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Original Article

Complete Genomic Sequence of a Strain of *Tomato Yellow Leaf Curl Virus* from Iran

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Abstract

Background and Aims: *Tomato yellow leaf curl virus* (TYLCV) is one of the most destructive viruses of tomato that leads to reduced tomato yield up to 100% in tropical and subtropical regions. In this study, the complete sequence of TYLCV isolate from Hormozgan province, Iran and its recombination evsent was determined.

Methods: TYLCV infected tomato was collected from Hormozgan province. Total DNA was extracted from infected tomato and whiteflies and used as template for amplification by PCR. Analyses of sequences data were done by Clustal W method and GeneDoc software and then phylogenic tree and bootstrapping was prepared by the Maximum likelihood method by Clustal X with 100 replication. Amino acids analysis was carried out by neighbor joining method and RDP3 with 1000 replication.

Results: A 670 bp fragment was amplified by using the specific primer for TYLCV and was then sequenced. Based on the 670 bp sequence, new primers were designed to amplify and characterize the next part in TYLCV circular genomeA 670 bp fragment was amplified by using the specific primer for TYLCV and was then sequenced. Based on the 670 bp sequence, new primers were designed to amplify and characterize the next part in TYLCV circular genome.

Conclusion: Gene analysis showed that the genome includes six ORFs with 94% identity relatives which were most close to TYLCV-Kahnooj. It also showed that this isolate has an additional part of sequences in the *rep* gene that has not been found in the other strains of TYLCV.

Keywords: Identification; nucleotide sequence; Tomato disease; TYLCV

Introduction

Tomato yellow leaf curl disease (TYLCD) caused by *Tomato yellow leaf curl virus* (TYLCV) a species of the genus *Begomovirus* in the family *Geminiviridae,* is one of the most serious viral diseases of tomato (*Solanum lycopersicum* L.) crops worldwide (16, 23, 28). Symptoms of disease include severe stunting of plant growth, erect shoots, miss shaped leaflets, inward and upward leaf curling and subsequently, developing leaves are severely chlorotic and show curling of the leaflets (9). In infected tomato, there is considerable drop of flowers, fruit fails to set, and marketable fruits are reduced (1). The Begomovirus genus is the largest genus of this family that is transmitted by whitefly (*Bemisia tabaci* Genn.), and infected seeds of dicotyledonous plants. The Begomovirus genome consists of one or two (DNA-A and DNA-B) circular single-stranded

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DNA (ssDNA) molecules. Each ssDNA is approximately 2.5– 2.8 Kb (20). Recently geminiviruses were reclassified and TYLCV was divided into several viral species (12). The TYLCV is a complex consisting of distinct geminivirus species which are associated with TYLCD (21). First observation of TYLCD from Iran was reported by Hajimorad *et al* (1996) The complete nucleotide sequence of the Iranian strain of Tomato yellow leaf curl virus- Iran (TYLCV-Ir) was determined and deposited in GenBank (5). Moreover, other Iranian isolates of TYLCV were sequenced completely (13, 30). Symptom variation among Iranian isolates of TYLCV on the same tomato varieties (14) showed there were different strains of TYLCV in Iran. Eighteen Iranian begomovirus isolates were compared according to 200 nt of their CP and 600 nt of their Rep gene N-terminal. It was concluded that Iranian monopartite isolates and the bipartite isolates are the closest to TYLCV-Gezira and Tomato leaf curl New Dehli virus (ToLCNDV), respectively (13). Based on these results, it is concluded that begomoviruses have present in the southern and southeastern regions of Iran originated from both Mediterranean basin and the Indian subcontinent. In the present work we report the complete sequence of a TYLCV isolate from an important tomato growing region in Iran, Hormozgan proveince, and its recombination event and we have also investigated its phylogeny and taxonomic situation.

Methods

Virus isolate and transmission tests

Samples were collected from the infected tomato of fields of Bandar-Abbas region in Hormozgan province, Iran. Pathogenecity tests were carried out under greenhouse conditions at (24°C ±2 and natural light). The isolated virus was first transmitted by whiteflies to tomato, common bean (*Phaseolus vulgaris* var Derakhshan), and *N. rustica* plants and identified based on the symptom development on these plants, and then the amplification of a TYLCV DNA fragment was performed by PCR, using a pair of TYLCV specific primers, TYLCV-F and TYLCV-R (27).

