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Geographic distribution and phylogenetic analysis of cucurbit yellow stunting disorder virus in Iran

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Summary. – Cucurbit yellow stunting disorder virus (CYSDV) is a destructive virus of cucurbits in Iran. During 2008–2012 growing seasons a total of 366 cucurbit samples including melon, cucumber, snakemelon and squash with typical symptoms of CYSDV infection were collected from ten Provinces in Iran. They were screened by ELISA and the infectivity of ELISA-positive samples was confirmed by RT-PCR. The results showed that 309 out of 366 samples were infected by CYSDV. The virus was present in many areas of southern and central Provinces of the country. Analyses of nucleotide and amino acid sequences of the CYSDV coat protein (CP) showed that Iranian isolates form a cluster and were placed in the Eastern subgroup of CYSDV. The Eastern subgroup of CYSDV was divided into two diverged subgroups including Iranian isolates and Saudi Arabian isolates. The identity among Iranian isolates was more than 99 %. Estimation of genetic distances showed that the number of nucleotide and amino acid substitutions per site from averaging overall Iranian sequence pairs were 0.004 and 0.008, respectively. Phylogenetic analyses and the estimation of genetic distance indicated that Iranian isolates have low genetic diversity.

Keywords: CYSDV; crinivirus; Closteroviridae; coat protein; sequence; Iran

Introduction

Whitefly-transmitted viruses extensively affect cucurbit crops in many tropical, sub-tropical and temperate regions (Wisler *et al*., 1998). Cucurbit yellow stunting disorder virus (CYSDV) is a whitefly-transmitted virus that has caused a major constraint on protected cucumber and melon crops in the world (Hassan and Duffus, 1991; Celix *et al*., 1996; Wisler *et al*., 1998; Abou-Jawdah *et al*., 2000; Desbiez *et al*., 2000; Kao *et al*., 2000, Louro *et al*., 2000; Desbiez *et al*., 2003; Brown *et al*., 2007; Kuo *et al*., 2007; Yakoubi *et al*., 2007; Liu *et al*., 2010). CYSDV is a member of the genus *Crinivirus* (the family *Closteroviridae*) and is transmitted in nature by the whitefly *Bemisia tabaci* (Gennadius) (Celix *et al*., 1996; Martelli *et al*., 2002). Its viral particles are flexible rods with the length between 750 to 800 nm (Liu *et al*., 2000). CYSDV was first reported in the United Arab Emirates (Hassan and Duffus, 1991), and later in the Middle East countries, Mediterranean region, Europe and North America (Hassan and Duffus, 1991; Celix *et al*., 1996; Wisler *et al*., 1998; Abou-Jawdah *et al*., 2000; Desbiez *et al*., 2000; Kao *et al*., 2000, Louro *et al*., 2000; Yakoubi *et al*., 2007). This virus induces interveinal chlorotic spots in mature leaves, which may enlarge and eventually fuse together producing yellowing of the entire leaf except for the veins that remain green (Celix *et al*., 1996). Yellowing symptoms are accompanied by reduction of fruit yield and quality and, therefore, this virus is of a high economic importance. Recently it has been shown that the virus is capable of infecting not only the members of the *Cucurbitaceae*, but also other species including Alfalfa (*Medicago sativa*), lettuce (*Lactuca sativa*), snap

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Abbreviations: CP = coat protein; CYSDV = cucurbit yellow stunting disorder virus; HSP70h = heat shock protein 70 homologue; ORF = open reading frame; TMGMV = tobacco mild green mosaic virus

bean (*Phaseolus vulgaris*), alkali mallow (*Silo hederacea*), Wright's groundcherry (*Physalis wrightii*) and 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 genetic diversity of CYSDV isolates from different areas of the world has been studied for the heat shock protein 70 homologue (HSP70h) and the coat protein genes of RNA 2. The genetic diversity of CYSDV isolates is unusually low compared to other members of the family *Closteroviridae*, although it is still possible to differentiate two genetic groups, the so-called Eastern and Western CYSDV subpopulations (Rubio *et al*., 1999, 2001). Analysis of nucleotide sequences of coding and non-coding regions of CYSDV isolates collected on a local scale over an 8-year period has indicated lower nucleotide diversity and less evenly distributed genome variability compared to other plant viruses. 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, 2004).

