

Natural Occurrence of *Tomato leaf curl New Delhi virus* in Iranian Cucurbit Crops

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(Received on October 6, 2015; Revised on January 1, 2016; Accepted on January 27, 2016)

The main areas for field-grown vegetable production in Iran were surveyed during the years of 2012–2014 to determine the occurrence of begomoviruses infecting these crops. A total of 787 leaf samples were collected from vegetables and some other host plants showing virus-like symptoms and tested by an enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies produced against *Tomato yellow leaf curl virus* (TYLCV). According to the ELISA results, 81 samples (10.3%) positively reacted with the virus antibodies. Begomovirus infections were confirmed by polymerase chain reaction (PCR) using previously described TYLCV-specific primer pair TYLCV-Sar/TYLCV-Isr or universal primer pair Begomo-F/Begomo-R. The PCR tests using the primer pair TYLCV-Sar/TYLCV-Isr resulted in the amplification of the expected fragments of *ca.* 0.67-kb in size for ELISA-positive samples tested from alfalfa, pepper, spinach and tomato plants, confirming the presence of TYLCV. For one melon sample, having a weak reaction in ELISA and no reaction in PCR using TYLCV-specific primers, the PCR reaction using the primer pair Begomo-F/Begomo-R resulted in the amplification

fragments of the expected size of *ca.* 2.8 kb. The nucleotide sequences of the DNA amplicons derived from the isolate, Kz-Me198, were determined and compared with other sequences available in GenBank. BLASTN analysis confirmed the begomovirus infection of the sample and showed 99% identities with *Tomato leaf curl New Delhi virus* (ToLCNDV); phylogenetic analysis supported the results of the database searches. This study reports the natural occurrence of TYLCV in different hosts in Iran. Our results also reveal the emergence of ToLCNDV in Iranian cucurbit crops.

Keywords : *Begomovirus*, phylogeny, *Tomato leaf curl New Delhi virus*, *Tomato yellow leaf curl virus*, vegetables

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With regarding to proper role of vegetables in ensuring human health, these crops are considered as one of the major food groups and grown all through the world (Dias, 2011). Based on the per capita consumption of vegetables in Iran, these products are widely grown in different regions of the country. The area harvested of vegetables in Iran was estimated over 876 thousand hectares in 2012 as compared with about 57 million hectares in the world (Food and Agriculture Organization of the United Nations, 2012). Diseases and pests are often cited as one of the major limiting factors for vegetable production, of which viral diseases account for more losses in such crops. Many viruses have been reported worldwide from vegetable plants (Brunt et al., 1995). Also, several viruses belonging to different virus genera and families such as *Cauliflower mosaic virus*, *Cucumber mosaic*

virus, *Tomato mosaic virus*, *Tomato yellow fruit ring virus* and *Watermelon mosaic virus* have been reported to infect Iranian vegetable crops (Farzadfar et al., 2005; Golnaraghi et al., 2013; Massumi et al., 2007, 2009; Yazdani-Khameneh et al., 2013).

The circular single stranded DNA viruses in the genus *Begomovirus*, family *Geminiviridae*, are amongst the most important vegetable viruses (Brown and Bird, 1992; Moriones and Navas-Castillo, 2000). This group of viruses known as one of the limiting factors of a number of economically important crops in many parts of the world, especially in tropical and sub-tropical regions (Zerbini et al., 2005). Begomoviruses infect only dicotyledonous plant species and are transmitted exclusively by the whitefly *Bemisia tabaci* (Homoptera: Aleyrodidae) in a non-circulative manner (Briddon, 2015; King et al., 2012). These viruses are considered as emerging plant viruses, due to their increasing incidence and the severity of the diseases they cause (Polston and Anderson, 1997), which are frequently being reported to occur on new hosts and spread to more geographical locations (Mansoor et al., 2003; Varma and Malathi, 2003). Recombination and mutation may play important roles in the establishment and emergence of new *Begomovirus* strains/species (Mansoor et al., 2006). Begomoviruses typically have genomes consisting of two components, referred to as DNA-A and DNA-B, which encodes all virus functions required for DNA replication, control of gene expression, insect transmission and movement in the plant (Hanley-Bowdoin et al., 1999). However, a small number of monopartite begomoviruses have been discovered. For these viruses, all essential virus functions are encoded by a single component that resembles DNA-A of the bipartite begomoviruses (Navot et al., 1991; Rojas et al., 2001). To date, more than two hundred confirmed and tentative species in the genus *Begomovirus* have been noted by the International Committee on Taxonomy of Viruses (ICTV; King et al., 2012).