To determine whether TYLCV-Ir2 isolate contained one or two partite genome, PCR reactions were performed using two specific DNA degenerate primers (PBL1v2040 and PCRc1) designed by Rojas *et al* 1993. In addition, DNA-A sequences from bipartite genome isolates were compared in phylogenic analysis.

Whitefly maintenance and plant inoculation

Whiteflies (*Bemisia tabaci*) biotype B were collected from greenhouse grown potato, Karaj, Iran. Whitefly biotype B colonies were established on cotton (*Gossypium hirsutum*) plants and then transferred, reared and maintained on cabbage (*Brassica oleracea*) plants inside insect-proof cages kept in greenhouse conditions at 25±2°C. After several generations, the new whitefly colonies were assessed by polymerase chain reaction (PCR) for TYLCV infections. These whiteflies were applied for transmission of the virus. TYLCV infection of plants and viruliferous whiteflies were assessed by PCR using TYLCV-F and TYLCV-R primers.

Viral DNA extraction

Total DNA was extracted from infected tomato and whiteflies according to Dellaporta *et al* (11) and Aljanabi *et al* (2), respectively and used as template for amplification by PCR. Leaf tissue (0.1 gr) was ground to a fine powder in liquid nitrogen and the homogenate was incubated in 600µl of extraction buffer (100 mM Tris-HCl pH: 8, 50 mM EDTA, 500mM NaCl, 10mM 2-β-mercaptoethanol, and 1% SDS) at 65°C for 10 min and then mixed with a half volume of Chloroform: Isoamyl alcohol (24:1). The mixture was centrifuged and the supernatant was transferred to another tube and DNA was precipitated by isopropanol. For whitefly DNA extraction, 20-30 whiteflies in 200µl of lysis buffer (10 mM Tris HCl [pH=8.0], 1 mM EDTA, 0.3% Triton X-100, 6 µgr/ml Proteinase-K) were homogenized. This suspension was incubated at 65°C for 15 min, then transferred on ice for 5 min and centrifuged.

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PCR amplification of viral DNA

TYLCV specific primers (TYLCV-F and TYLCV-R) amplifying a 670 bp fragment, were designed according to the conserved sequence of TYLCV-sar (EU143757), TYLCV-Is (DQ845787.1)(27). To continue the completion of sequence another primer pairs (TY2-F, TY2-R, e-F, e-R) was designed based on our sequence data and Oligo5 software (Table 1). TY2-F and TY2-R amplified 2310 bp fragment and e-F, e-R primers amplified 1317 bp fragment. For investigation of DNA genome being one or two partite, we used a pair of degenerate primers (PBL1v2040 and PCRc1), specific for DNA-B, that amplified 600 bp fragment (29)(Table 1). The optimized PCR amplification was performed in a 25 µl reaction volume containing one unit of *Taq* DNA polymerase (Fermentas, Lithuania), 50 ng of plant DNA, 2.5µl of 10x PCR buffer (500 mM KCl, Tris-HCl pH 8.4), 1.5 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 25 µmole of each primer and deionized H_2O up to 25 µl. The mixture was incubated at 94°C for 4 min, followed by 25 amplification cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min and final extension was at 72°C for 10 min. PCR reaction was carried out on thermocycler machine (Mastercycler ep Gradient) supplied by Eppendorff (Germany). PCR products were fractionated and assessed on 1.5% TAE agarose gels. PCR products were purified by a kit (Fermentas, Lithuania) and then sequenced by Gene service company (England). The 670 bp fragment was amplified by primers (TYLCV-F and TYLCV-R).

Data analysis

Analyses of sequences data first was carried out by blasting search in NCBI and then sequence alignment and percent identities of DNA were done by Clustal W method and GeneDoc software and then phylogenic tree and bootstrapping was prepared by the Maximum likelihood method by Clustal X with 100 replication. Amino acids analysis was carried out by neighbor joining method and RDP3 with 1000 replication. For analysis of amino acids we chose Iranian isolates (EU085423, EU635776, FJ355946, AJ132711) and closest strains of TYLCV with TYLCV-Ir2 (AY044138 and EF054894). Recombination analysis was done with GENECONV and 3Seq methods by RDP3 software (17). Tomato leaf curl Sudan virus (TLCSV)-Gezira strain (NC_005855) was used as an out-group.