Although CYSDV has been detected in Bushehr Province of Iran in 2005 (Keshavarz and Izadpanah, 2005), no information is available on its presence in other cucurbit growing regions of this country. Likewise, there is no information on the molecular characteristics of Iranian isolates of the virus. This paper reports the results of a more extensive survey for the virus and genetic assessment of CYSDV isolates in Iran.

Materials and Methods

Sampling. During a 4-year period (2008–2012), squash (*Cucurbita pepo* L.), cucumber (*Cucumis sativus* L.), snakemelon and melon (*Cucumis melo* L.) growing fields and greenhouses in various regions of Iran were surveyed and a total of 366 leaf samples showing CYSDV symptoms were collected (Table 1).

ELISA. All samples were screened for the presence of CYSDV by indirect-ELISA (Converse and Martin, 1990) using the CYSDV specific polyclonal antibody (Keshavarz, 2003) and goat anti-rabbit alkaline phosphatase conjugate. Each sample was tested in duplicate. Samples were considered positive if their absorbance at 405 nm was equal or greater than threefold of healthy control mean values.

RNA extraction and RT-PCR. Total RNA was extracted from the ELISA-positive leaves using Tri Pure isolation reagent (Roche Chemical, St Louis, MO, USA) and was used as a template for RT-PCR. Specific oligonucleotide primers CYSCPf (5'-ATGGCG AGTTCGAGTGAGAATAA-3') and CYSCPr (5'-ATTACCACAGC CACCTGGTGCTA-3') (Rubio *et al*., 2001) corresponding to both ends of the CYSDV CP gene were used in RT-PCR. Approximately 300 ng of total RNA was subjected to reverse transcription for 1 hr at 42°C in a 20-µl reaction mixture with RevertAid™ M-MuLV reverse transcriptase (Fermentas, Lithuania) and 20 pmol of the reverse primer. PCR was performed in a 25 µl reaction mixture containing PCR buffer (50 mmol/l KCl; 10 mmol/l Tris-HCl, pH 9.0; and 1.0% Triton X-100), 1.5 mmol/l $MgCl_2$, 1 mmol/l each of the four dNTPs, 2.5 units of *Taq* DNA polymerase (Fermentas, Lithuania), and 20 pmol of each primer. After an initial denaturation step at 94°C for 4 min, PCR was performed for 30 cycles, each at 94°C for 30 sec, 57°C for 30 sec, and 72°C for 1.5 min, followed by an extension step at 72°C for 7 min. RT-PCR products were separated by electrophoresis in 1% agarose gel and stained by ethidium bromide (0.5 µg/ml). 1Kb DNA molecular weight markers (Fermentas, Lithuania) were used to determine the size of the amplified fragments.

Phylogenetic analyses and population genetic distance estimation. Molecular variability of CYSDV isolates was studied using a set of 11 CYSDV isolates representing the regional and host diversity. The CP fragment of each isolate amplified by RT-PCR was ligated into the pTZ57R/T vector (Fermentas, Lithuania) following the manufacturer's instructions and transformed into *Escherichia coli* DH5α cells by heat shock (Sambrook and Russell, 2001). Selection of clones with appropriate inserts was carried out by colony PCR using the same primers as those used for RT-PCR and digestion with restriction enzyme *Bam*HI, followed by gel electrophoresis.

Sequences of the cDNAs were determined in both directions for two clones of each isolate (Macrogene Inc., Seoul, South Korea).

Table 1. Sampling regions, hosts, and the number of CYSDV-infected samples in each region as determined by indirect ELISA

Region	Host	No. infected / No. tested (infection percentage)	Sampling year
Isfahan(Badrood and Gorgab)	Melon	18/35(51.42)	2008, 2010
Bushehr	Melon, snakemelon, squash, cucumber	124/124(100)	2008–2012
Kerman (Jirfoft)	Greenhouse cucumber	24/28(85.71)	2008
Khouzistan(Dezfool)	Melon, watermelon	28/30(93.3)	2008
Hormozgan	Melon	18/31(58)	2009
Tehran	Melon	32/37(86.48)	2009, 2011
Varamin	Melon, cucumber (greenhouse)	28/31(90.3)	2008, 2010
Markazi (Saveh)	Melon	6/15(40)	2011
Qom	Cucumber	6/10(60)	2011
Garmsar(Ivanakay)	Melon	25/25(100)	2012
Total		309/366(84.4)	

The nucleotide sequences of CP coding region were submitted to the GenBank (http:// www.ncbi.nlm.nih.gov/) and were compared with 36 published sequences from other countries (Fig. 1).