In Iran, several begomoviruses have been reported to occur on vegetable crops in recent years; some of them infect economically important crops such as tomato and cucurbits (Fazeli et al., 2009; Heydarnejad et al., 2009; Kheyr-Pour et al., 2000; Massumi et al., 2009), and have capacities to cause epidemics in and significant yield losses to these crops, e.g., *Tomato yellow leaf curl virus* (TYLCV) (Moriones and Navas-Castillo, 2000). In this paper, we detect TYLCV occurring in vegetables and some other host plants in the country. We also detect and describe the partial molecular characterization of *Tomato leaf curl New Delhi virus* (ToLCNDV) infecting melon (*Cucumis melo*; family *Cucurbitaceae*) in Khuzestan

province, Iran.

Materials and Methods

Surveys and sample collection. During the growing seasons of 2012–2014, the main areas for field-grown vegetable crops in Iran, including Khuzestan and Tehran provinces were surveyed to determine the occurrence of TYLCV in these crops. Overall, 86 fields were surveyed and 777 leaf samples collected from different crops, especially vegetables, showing virus-like symptoms of mosaic, mottling, chlorosis, dwarfing, yellowing, leaf deformation, leaf curling, leaf rolling and necrosis (Table 1). We also collected 10 symptomatic weed samples in or around fields surveyed. The collected leaves were immediately placed in plastic bags and transported to the laboratory and kept at 4°C. For long-term storage, leaf samples were dried by using silica gel or a freeze-dryer and stored at –20°C.

Serological assays. Samples were tested by a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977) using TYLCV-polyclonal antibodies (Bioreba, Reinach, Switzerland) according to the manufacturer's instructions. All buffers as well as negative and positive controls used in these assays were provided by the company. Briefly, microtiter plates were coated with 100 µl of 1:1,000 dilution of TYLCV-immunoglobulin G (IgG) in carbonate coating buffer and incubated overnight at 4°C. Plates were washed with washing buffer and then, leaf extracts were added to the plate wells (100 µl in each well); the plates were incubated overnight at 4°C. After washing the plates, 100 µl of alkaline phosphatase-conjugated TYLCV-IgG diluted in conjugate buffer (1:1,000) was added and incubated 3 hours at 37°C. Wells were washed and incubated with 100 µl of substrate (1 mg/ml of *p*-nitrophenyl phosphate in substrate buffer) at room temperature. Color reactions were measured at 405 nm using a microtiter plate reader (EL800; Bio-Tek Instruments, Winooski, VT, USA). A sample was considered virus-positive if its optical density (OD) was equal to or exceeded three times of the mean OD of the negative controls (healthy).

Polymerase chain reaction (PCR). Total DNA was extracted from 0.04 g of dried leaf tissue using the CTAB protocol described earlier (Dellaporta et al., 1983) or DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to those that recommended by the supplier; finally, DNA pellets were suspended in 25 µl of diethylpyrocarbonate (DEPC)-treated water and stored

Table 1. Occurrence of *Tomato yellow leaf curl virus* (TYLCV) in different crops in the provinces surveyed*