Results

Tomato plants showing TYLCV like symptoms such as leaf curling, yellowing and stunting were collected from a field in Hormozgan province, southern Iran. A 670 bp DNA was amplified in collected tomato plants by PCR using TYLCV-F and TYLCV-R (Fig 1). Three PCR products fragments (670 bp, 2310 bp, and 1317 bp) were obtained using three pairs of specific primers (Table 1). The isolate was named TYLCV-Ir2 and its nucleotide sequence was registered in NCBI

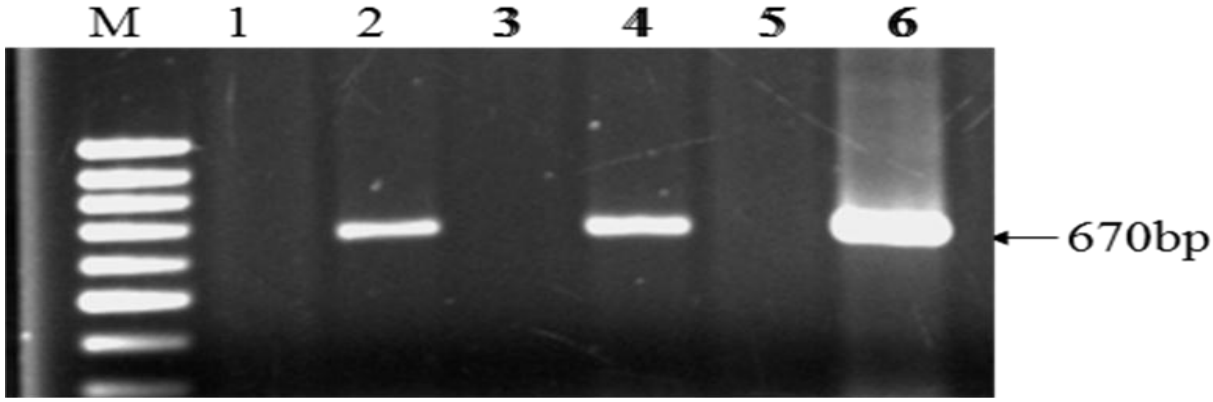

Fig. 1. PCR Detection of TYLCV-Ir2 specific 670bp band by using TYLCV-F and TYLCV-R:
M) Marker (GenRuler^{TM} 100bp DNA Ladder, Fermentase) Healthy plant 2) Tomato infected by *Bemisia tabaci* 3) nonviruliferous *Bemisia tabaci* 4) viruliferous *Bemisia tabaci* 5) Cauliflower, host of *Bemisia . tabaci* 6) tomato plants infected in field (original source, Bandar-Abbas).