Multiple sequence alignment of nucleotide sequences was performed using the Muscle (Edgar, 2004) with the default parameters. The most appropriate model was determined using the Bayesian Information Criterion (BIC) implement in MEGA version 5.0 program (Tamura *et al.*, 2011). The nucleotide and the deduced amino acid sequence alignments were used to calculate genetic distance by T92 (Tamura, 1992) and JTT+G methods (Jones *et al.*, 1992), respectively, which was selected by the lowest BIC. Phylogenetic analyses were carried out by the Neighbor-joining method using MEGA 5.0 under assumption of the substitution model proposed. Robustness of inferred evolutionary relationships was assessed by 500 bootstrap replicates. Branches of less than 50% bootstrap support were collapsed.

Potential recombination events between nucleotide sequences of CP coding regions were assayed with SimPlot 3.5.1 (Lole *et al.*, 1999). Total population of CYSDV isolates was divided into five subpopulations based on different sampling regions (Table 4). Diversities within and between populations were estimated according to Nei (1987) based on the T92 model. The numbers of base substitutions per site from averaging overall sequence pairs within each population and between populations were calculated and standard errors were obtained by a bootstrap procedure (500 replicates).

Results

Detection and geographic distribution of CYSDV

Three hundred and nine out of 366 samples showed positive reaction with the CYSDV specific polyclonal antibody in indirect-ELISA (Table 1). RT-PCR on total RNA from the ELISA positive samples resulted in amplification of an expected 750 bp DNA fragment.

Table 2. List of the Iranian CYSDV isolates used in this study

Isolate	Geographical origin	Host	Year of isolation	Acc. No.
BU	Bushehr(Chakoota)	Melon	2005	AY730779.1
BS	Bushehr	Squash	2011	KC469990
DM	Dezfool	Melon	2008	KC469991
VM	Varamin	Melon	2008	KC469992
BM1	Bushehr	Snakemelon	2008	KC469993
BC	Bushehr	Cucumber	2009	KC469994
BM2	Bushehr	Melon	2011	KC469995
BM3	Bushehr	Melon	2012	KC469996
VC	Varamin	Cucumber	2011	KC469997
TM1	Tehran	Melon	2009	KC469998
TM2	Tehran	Snakemelon	2011	KC469999
JCU	Jiroft	Cucumber	2008	KC470000

Phylogenetic and sequence analyses

The nucleotide sequences of full-length CP (756 nt) of 11 isolates were determined and deposited in GenBank database (Table 2). Estimation of genetic distances showed that the numbers of nucleotide and amino acid substitutions per site from averaging overall Iranian sequence pairs were