Province	Host (common name)	No. of fields, infected/visited (%) [†]	No. of samples, infected/collected (%) [‡]	Collection year
Khuzestan	<i>Abelmoschus esculentus</i> (okra)	1/5 (20.0)	1/10 (10.0)	2012
	<i>Citrullus vulgaris</i> (watermelon)	0/1 (0.0)	0/7 (0.0)	2012
	<i>Cucumis melo</i> (melon)	1/2 (50.0)	2/25 (8.0)	2012
	<i>Cucumis sativus</i> (cucumber)	1/5 (20.0)	1/5 (20.0)	2012
	<i>Cucurbita pepo</i> (zucchini)	1/9 (11.1)	2/72 (2.8)	2012, 2013
	<i>Lactuca sativa</i> (lettuce)	0/2 (0.0)	0/8 (0.0)	2012
	<i>Solanum lycopersicum</i> (tomato)	6/15 (40.0)	10/148 (6.8)	2012, 2013
	<i>Solanum melongena</i> (eggplant)	0/1 (0.0)	0/3 (0.0)	2012
	<i>Solanum tuberosum</i> (potato)	2/7 (28.6)	2/16 (12.5)	2012, 2013
	<i>Spinacia oleracea</i> (spinach)	1/1 (100.0)	1/9 (11.1)	2012
	<i>Trigonella foenum-graecum</i> (fenugreek)	0/1 (0.0)	0/7 (0.0)	2012
	<i>Vicia faba</i> (broad bean)	1/4 (25.0)	1/12 (8.3)	2012, 2013
Tehran	<i>Capsicum annuum</i> (pepper)	6/9 (66.7)	25/110 (22.7)	2012, 2013, 2014
	<i>Medicago sativa</i> (alfalfa)	3/6 (50.0)	4/110 (3.6)	2012, 2013, 2014
	<i>Solanum melongena</i> (eggplant)	9/12 (75.0)	25/109 (22.9)	2012, 2013, 2014
	<i>Spinacia oleracea</i> (spinach)	4/6 (66.7)	6/126 (4.8)	2012, 2013
Total [§]	36/86 (41.9)	80/777 (10.3)		

*Identification is based on serological reactions (enzyme-linked immunosorbent assay).

[†]Percent of virus-infected fields.

[‡]Percent of virus infection rate in the symptomatic samples collected.

[§]Average of virus infection.

Table 2. List of primers used in this study

Primers	Sequences* (5'-3')	Reference
TYLCV-specific		Pico et al., 1999
TYLCV-SAR	GCCATATACAATAACAAGGC	
TYLCV-ISR	CGCCCGTCTCGAAGGTTC	
Begomovirus-universal		Akhter et al., 2009
Begomo-F	ACGCGTGCCGTGCTGCTGCCCCATTGTCC	
Begomo-R	ACGCGTATGGGCTGYCGAAGTTSAGAC	
KzMe198-specific		This study
Begomo-F1	GTGCTGCTGCCCCATTGTC	
Begomo-F2	CATTAGTTAGGAAGTTTGTAGG	
Begomo-R3	CGCCGAATCAAAACGACAAG	
Begomo-R4	CCCATAAGCATAGTCATAGAG	

*In the primer sequences, Y = C/T and S = C/G.

at -20°C . PCR amplifications were carried out using *SmarTaq* DNA polymerase (CinnaGen, Tehran, Iran), and thermocycling was done in a MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA). All the primers used were synthesized by Metabion (Martinsried, Germany).

As a first step, to confirm the presence of TYLCV, 33 leaf samples with a positive reaction in the serological assays with the virus were tested by PCR using primer pairs TYLCV-Sar and TYLCV-Isr designed for the

specific detection of the virus (Pico et al., 1999; Table 2). The cycling profile was 10 minutes of melting at 95°C followed by 35 cycles of melting, annealing, and DNA extension of 1 minute at 95°C , 45 seconds at 50°C , and 2 minutes at 72°C , respectively, and a final extension for 10 minutes at 72°C .

In the second step, the forward and reverse degenerate primers Begomo-F and Begomo-R (Table 2) were used to detect the possible begomovirus(es) in 14 ELISA-

positive leaf samples negatively reacted in PCR using the TYLCV-specific primers. The universal primer pair has been designed for the amplification of DNA-A of begomoviruses (Akhter et al., 2009). The PCR program was as follows: 95°C for 10 minutes, 38 cycles of 95°C for 1 minute, 50°C for 45 seconds, 72°C for 2 minutes, and finally 72°C for 10 minutes.

PCR products and DNA ladder (Fermentas, Lithuania) were loaded onto 1.2% agarose gel in the presence of 1 µg/ml ethidium bromide using 1X Tris-borate ethylenediaminetetraacetic acid (TBE) buffer (Sambrook et al., 1989). Gels were visualized and photographed with ultraviolet-illumination.