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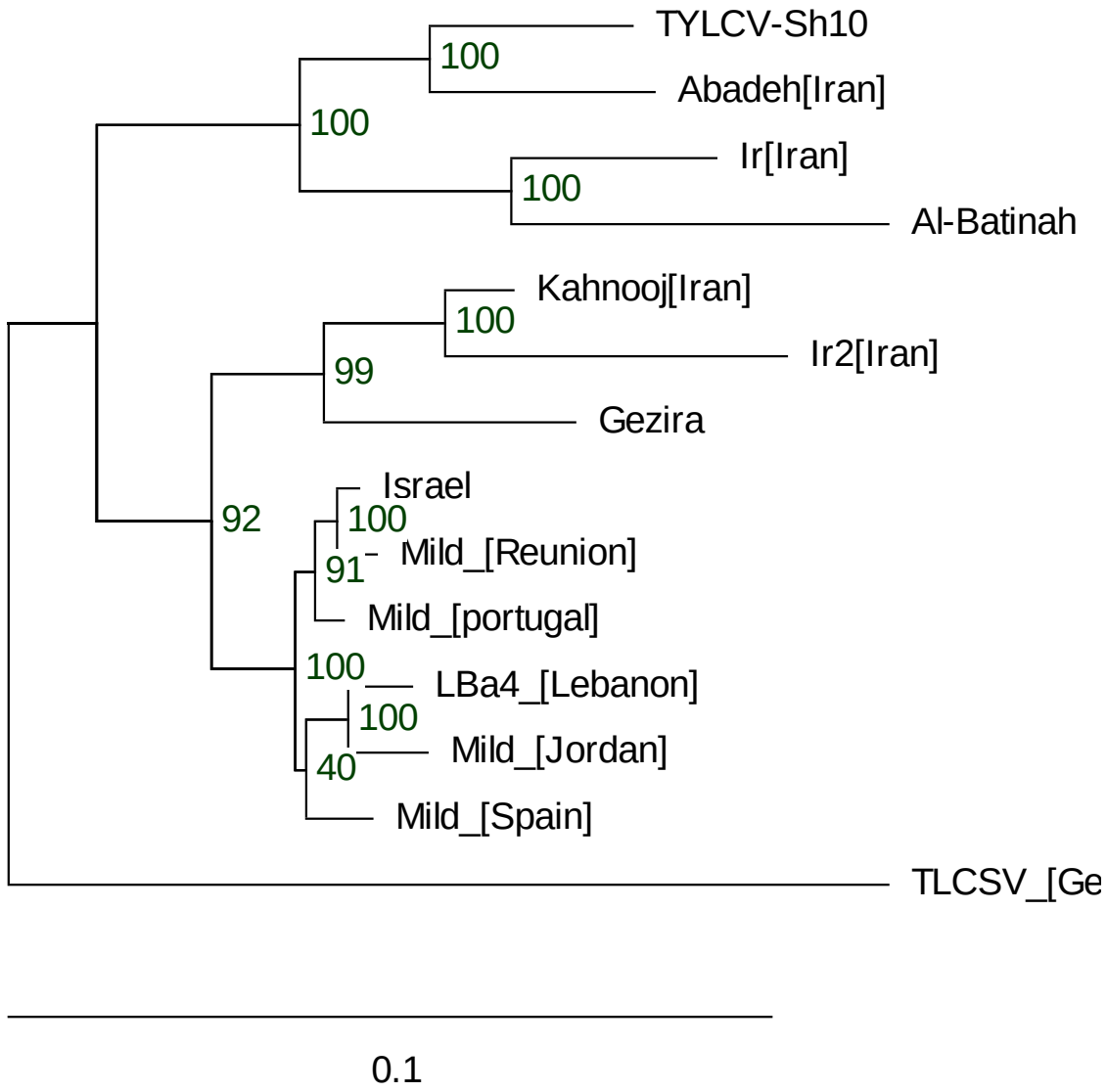

Fig. 2. Phylogenetic relationship analysis by the Maximum likelihood method based on complete nucleotide sequences of TYLCV. The numbers appearing at each node indicate the frequency of the cluster after bootstrap analysis. The scale bar shows the number of nucleotide substitutions in the aligned sequence. Virus abbreviations and GenBank accession numbers are as follows: Mild isolate Reunion (AJ865337), Mild[Portugal] (AF105975), Gezira (AY044138*)*,Iran, Abadeh (FJ355946), Iran, Ir (AJ132711), Iran, Kahnooj (EU635776), Mild [Spain] (AJ519441), Ir2 (EU085423), Mild, Jordan (EF054894), LBa4, Lebanon (EF185318), Al-Batina (DQ644565), Mild[Israil] (X76319), TYLCV_Sh10 (EU031444), TLCSV-Gezira (NC_005855).

under the accession NO. EU085423. A fragment related to DNA-B was not amplified by PBL1v2040 and PCRc1, showing that TYLCV-Ir2 has one DNA genome. All PCR products were sequenced with four replications at least.

The complete nucleotide sequence of the TYLCV-Ir2 isolate showed that the sequences consisted of 2776 nt, and was submitted to NCBI Genebank database under the accession number of EU085423. Analysis of the TYLCV-Ir2 sequence confirmed that the genome organization was typical single partite genome of begomoviruses with six ORFs whose positions of ORFs were determined (Table 2). In viral sense two ORFs (V1 and V2) were partially overlapped. In complementary sense, three of the four ORFs, (Cl, C2 and C3) are partially overlap and ORF C4 was entirely overlapped by ORF C1. The Genome sequence of TYLCV-Ir2 was closely

