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Short Communication

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

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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 RNA 1 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.

مقاله‌ی کوتاه علمی

آنالیز فیلوژنتیکی چهار جدایه های ایرانی ویروس کوتولگی زرد کدوییان بر اساس انتهای ۳ آر. ان. ای شماره ۱

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چکیده

به منظور آنالیز فیلوژنتیکی جدایه های ایرانی ویروس کوتولگی زرد کدوییان (*Cucurbit yellow stunting disorder virus*, CYSDV) ناحیه انتهای ۳′ RNA_1 (به طول ۵۶۰ نوکلئوتید) از چهار جدایه ویروس کوتولگی زرد کدوییان جمع آوری شده از استان های بوشهر، کرمان (جیرفت)، خوزستان(دزفول) و تهران با واکنش زنجیره ای پلی مراز و با استفاده از آغازگرهای اختصاصی تکثیر شد. ترادف نوکلئوتیدی ناحیه مذکورتعیین و با توالی‌های موجود در Gen Bank مقایسه شد. نتایج نشان دهنده تشابه ترادف نوکلئوتیدی بالا (۹۸-۹۹٪) بین جدایه‌های CYSDV بود و تشابه ترادف بین جدایه های ایرانی ۹۹ درصد بود. درخت فیلوژنتیکی ترسیم شده به روش نزدیک‌ترین همسایه با استفاده از ترادف نوکلئوتیدی ناحیه تکثیر شده نشان داد که تمامی جدایه های CYSDV در یک گروه قرار گرفته و جدایه های بوشهر، خوزستان و تهران در زیر گروه مجزا نسبت به سایر جدایه های مورد بررسی قرار گرفتند.

واژه‌های کلیدی: خصوصیات مولکولی، واکنش زنجیره‌ای پلی مراز، ویروس کوتولگی زرد کدوییان.

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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 $MgCl_2$ (50 mM) , 1*µl* of dNTPs (10 mM), 2.5 units of *Taq* DNA polymerase (Fermentas, Lithuania), and 20 pmol of each primer. Amplification was performed in an 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 ethidum 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, Lithuanai).

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* (BPYV, 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

Country	Host	Accession number
Iran (Bushehr)	*C. melo*	KU904303
Iran (Khuzestan)	*C. melo*	KU904305
Iran (Kerman)	*C. sativus*	KU904304
Iran (Tehran)	*C. melo*	KU904306

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).

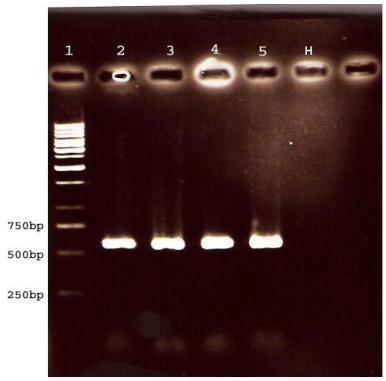

Fig. 1. Agarose gel electrophoresis pattern of RT-PCR products using CYSDV infected plants total RNA extract, 1, molecular marker (1 kb DNA ladder, Fermentase); 2-5, isolates Kerman(Jiroft), Khuzestan (Dezfool), Bushehr (Borazjan) and Tehran (Varamin), respectively and H, healthy plant.

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 (Garcı´a-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.

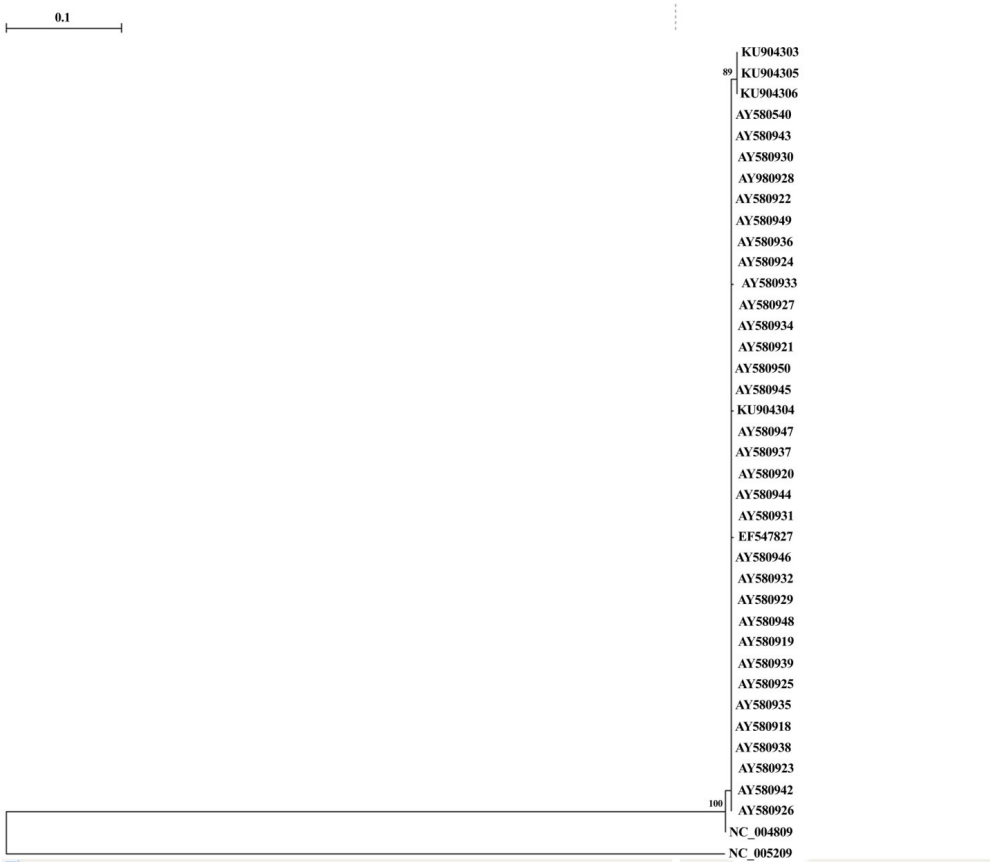

Fig. 2. Neighbor-joining tree of CYSDV isolates generated by Mega 5 based on RNA$_1$3' sequences of worldwide collected isolates.