Sequencing of PCR products. To determine the nucleotide sequence of the PCR product for one sample tested, amplified using primers Begomo-F and Begomo-R, the expected fragments were excised from the gel and cleaned by the sequencing service of Bioneer (Daejeon, Korea). DNA sequencing was done by primer walking in both directions using the amplification primers and other designed primers (Table 2). Nucleotide sequence data were assembled using Bio-Edit version 7.2.5 (Hall, 1999). The GenBank non-redundant nucleotide database was searched using BLASTN to identify the sequences that most closely matched sequences obtained for the sample (Altschul et al., 1997). The results of database searches were confirmed by phylogenetic analysis.

Phylogenetic analysis. In this study, the sequence obtained for the isolate Kz-Me198 and 59 representative sequences from different species of the genus *Begomovirus*, including those isolated from Iran and India, as well as representative sequences of worldwide isolates of ToLCNDV and two Iranian isolates of the virus available from the international sequence databases were used. All the sequences were first aligned using CLUSTAL X (Jeanmougin et al., 1998) or CLUSTAL W (Thompson et al., 1994). However, this procedure resulted in some gaps, which were subsequently deleted manually using Bio-Edit program. The sequences were subsequently analyzed to find the best nucleotide substitution model. Tamura-Nei model (Tamura and Nei, 1993) was estimated as the best model with the lowest Bayesian information criterion (BIC) and Akaike information criterion, corrected (AICc) scores (Nei and Kumar, 2000). This model was used, with a proportion of invariant sites and a gamma distribution of site-rate variants across five categories, to estimate the phylogenetic relationships. Phylogenetic analysis was done by the maximum-likelihood (ML) algorithm (Nei and Kumar, 2000) with 1,000 bootstraps replicates to estimate node significances (Efron et al., 1996). The ML

tree was found by subtree pruning and regrafting trees. All these analyses were done by MEGA 6.0 software (Tamura et al., 2013).

Results

Surveys and detection of TYLCV by DAS-ELISA. A total of 787 leaf samples collected from vegetables and other host plants during 2012–2014 were tested by DAS-ELISA. Among those tested, 81 samples (10.3%) from broad bean (*Vicia faba*; 1/12, 8.3%), cucumber (*Cucumis sativus*; 1/5, 20.0%), eggplant (*Solanum melongena*; 25/112, 22.3%), melon (*Cucumis melo*; 2/25, 8.0%), okra (*Abelmoschus esculentus*; 1/10, 10.0%), pepper (*Capsicum annuum*; 25/110, 22.7%), potato (*Solanum tuberosum*; 2/16, 12.5%), spinach (*Spinacia oleracea*; 7/135, 3.7%), zucchini (*Cucurbita pepo*; 2/72, 2.8%), tomato (*Solanum lycopersicum*; 10/148, 6.8%) and alfalfa (*Medicago sativa*; 4/110, 3.6%) were found to be infected with TYLCV (Table 1); the virus was also detected in one common mallow sample (*Malva neglecta*) collected from Khuzestan province. Begomovirus infections were found in 36 fields (41.9%) surveyed. Mosaic, leaf rolling, leaf curling, leaf deformation and dwarfing symptoms were associated with the virus infection.

PCR. The expected fragments of approximately 0.67 kb (Pico et al., 1999) were amplified by PCR from total DNAs extracted from 19 ELISA-positives samples using the specific primers TYLCV-Sar and TYLCV-Isr. The results of PCR analysis confirmed the presence of TYLCV in samples of pepper, spinach, tomato and alfalfa. However, amplicons were not obtained from the other 14 samples. The DNAs extracted from these samples were subsequently tested by PCR using the begomovirus universal primers Begomo-F and Begomo-R; an amplicon with the expected size of ca. 2.8 kb (Akhter et al., 2009) was obtained for one sample tested, namely Kz-Me198, from a melon plant in Hamidiyeh (Ahwaz, Khuzestan, Iran) showing mosaic and leaf deformation symptoms (Fig. 1A). The sample had a weak reaction in ELISA to TYLCV antibodies. To confirm these observations, nucleotide sequences of the DNA amplicon derived from the isolate was determined as follows.