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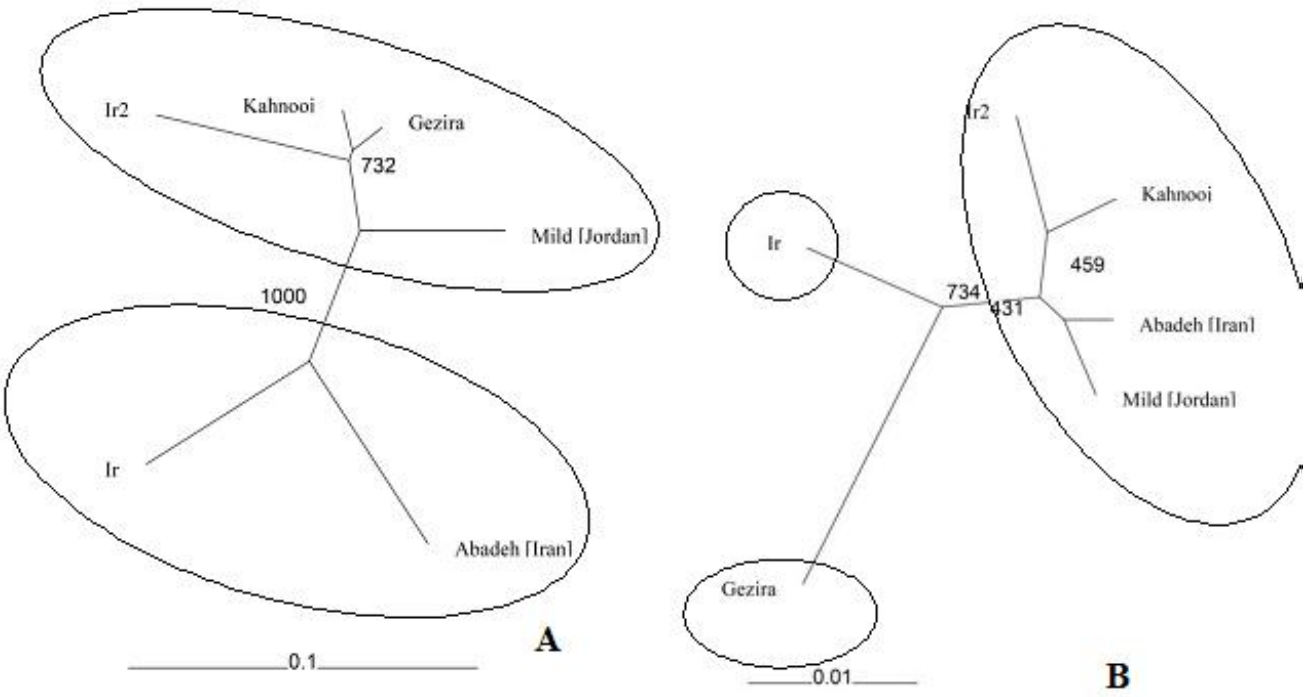

Fig. 3. Amino acids Maximum Niberjoining method Phylogram (Constracted by Clustal X, supported by bootstrapping 1000 replicate) A) C1 amino acids phylogram, B) V1 amino acids phylogram. Virus abbreviations and GenBank accession numbers are as follows: Gezira (AY044138), Iran, Abadeh (FJ355946), Iran, Ir (AJ132711), Iran, Kahnooj (EU635776), Jordan (EF054894), Ir2 (EU085423).