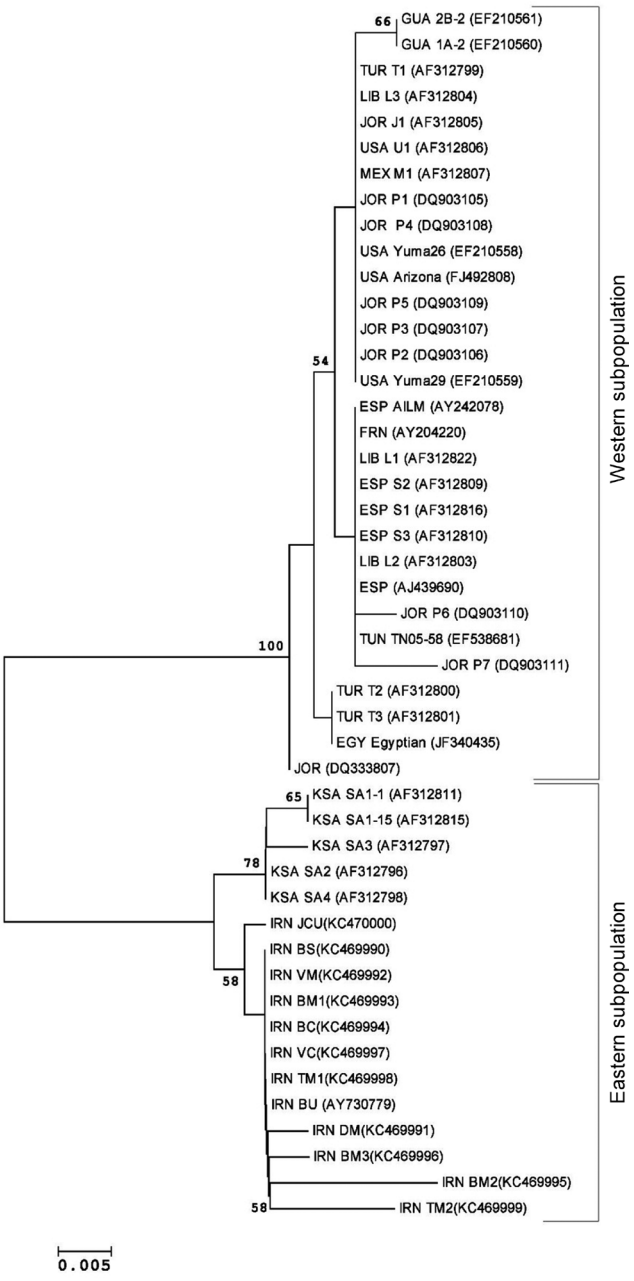

Fig. 1

Neighbor-joining tree of CYSDV isolates generated by Mega 5 based on CP amino acid sequences of worldwide collected isolates. The bootstrap values > 50% are shown near each node

Table 3. Estimates of average evolutionary divergence of overall sequence pairs in different regions

Subpopulation	Genetic distances	
	Nucleotide	Amino acid
Iranian isolates	0.004 (0.002)	0.008 (0.003)
Saudi Arabian isolates	0.034 (0.004)	0.021 (0.005)
Subpopulation west	0.003 (0.001)	0.004 (0.002)
Subpopulation east	0.013 (0.002)	0.011 (0.003)
Overall	0.056 (0.006)	0.033 (0.005)

0.004 and 0.008, respectively and more than 99% identity was obtained between CP sequences of 11 Iranian CYSDV isolates (Table 3). In comparison with other CYSDV isolates, the Iranian isolates showed the highest (98.8%) and the lowest (92.2%) identity to Saudi Arabian isolate (AF312798) and Guatemalan isolate (EF021561), respectively.

Constructed phylogenetic tree (Fig. 1) indicated that CYSDV isolates were placed in two groups: group I including isolates from Spain, France, USA, Jordan, Lebanon and Turkey, and group II including all isolates from Iran and Saudi Arabia. Iranian isolates were clustered in a distinct clade with respect to Saudi Arabian isolates. These findings indicated that Iranian isolates are slightly different from Saudi Arabian isolates. Among Iranian isolates, Jiroft isolates collected in a greenhouse were also slightly different. However, isolates collected from different hosts, different geographical regions and in different years were not significantly different. The mean genetic distance between CYSDV isolates collected all over the world was 0.056 and 0.033 based on nucleotide and amino acid sequence analyzes. No recombination events were detected in the CP coding region of Iranian CYSDV isolates.

Population genetic analyses

The mean genetic distance for each subpopulation is shown in Table 3. The smallest and the greatest mean genetic distances were observed in the Western subpopulation and Saudi Arabian subpopulation, respectively, in distances calculated from both the nucleotide and the amino acid sequences data.

All sequenced CYSDV CP collected from all over the world were grouped into five subpopulations based on the geographical distribution (Table 4). The highest and the lowest distance between populations were found between Saudi Arabian and Jordanian isolates and between American, Jordanian and European isolates, respectively. Estimation of evolutionary divergence over sequence pairs between groups showed that the populations from Saudi Arabia were more divergent compared to other populations (Table 3).

The number of base substitutions per site obtained by averaging overall sequence pairs was 0.056 ± 0.006. The mean evolutionary diversity was 0.003 ± 0.001 within subpopulations and 0.053 between populations. The coefficient of evolutionary differentiation was 0.942 ± 0.011.