Nucleotide sequence. The nucleotide sequence of the amplified DNA derived from the isolate Kz-Me198, after trimming and excluding the primer sequences, was 2,643 nt long. The results indicated that this sequence encompassed regions encoding AV1 and AV2 genes in the viral sense, and AC1, AC2, AC3, AC4, and AC5 genes in the complementary sense, and an intergenic

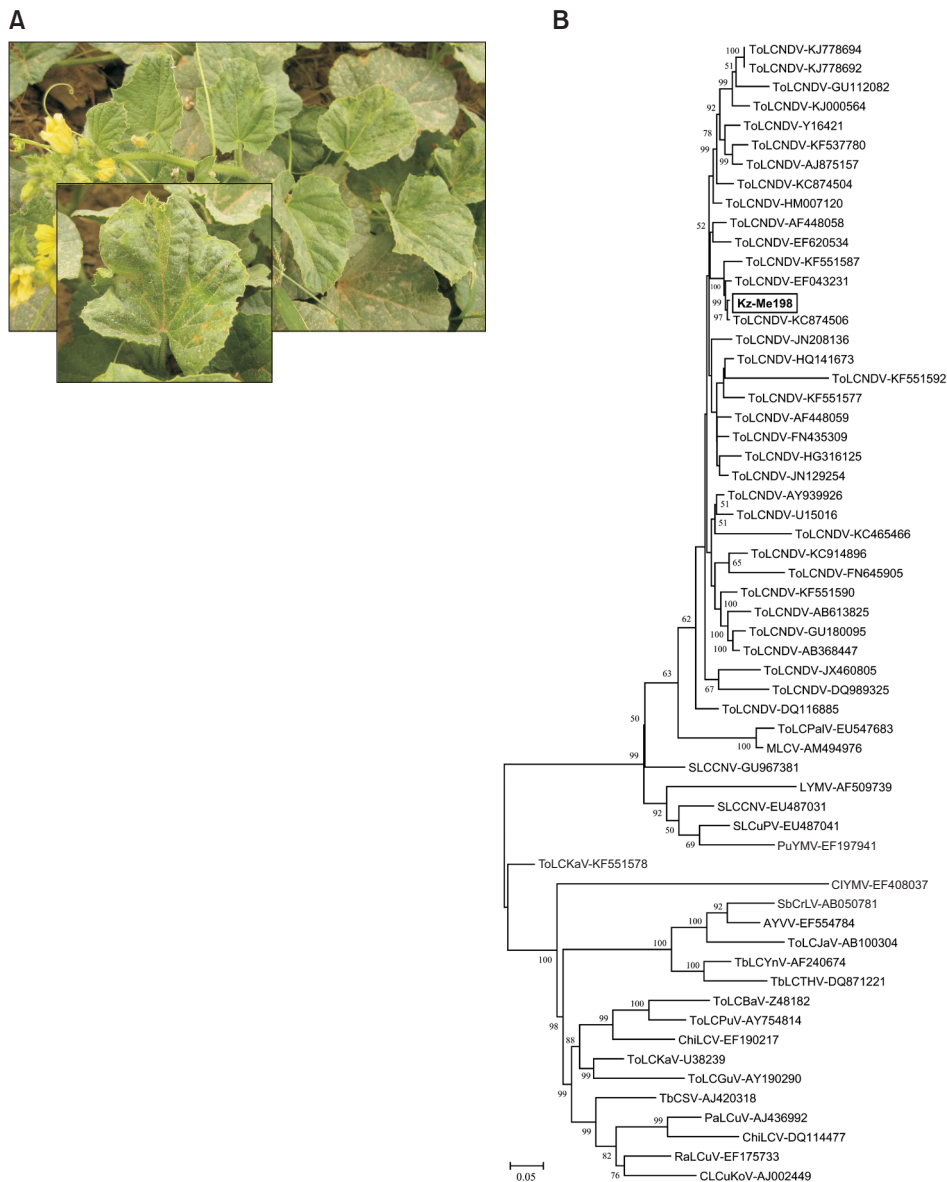


Fig. 1. (A) Mosaic and leaf deformation symptoms associated with the begomovirus infection on melon. (B) Maximum-likelihood trees indicating the relationships between the nucleotide sequence for the isolate Kz-Me198 compared to various representative sequences of begomoviruses. Numbers at each node indicate the percentage of supporting bootstrap samples (only values equal to or more than 50% are shown). Horizontal branch lengths are drawn to scale with the bar indicating 0.05 nt replacements per site. The abbreviation name of each begomovirus species and accession code in the international gene sequence database are listed. The begomovirus species referred to are: ToLCNDV, *Tomato leaf curl New Delhi virus*; ToLCPaV, *Tomato leaf curl Palampur virus*; MLCV, *Melon leaf curl virus*; SLCCNV, *Squash leaf curl China virus*; LYMV, *Luffa yellow mosaic virus*; SLCuPV, *Squash leaf curl Philippines virus*; PuYMV, *Pumpkin yellow mosaic virus*; ToLCKaV, *Tomato leaf curl Karnataka virus*; CIYMV, *Clerodendron yellow mosaic virus*; SbCrLV, *Soybean crinkle leaf virus*; AYVV, *Ageratum yellow vein virus*; ToLCJaV, *Tomato leaf curl Java virus*; TbLCYnV, *Tobacco leaf curl Yunnan virus*; TbLCTHV, *Tobacco leaf curl Thailand virus*; ToLCBaV, *Tomato leaf curl Bangalore virus*; ToLCPuV, *Tomato leaf curl Pune virus*; ChiLCV, *Chilli leaf curl virus*; ToLCGuV, *Tomato leaf curl Gujarat virus*; TbCSV, *Tobacco curly shoot virus*; PaLCuV, *Papaya leaf curl virus*; RaLCuV, *Radish leaf curl virus*; CLCuKoV, *Cotton leaf curl Kokhran virus*.

region located between AC1 and AV2. This sequence also contained a potential stem-loop forming region (5'-GC GGCCATTCGTATAATATTACCGAATGGCCGCG-3')