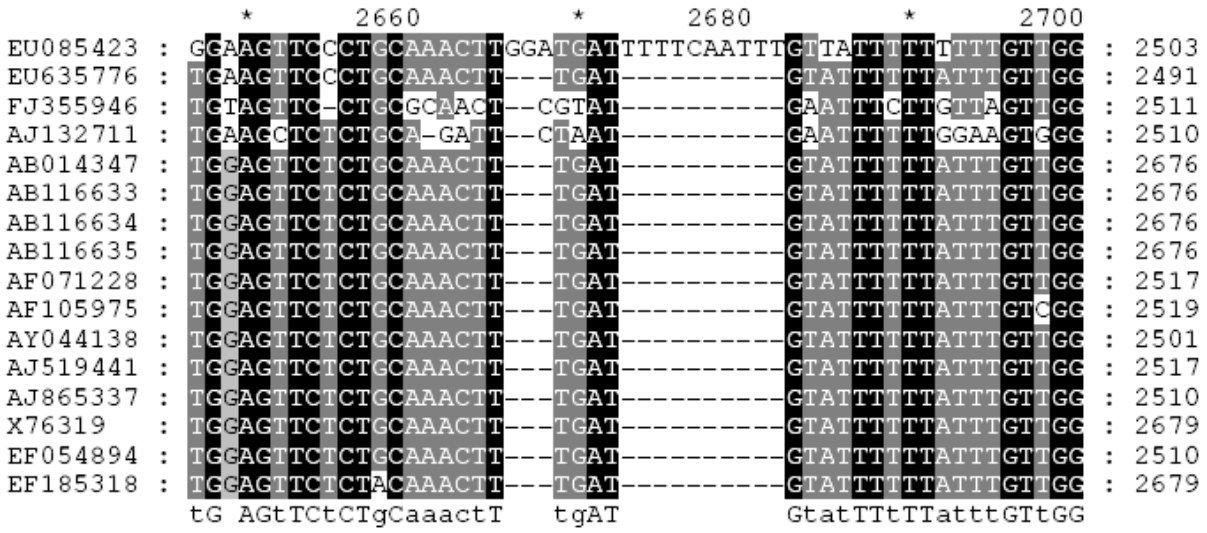

Fig. 4. The nucleotide alignment of TYLCV showing two insertion parts in TYLCV-Ir2 that located in C1 ORF.

Fig. 5. Blasting search of the additional part in C1: nucleotide blasting shown identity with a part of tomato genome (AK326491) for 21 nt.

related to TYLCV-Kahnooj (EU635776) by

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Fig. 6. Location of recombination breakpoints detected in the C1 region.

Table 1. Sequences of the four pairs of primers used for PCR in this study.

Name	Sequence	Size of amplified fragment (bp)	Ref.
TYLCV-F	5'- CGC CCG TCT CGA AGG TTC-3'	670	Pico *et al*., 1999
TYLCV-R	5'- GCC ATA TAC AAT AAC AAG GC-3'		
TY2-F	5'- GGA ACA GGC ATT AGT TAA GAG-3'	2310	This study
TY2-R	5'- TGT TTA TGC CTT GGA CAA TGG -3'		
e-F	5'- GGA CAA CTA ACC AAT TAT CTA AC-3'	1317	This study
e-R	5'- GTC TTC GCT ATG CGG TGT TGG -3'		
PBL1v 2040	5´-GCC TCT GCA GCA RTG RTC KAT CTT CAT ACA-3´	600	Rojas *et al*., 1993
PCRc1	5´-CTA GCT GCA GCA TAT TTA CRA RWA TGC CA-3´ (B=C, G K=G, T R=A, G W=A, T Y=C, T)		

94.6% identity (Table 3). This result based on ICTV showed that TYLCV-Ir2 was a new strain of TYLCV. The amino acid sequences of the TYLCV-Ir2 ORFs was analyzed (Table 2 and 3) and results showed that V1 of TYLCV-Ir2 was most closely related to TYLCV isolates Kahnooj (EU635776) and Abadeh (FJ355946) (98.8% identity), Reunion (AJ865337) (98.4%) (Fig 3 and Table 3). However, the amino acid sequences of the V2 more relevant to Tomato yellow leaf curl virus Isolates Kahnooj (EU 635776), Mild (Israel) (X76319) and Mild [Spain] (AJ519441) (97.4%), C1 amino acid showed 90.8% milarity to the isolates TYLCV-Kahnooj (EU635776) and TYLCV-Geizira (AY044138) (Table 3 and Fig 3). C2 amino acid sequence was more closely related to isolates Geizira (AY044138), Mild [Japan:Kisozaki] (AB116634), Mild[Aichi] (AB014347), AB116633 (Japan[Atumi]), Mild[Portugal] (AF105975), and Mild [Japan:Daito] (AB116635), C3 showed 87.3% similarity with Tomato leaf curl virus Kahnooj (EU635776). C4 was related to TYLCV-Kahnooj (96%) and Geizira (AY044138) (90%) (Table3). In order to analyze the phylogenetic relationships among TYLCV-Ir2and other isolates of TYLCV, the relationship between TYLCV-Ir2 and the complete nt sequences of 13 closely related TYLCV isolates from NCBI were compared. As a result, in nucleotide phylogenic relationship analysis (Fig 2) TYLCV-Ir2, TYLCV-Kahnooj (EU635776), and TYLCV-Gezira (AY044138) were placed in a closely related cluster. Other Iranian Isolates like TYLCV-Abadeh (Fj355946) and TYLCV- Iran (AJ132711) were introduced into separated cluster.