References

ABOU-JAWDAH, Y., H. SOBH, A. FAYAD, H. LECOQ, B. DELECOLLE and J. TRAD-FERRE, 2000. *Cucurbit yellow stunting disorder virus*. A new threat to cucurbits in Lebanon. Journal of Plant Pathology, 82: 55-60.

AGUILAR, J. M., M. FRANCO, C. F. MARCO, B. BERDIALES, E. RODRIGUEZ-CEREZO, V. TRUNIGER and M. A. ARANDA, 2003. Further variability within the genus Crinivirus, as revealed by determination of the complete RNA genome sequence of *Cucurbit yellow stunting disorder virus*. Journal of General Virology, 84: 2555–2564.

BROWN, J. K., J. C. GUERRERO, M. MATHERON, M. OLSEN and A. M. IDRIS, 2007. Widespread outbreak of *Cucurbit yellow stunting disorder virus* in melon, squash, and watermelon crops in the Sonoran Desert of Arizona and Sonora, Mexico. Plant Disease, 91: 773.

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

GOMEZ-GUILLAMON and E. RODRIGUEZ-CEREZO, 1996. Characterization of *Cucurbit yellow stunting disorder virus*, a *Bemisia tabaci* -transmitted closterovirus. Phytopathology. 86: 1370–1376.

EDGAR, R. C. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatic, 19: 113.

GARCIA-ARENAL, F., A. FRAILE and J. M. MALPICA, 2001. Variability and genetic structure of plant virus population. Annual Review of Phytopathology, 39: 157–186.

HASSAN, A. and J. E. DUFFUS, 1991. A review of a yellowing and stunting disorder in the United Arab Emirates. Emirates journal of agricultural science, 2: 1–16.

KAO, J., L. JIA, T. TIAN, L. RUBIOand B. W. FALK, 2000. First report of *Cucurbit yellow stunting disorder virus* (genus *Crinivirus*) in North America. Plant Disease, 84: 101.

KESHAVARZ, T. and K. IZADPANAH, 2005. Etiology of cucurbit yellows in Bushehr Province, Iran. Iranian Journal of Plant Pathology, 41: 107–121.

KESHAVARZ, T., M. SHAMS-BAKHSH and K. IZADPANAH, 2013. Geographic Distribution and phylogenetic Analysis of *Cucurbit yellow stunting disorder virus* in Iran. Acta Virologica, 57(4): 415-420.

KUO, Y. W., M. R. ROJAS, R. L. GILBERTSON and W. M. WINTERMANTEL, 2007. First report of *Cucurbit yellow stunting disorder virus* in California and Arizona, in association with *Cucurbit leaf crumple virus* and *Squash leaf curl virus.* Plant Disease, 91: 330.

LECOQ, H. and C. DESBIEZ, 2012. Viruses of cucurbit crops in the Mediterranean region: an ever-changing picture. Advances in *Virus* Research, 84, 67–126.

MARCO, C. F. and M. A. ARANDA, 2005. Genetic diversity of a natural population of *Cucurbit yellow stunting disorder virus*. Journal of General Virology,86: 815–822.

MARTELLI, G. P., A. A. AGRRANOVSKY, M. BAR-JOSEPH, D. BOSCIA, T. CANDRESSE, R. H. A. COUTTS, V. V. DOLJA, B. W. FALK, D. GONSALVES, W. JELKMANN, A. V. KARASEV, A. MINAFRA, S NAMBA, H. J. VETTEN, G. C. WISLER and N. YOSHIJAWA, 2002: The family *Closteroviridae* revised. Archives of Virology, 147: 2039–2044.

POLSTON, J. E., L. L. HLADKY, F. AKAD and W. M. WINTERMANTEL, 2008. First report of *Cucurbit yellow stunting disorder virus* in cucurbits in Florida. Plant Disease, 92:1251.

RUBIO, L., J. SOONG, J. KAOAND and B. W. FALK, 1999. Geographic distribution and molecular variation of isolates of three whitefly -born closteroviruses of cucurbits: *lettuce infectious yellows virus, Cucurbit yellow stunting disorder virus* and beet pseudo yellows virus. Phytopathology, 89: 707–711.

RUBIO, L., Y. ABOU-JAWDAH, H. X. LIN and B. W. FALK. 2001. Geographically distant isolates of the crinivirus *Cucurbit yellow stunting disorder virus* show very low genetic diversity in the coat protein gene. Journal of General Virology, 82: 929–933.

TAMURA, K., D. PETERSON, N. PETERSON, G. STECHER, M. NEIAND and S. KUMAR, 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28: 2731–2739.

TZANETAKIS, I. E., R. R. MARTIN and W. M. WINTERMANTEL, 2013. Epidemiology of criniviruses: an emerging problem in world agriculture. Front Microbiology, doi: 10.3389/fmicb.2013.00119.

WINTERMANTEL, W. M., L. L. HLADKY, A. A. CORTEZ and E. T. NATWICK, 2009. A new expanded host range of *Cucurbit yellow stunting disorder virus* Includes Three Agricultural Crops. Plant Dissease, 93: 685–690.