Discussion

In this study the indirect ELISA on 366 cucurbit samples showed that CYSDV occurred in all surveyed cucurbit-growing regions in Iran. This is the first report of occurrence of CYSDV in Isfahan (Badrood and Gorgab), Kerman (Jiroft), Tehran (Tehran and Varamin), Khouzestan (Dezfool), Hormozgan, Markazi (Saveh), Qom and Semnan (Garmsar, Ivanakay) Provinces. The higher incidence of the virus in warmer southern Provinces (Bushehr, Khouzestan, Kerman and Hormozgan) may be due to more favorable conditions for the activity of whitefly vector in these regions. As it has been seen in Portugal, the first symptoms of CYSDV in a field plot of cucumber were associated with heavy infestations of *B. tabaci* (Louro *et al.*, 2000). High populations were also associated with symptoms on melon in the USA (Kao *et al.*, 2000). Some symptomatic samples, which did not react with CYSDV antibody, were possibly infected by other yellows-inducing viruses such as cucurbit aphid born yellows virus (Bananej *et al.*, 2006).

In this study the nucleotide sequence of CYSDV CP gene was used to estimate the genetic variability of Iranian isolates in comparison with published records from other countries.

Table 4. Genetic distances in the CP coding region between populations of CYSDV

	Between population mean distances				
Population	Europe	Jordan	America	Iran	Saudi Arabia
Europe	–	0.001	0.001	0.012	0.012
Jordan	0.004	–	0.001	0.013	0.012
America	0.003	0.003	–	0.013	0.012
Iran	0.107	0.109	0.110	–	0.005
Saudi Arabia	0.110	0.112	0.111	0.023	–

The distances and their corresponding standard errors are shown below and above the diameter, respectively.

Nucleotide sequences of CP gene have been applied in phylogenetic analyses by others (Rubio *et al.*, 1999, 2001; Marco and Aranda, 2004; Sweiss *et al.*, 2007). The constructed phylogenetic tree indicated that CYSDV isolates could be placed into two groups, isolates from Iran and Saudi Arabia in one and isolates from other countries in the second. Rubio *et al.* recognized two CYSDV subpopulations: Western (including those of Spain, Jordan, Turkey, Lebanon, and North America), and Eastern consisting of Saudi Arabian isolates (Rubio *et al.*, 2001). The results of the present study showed that Iranian isolates were clustered with Saudi Arabian isolates. However, within the Eastern group, the Iranian isolates formed a distinct subgroup with more than 99 % identity. Estimation of genetic distances showed that the number of nucleotide and amino acid substitutions per site from averaging overall Iranian sequence pairs were 0.004 and 0.008, respectively. As shown by Marco and Aranda (2004) the CYSDV population is extremely uniform. Thus, the value of the nucleotide diversity at synonymous positions in the coding regions of the CYSDV genome was 30-times smaller than an equivalent estimate for a population of tobacco mild green mosaic virus (TMGMV), a tobamovirus that is typically considered to be very stable (Fraile *et al.*, 1996). The low genetic diversity found in the CYSDV isolates could be due to the rapid expansion of CYSDV, related to the explosion of CYSDV vector populations into new areas (Rubio *et al.*, 2001). As shown by the other researchers, genetic variability of other viruses can be reduced by evolutionary forces such as selection, genetic drift and bottleneck events (Garcia-Arenal *et al.*, 2001). In fact, for most of the cases analyzed, plant virus populations have been shown to be genetically stable (Roossinck, 1997; Holland and Domingo, 1998). Estimation of evolutionary divergence of different populations of CYSDV showed that Iranian populations had lower diversity compared to overall world populations. The populations of Saudi Arabia were more diverse than others (Table 3). Also, our results showed that three populations, which were grouped by Rubio *et al.* (2001) as Western subpopulation (European, Jordanian and American), show very low genetic distances between each other, although, they have been reported from geographically distant regions.

It can be concluded that Iranian CYSDV isolates have low genetic diversity and, therefore, it can be suggested that hpRNA silencing strategy could be appropriate to generate a transgenic plant with broad-spectrum resistance to all Iranian CYSDV isolates.

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