Results from multiple nucleotide and amino acids analysis showed that TYLCV-Ir2 had an inserted site in 5' terminal nucleotide of *Rep* gene (C1) that is not found in other strains (Fig 4). These additional nucleotide sequences were located in two parts close to each other, one with three nucleotides and another with 10 nucleotides that were rich in T (Fig 4). Blasting analysis in NCBI showed that 21 nucleotides are common in this part that covered both insertion sites completely the same as part of *Solanoum lycopersicom* (important host of TYLCV) cDNA HTC (AK326491) in fruit that was sequenced by Aoki *et al* (3) (Fig 5).

In addition, another recombination was found at 3' end of C1 that is very different from the other TYLCV strains, also TYLCV Ir2 gets to stop codon sooner than other strains and has

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Table 1. Open reading frames (ORFs) and positions of Iranian and some other countries Strains.

ORF		Ir2	Ir	Abadeh	Kahnooj	Mild[Jordan]	**Mild [Spain]**
V1	Position	289-1065	308-1084	308- 1084	289-1065	308-1084	315-1091
	Number of amino acids	258	258	258	258	258	258
V2	Position	129- 479	148-498	148- 498	129-479	148-498	155-505
	Number of amino acids	116	116	116	116	116	116
C1	Position	1538- 2614	1542-2621	1543- 2616	1523-2602	1548-2621	1549-2628
	Number of amino acids	358	359	357	359	357	359
C2	Position	1207- 1614	1226-1633	1227- 1634	1207-1614	1226-1633	1233-1640
	Number of amino acids	135	135	135	135	135	135
C3	Position	1062- 1466	1081-1485	1103- 1486	1062-1466	1081-1485	1088-1492
	Number of amino acids	134	134	127	134	134	134
C4	Position	2143- 2445	2171-2470	2172- 2471	2143-2445	2162-2464	2169-2471
	Number of amino acids	100	99	99	100	100	100

five amino acids less than the others. Blasting search showed that a 35 nt was common among several Iranian isolates which are recently sequenced (GU076442, GU076452, GU076451) and also in some isolates from Oman (DQ644565, FJ956705, FJ956704). Recombination analysis by RDP3 software (17) showed that TYLCV-Ir2 was a recombinant strain, that its 5' part of C1 ORF from nt 1538- 1573 is identical to the same ORF in TLCSV-Gezira and it has also two unknown parts at its 3' end (Fig 6). The alignment of Rep amino acids showed that it was very variable gene and that TYLCV-Ir2 was more different to other strains from 5' and 3' part of Rep.

Discussion

TYLCV has been reported as an important virus in Mediterranean region and recently in Iran. In this study the isolate of TYLCV from Hormozgan province was sequenced and taxonomic position of this isolate was analyzed. The 670 bp fragment from PCR amplification was sequenced and then other primers based on this sequence were designed until this isolate was completely sequenced. Our results showed that TYLCV-Ir2 isolate was different to the other TYLCV isolates that were completely sequenced and whose sequences are available in NCBI, suggesting that TYLCV is a diverse virus in Iran. TYLCV-Ir2 complete sequence showed it was close to Kahnooj isolate than TYLCV-Ir and Abadeh isolates (Table 3).

In the analysis of ORFs, we found that C4 has more diversity compared to other ORFs, while the recombination was not found in V1 (Table 3). The CP of Geminiviruses play an important role in the transmission specificity by the insect vector (6) and the essential role of amino acids of the TYLCsV-CP for transmission by *B. tabaci* were mapped (24). However, the result of alignment from this study showed conservation in CP and strongly supported the important role of CP for TYLCV as mentioned by others (6, 21, 25). Amino acid analysis of C1 showed that TYLCV-Ir2 was located in one

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Table 3. Percentage nucleotide (complete genome) and amino acid (ORFs) identities for TYLCV-Ir2 with the most closely isolates from NCBI.

