extension for 7 min at 72°C.

The amplified fragments were stained with ethidium bromide (0.5 µg/ml), visualized and photographed using the Gel DOC IMAGO System (B&L Systems, the Netherlands). The size of fragments was determined by comparison with GeneRuler 1 kb DNA Ladder (Fermentas, Lithuania).

PCR products were purified by agarose gel DNA extraction kit (Roche) and sequenced in both directions (Gene Service, UK). The nucleotide sequences of RNA1 3' coding region were submitted to the GenBank (http://www.ncbi.nlm.nih.gov/) (Table 1) and were compared with 33 published sequences from Spain including 5 isolates of watermelon (AY580947-50, AY580931), 12 isolates of melon (AY580918-19, AY580926-29, AY580932-33, AY580944-46, NC_004809) 16 isolates of cucumber (AY580920-25, AY580930, AY580934-40, AY580942-43) and one melon isolate from America (AY547827) (available sequences).

Alignment of nucleotide sequences was performed using MUSCLE (Edgar, 2004). The most appropriate model was determined using the Bayesian Information Criterion (BIC) implemented in MEGA, version 5.0 program (Tamura et al. 2011), then phylogenetic tree was reconstructed with the neighbor-joining (NJ) method using the MEGA5. Beet pseudo-yellows virus (BPYYV, NC_005210) was defined as out group.

To verify the statistical validity of the clusters, a bootstrap value was calculated on 1,000 trials.

### Table 1. List of Iranian CYSDV isolates used in sequence analysis

<table>
<thead>
<tr>
<th>Country</th>
<th>Host</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iran (Bushehr)</td>
<td>C. melo</td>
<td>KU904303</td>
</tr>
<tr>
<td>Iran (Khuzestan)</td>
<td>C. melo</td>
<td>KU904305</td>
</tr>
<tr>
<td>Iran (Kerman)</td>
<td>C. sativus</td>
<td>KU904304</td>
</tr>
<tr>
<td>Iran (Tehran)</td>
<td>C. melo</td>
<td>KU904306</td>
</tr>
</tbody>
</table>

### Result and discussion

In this study some molecular properties of Iranian CYSDV isolates were studied based on the coding region of 3'-terminal region of RNA 1. RT-PCR on total RNA from Iranian isolates resulted in amplification of an expected 560 bp DNA fragment, while no amplification of the product was observed from the healthy plant extracts (Fig. 1).

![Fig. 1. Agarose gel electrophoresis pattern of RT-PCR products using CYSDV infected plants total RNA extract, 1, molecular marker (1 kb DNA ladder, Fermentase); 2-5, isolates Kerman (Jiroft), Khuzestan (Dezfool), Bushehr (Borazjan) and Tehran (Varamin), respectively and H, healthy plant.](image)

Obtained nucleotide sequences were deposited in NCBI GenBank under accession numbers of KU904303-KU904306. In multiple alignment nucleotide sequences of four Iranian and 34 CYSDV isolates from GenBank, 98-99% identity was obtained among Iranian and other isolates, whereas isolates from other countries including Spain and USA had 100% identity. Among Iranian isolates Kerman (Jiroft) isolate showed the highest identity to isolates from other countries including Spain and America (Fig. 2). Phylogenetic tree constructed by neighbor-joining method (Fig. 2) indicated that except Kerman (Jiroft) isolate, other three Iranian isolates were clustered in a distinct subgroup.
This difference may be related to the original hosts of virus isolates, as the Kerman (Jiroft) isolate was collected from greenhouse cucumbers, whereas the other Iranian isolates were from melon plants.

Marco and Aranda (2005) showed that mean nucleotide diversity in the 3’ terminal region of CYDV RNA1 (the same region which amplified in this study) was very low. The high genetic stability of the CYSDV could be attributed to selection pressures, including the maintenance of structural features of the virus or interaction with host and vector factors (García-Arenal et al., 2001). According of results obtained in this study, it can be concluded that Iranian CYSDV isolates have low genetic diversity and confirmed the results which previously presented by Keshavarz et al. 2013.

![Fig. 2. Neighbor-joining tree of CYSDV isolates generated by Mega 5 based on RNA1_3' sequences of worldwide collected isolates.](image)

References


CELIX, A., A. LOPEZ-SESE, N. ALMARZA, M. L.


Keshavarz and Bananej: Phylogenetic analysis of four Iranian *Cucurbit yellow stunting disorder virus* isolates …