which included the conserved nonanucleotide sequence TAATATTAC present within the replication origin of almost all geminiviruses. BLASTN analysis showed

that the sequence shared the highest identity (99%) with the DNA-A segment of ToLCNDV (2,628/2,643 bases; accession No. KC874506 from potato in India). The genomic sequence of Kz-Me198 determined in this study was deposited in GenBank database (accession No. KP793719).

Phylogenetic analysis. The phylogenetic relationships among Kz-Me198 and begomoviruses were investigated by ML method; the tree is shown in Fig. 1B. The tree was calculated from the DNA-A sequences of 59 begomovirus isolates, including 34 sequences from ToLCNDV. Based on the results, the isolate Kz-Me198 fell into the ToLCNDV cluster which confirmed the presence of the virus; this was similar to those that found for the other two Iranian isolates of the virus (accession Nos. KJ778692 and KJ778694) from eggplant and pepper in south-east of Iran (Sistan-va-Balouchestan province), respectively. Multiple sequence alignment using the CLUSTAL W program showed that the nucleotide sequence of Kz-Me198 shared 93.9% and 84.9–99.4% identities with Iranian and worldwide isolates of the virus, respectively.

Discussion

Begomoviruses have become the most destructive group of plant viruses. These viruses have become a main constraint on crop production all through the world due to the worldwide increase in their population and distribution of insect vectors, and movement of plant material globally (Seal et al., 2006). In the present work, the main vegetable producing areas in Khuzestan and Tehran provinces of Iran were surveyed in the period of 2012–2014 for the presence of TYLCV and the virus was found to be widespread in these regions, similar observations have been reported previously (Bananej et al., 2009; Shirazi et al., 2014); this may reflect both a very high inoculum reservoir and the prevalence and efficiency of its whitefly vectors. This study also reports the natural occurrence of TYLCV on alfalfa, pepper and spinach plants for the first time in Iran; these hosts may play an important role in the spread of the virus in these regions.