Strains	**Complete Genome**	**V1**	**V2**	**C1**	**C2**	**C3**	C4
Iran [Ir]	86.3	96.9	96.6	81.6	80	85.1	42.4
Iran [Kahnooj]	94.6	98.8	97.4	90.8	80	87.3	96
Gezira	92	96.5	95.7	90.8	80.7	82.8	90
Japan [Kisozaki]	86.3	97.7	96.6	87.4	80.7	85.8	74
Mild [Aichi]	86.3	97.7	96.6	87.4	80.7	85.8	74
Japan [Atumi]	86.1	97.3	96.6	87.2	80.7	85.8	74
Mild [Reunion]	91.1	98.4	96.6	87.4	79.3	84.3	74
Mild[Spain7297]	90.7	97.7	95.7	87.7	80	85.8	74
Mild [Spain]	90.9	98.1	97.4	86.6	78.5	85.8	75
Iran [Abadeh]	86.7	98.8	96.6	80.4	79.3	76.4	36.4
TYLCVV	76.6	79	74.1	79.1	60.7	69.4	64.6
TLCV[Ir]	70.8	74.2	74.8	74.6	61.2	67.2	41.4
Mild[Portugal]	91.2	98.1	96.6	87.2	80.7	85.8	73
Mild [Jordan]	90.6	98.1	95.7	86	79.3	84.3	73
Mild [Is]	86.2	98.8	97.4	87.4	79.3	85.1	73
Japan[Daito]	86.2	97.7	96.6	87.2	80.7	85.8	74
LBa4 [Lebanon]	85.6	98.1	96.6	86.9	80	85.8	73.4

cluster with Gezira, Kahnooj and Mild (Jordan) strains. Our analysis showed that TYLCV is more variable in the nucleotide sequence C1 and intergenic region compare to its V1 and V2 (8, 24).

To study the number of the TYLCV-Ir2 partite genome, PCR was carried out using degenerate primers (PBL1v2040 and PCRc1), specific for DNA-B. In addition, the comparison of TYLCV-Ir2 genome with other TYLCV genomes available in NCBI, showed that TYLCV-Ir2 was closer to TYLCV one partite genome.

Our results presented here showed that the *rep* gene was very variable and more recombinant acceptable from this part and the new recombination from TYLCV-Ir2 was found at this part. This result is in agreement with the work of Harrison and Robinson (15) that suggests the rep- intergenic region of the type and Mild strains of TYLCV were acquired from differential parental viruses that could not be identified at that time.

In this study, an additional nucleotide from 5' *Rep* was found that is identical with a part of tomato (*S. lycopersicom*) genome (AK326491) which might have originated from tomato host plant and recombined with the virus genome. Recombination between members of the family *Geminiviridae* is common (26). In this group of viruses, recombinations contribute greatly to the genetic diversification of the viral populations (18). Begomoviruses recombinations play a strong role for the ecological fitness of these viruses and their rapid adaptation to changing environmental conditions (18). During mix infections, recombination and reassortment of segments between viruses are common and these genetic exchanges help them to quickly evolve (10, 22). Recombination in viruses is recognized as a frequent phenomenon in nature and it plays a key role in the virus evolution (4, 7, 19). Our results suggest that TYLCV-Ir2 and Kahnooj isolates are originated from same isolates and they are closely related to TYLCV Gezira.

Fazeli *et al* (13) reported that one partite Iranian begomoviruses had originated from Mediterranean basin and bipartite of these viruses originated from Indian isolates but our results strongly suggest that TYLCV-Ir2 is distinct from neighbor countries strains of Iran. However the nucleotide sequence and phylogenetic relationship analyses of the virus indicated that TYLCV in Iran has existed for a long time or had been native in the area for a long time span so that could acquire a lot of

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diversity. Fazeli *et al* (13) have reported some natural infection of weeds by TYLCV in Iran. These results support the theory that TYLCV might have been native in Iran. We are not in agreement with Fazeli *et al* (13) who reported that Iranian isolate originated from Mediterranean and Indian Isolates. Finally we suggest that for introducing resistant cultivars to TYLCV, we should further investigate the nature of the diversity of TYLCV isolates in Iran and recombination possibilities among them.

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