We also found a melon sample (Kz-Me198) infected with a new begomovirus in Khuzestan province. Mosaic and leaf deformation symptoms were associated with the virus infection of the sample; several begomovirus species have been reported to cause similar symptoms in many host plants (Zerbini et al., 2005). These symptoms are different from those that observed for other begomoviruses previously reported to infect cucurbit crops in Iran: *Watermelon chlorotic stunt virus* and *Tomato leaf curl Palampur virus*; these viruses induce

chlorosis, yellowing, stunting and leaf curl symptoms on the affected plants (Heydarnejad et al., 2009; Kheyr-Pour et al., 2000). Mosaic and leaf deformation also can be observed followed by the infection of cucurbit plants with several other viruses (Bananej and Vahdat, 2008); this makes it more difficult to detect the begomovirus infections only on the basis of visual and field inspections.

The virus sample, Kz-Me198, had a weak reaction with TYLCV-polyclonal antibodies; serological relationships among different begomovirus species have been reported previously (Rishi, 2004). The expected DNA fragments were not amplified by PCR using the TYLCV-specific primers. However, the use of begomovirus universal primers Begomo-F and Begomo-R resulted in the amplification of DNAs with the expected size; the nucleotide sequence of the DNA amplicons was subsequently determined. The results of BLASTN analysis showed that the sequence obtained for the isolate closely matched that of the DNA-A of ToLCNDV (identity score: 99%). Phylogenetic analysis confirmed the database searches results and showed a clustering of Kz-Me198 with other ToLCNDV isolates with a high bootstrap support. Several taxonomic criteria have been proposed by the ICTV for the species demarcation of the genus *Begomovirus*; the most important of which is the nucleotide sequence identity threshold of 89% between DNA-A components of begomoviruses (ICTV; King et al., 2012). Therefore, our results unequivocally indicated the presence of ToLCNDV in the melon sample.

The discovery of ToLCNDV infecting Iranian melons adds another virus to the list of species that have been reported to infect cucurbit crops in the country. The virus has a wide host range, including pepper, potato, tomato and cucurbit plants (Hussain et al., 2005). ToLCNDV virus has been reported to induce severe symptoms, yellow mosaic, on various cucurbit plants under natural conditions (Ito et al., 2008; Mnari-Hattab et al., 2015; Tiwari et al., 2010, 2012), which are different from the milder symptoms associated with the virus infection observed in this study. Further studies on the pathogenicity of Iranian isolates of the virus seem necessary.

ToLCNDV has been found to infect some solanaceous crops in south-east of Iran, Sistan-va-Balouchestan province (accession Nos. KJ778692 and KJ778694). Moreover, three ToLCNDV-like isolates have been reported from two weeds and tomato in Kerman and Hormozgan provinces in south-east and south of the country (Fazeli et al., 2009), respectively, and now, we report the virus on melon in the south-west regions (Khuzestan province). In addition, the Iranian isolates used in the phylogenetic analysis were clustered

together with different virus isolates from the Indian subcontinent. Therefore, we postulate that ToLCNDV has been introduced from the neighboring country, Pakistan, to Iran; the virus probably is spreading to the other geographical areas and might have more hosts in the country. This may signal the emergence of a serious new threat to agricultural production in the mid-Eurasian region. In this respect, it is necessary to study on the occurrence and incidence of the virus in different host plants and locations in Iran.

This study reports the natural occurrence of TYLCV in different host plants and ToLCNDV in melon in Iran. However, we found 13 samples with positive reactions in DAS-ELISA to TYLCV antibodies which did react neither with the virus-specific primers nor with the begomovirus universal primers in PCR, indicating a possible presence of new *Begomovirus* species or strains in the country, similar to the earlier reports (Fazeli et al., 2009). In this respect, it is necessary to study on ToLCNDV and other possible emerging begomoviruses to manage and control their potential epidemics.

Acknowledgments

The authors wish to thank the Science and Research Branch (Tehran) and Varamin-Pishva Branch (Varamin) of IAU for supporting this project. This work also was supported by grants from the Iranian Group for the Promotion of Science, IGPS, Nos. 91001002 and 92001001-4.

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