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Original Contribution

EMERGING TWO DISTINCT GROUPS OF THE *TOMATO YELLOW LEAF CURL VIRUS*- SEVERE STRAIN (TYLCV-IL) VARIANTS IN IRAN

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ABSTRACT

Since Iran is more likely close to the global center of *Tomato Yellow leaf curl virus* (TYLCV) current genomic diversity and its ongoing intensive evolutionary dynamics, the presence of five TYLCV stains including the most notorious one, TYLCV-IL, inside country epidemiologically requires surveillance of the genetic variation inside the Iranian TYLCV-IL populations. Five new full length genomes of TYLCV-IL were isolated from tomato plants in Bojnurd, North Khorasan, Iran. This is the first report of the TYLCV occurrence in North Khorasan, Iran. These complete genomes together with the all publicly available isolates previously reported from Iran including seven full genome sequences of the virus strain were introduced into the phylogenetic analysis. Percent nucleotide sequence identity of aligned full genome plus non-coding and coding regions was pairwisely compared. Full length genome of all isolates shared 97.3-99.9% identity with each other with the exception of TYLCV-IL[IR:Sh:40:07]; GU076444, originated from Shiraz, Iran, which shared the lowest full genome percentage identity of 94.8-95.1% with the rest of Iranian isolates. Reversely, this isolate showed the maximum percent complete sequence identity of 96.7% with TYLCV-IL [Israel:rehovot]; X15656. Therefore, it could be considered as an evolutionary bridge between the TYLCV-IL isolates of Meditranean basin and those of Iran. Full length genomes of the Iranian isolates showed maximum percentage identity of 94.6-96.7% with both TYLCV-IL [Israel:rehovot] and TYLCV-IL [Almeria]; AJ489258. Phylogenetic analysis based on the complete genome of Iranian TYLCV-IL isolates either revealed two distinct and divergent clusters of TYLCV-IL variants, denominated as group A and B or fully differentiated them based on their geographical origins. In our analysis, group A and B of variants which are first-time reported here represent two distinct groups of emerging TYLCV-IL variants in Iran. Phylogenetic tree depicted from the sequence alignment of the intergenic region (IR) of nineteen Iranian TYLCV-IL genomes showed five divergent clusters of the variants grouped 1-5 irrespective of their geographical origin.

Key words: Iran, Phylogenetic analysis, TYLCV-IL variants

INTRODUCTION

Tomato yellow leaf curl disease (TYLCD) is one of the most destructive tomato (*Solanum lycopersicum* L.) diseases in the tropical, subtropical and temperate region worldwide (1, 2). This disease is caused by the type species of *Tomato yellow leaf curl virus* (TYLCV), TYLCV-Is, plus a complex of 10 other accepted species and their related strains which are denominated as TYLCV-like viruses (TYLCVs) (3). These viruses taxonomically belong to the genus of *Begomovirus* in the family of *Gimini viridae* and their nomenclatures are according to the demarcation criteria of International Committee on Taxonomy of Viruses (ICTV) (4). They are naturally transferred by the whitefly, *Bemesia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), biotypes particularly the biotype B in a circulative persistent manner and possess a monopartite genome about 2.8 kb (except for *Tomato yellow leaf curl Thailand virus* and *Tomato yellow leaf curl Kanchanaburi virus* which have bipartite genomes) of the circular single strand DNA encapsidated inside a twinned isometric (quasi-icosahedral) particles (ca. 18×30 nm), infecting the dicotyledonous plants including tomato, pepper, bean, cucurbits and tobacco crops worldwide (1, 3). Their genome consist of the

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six partially overlapping open reading frames (ORFs) organized bidirectionally on the viral and complementary sense strands and separated by a non-coding sequence of about 300 nucleotides that is located between the C1 and V2/V1 ORFs (intergenic region; IR) and contains a conserved stem–loop structure which in turn bears the nonanucleotide sequence of the loop, i.e. TAATATTAC that is conserved across all geminiviruses , functions as a portion of the origin of DNA replication (*ori)* sequence and is nicked by the replication-associated protein (Rep) between the thymine and adenine residues(TAATATT/AC) (5-8). The arrangement of the ORFs represents two genes on the viral sense strand including V1 and V2 which encode the coat protein (CP) and a movement like protein.

The four ORFs of the complementary sense strand are C1, C2, C3 and a small C4 embedded within C1, encoding for a replication-associated protein (Rep), a transcription activator protein (TrAP), a replication enhancer protein (REn) and a small C4 protein, respectively (7, 9, 10). Historically, the severe incidence of tomato yellow leaf curl disease was recorded in late 1920s in Jordan Valley, Israel and subsequently tomato yellow leaf curl virus-like viruses were first described in the region in early 1960s (11, 12). In early 1990s, two begomovirus strains with the different severities associated with the TYLCD were cloned, sequenced and denominated as TYLCV-IL (TYLCV-IL [IL: Reo: 86]-X15656) (severe strain) and TYLCV-Mld (TYLCV-Mld[IL:93]– X76319) (mild strain) in Israel (6, 12). Later on it was known that the severe strain, TYLCV-IL, was a strain-species recombinant resulting from the recombination of the mild strain, TYLCV-Mld, as a recipient with another begomovirus species closely related to *Tomato leaf curl Karnataka virus* (ToLCVKV) as a donor. The large portion of TYLCV-IL genome originated from TYLCV-Mld but the 5◌ part of its Rep gene shows high similarity to that of ToLCKV (13).

To date, overall seven TYLCV strains which mostly arisen through interspecies recombination have been identified including TYLCV-IL from both Israel and Iran, TYLCV-Mld from Israel, TYLCV-IR, TYLCV-Bou and TYLCV-Ker from Iran, TYLCV-Gez from Sudan and TYLCV-OM from both Iran and Oman (14-17). Hence, Iran has the greatest number of diverse molecularly characterized TYLCV stains, i.e. five strains (17). TYLCV-IL and TYLCV-Mld, however, have the widest geographical distribution ranging from the Old World to the New World (18-21). Nevertheless, TYLCV-IL either possesses the broadest geographical distribution amongst all geminiviruses or causes the most intensive tomato disease (17, 22). This stain has introduced twice into the New World in the early 1990s and 2000s from the Mediterranean basin and Asia, respectively (23, 24) and is spreading towards North and South Americas (25, 26).

Presence of the world most devastating strain of TYLCV ,TYLCV-IL, in Iran has been proven by the numerous works done on the Molecular characterization of TYLCV-IL isolates (17, 27, 28) **(Table 1)** in different part of country and depicts an inflow rout of the virus variants from the south to the north-eastern parts **(Figure 1)**. Since the regions around Iran is considered to be the center of recent TYLCV diversity and its ongoing intensive evolution, the presence of five TYLCV stains including the most notorious stain, TYLCV-IL, inside country suggests that Iran is more likely close to the global center of TYLCV diversity (17). It seems that TYLCV-IL variants available in the Iran geography might pose a great threat and a large limitation to the production of tomato and other host crop in this country and the surrounding neighbors. Therefore, here for the first time we undertook this comprehensive genomic analyzing study to reveal the phylogenetic relatedness of Iranian TYLCV-IL isolates in different parts of country from south to north and to differentiate and recognize the potential new variants. In our analysis we have used five new full genomes of TYLCV-IL isolated from North Khorasan province, Bojnurd, Iran along with the to-date six publicly available full genomes of Iranian TYLCV-IL isolates from southern parts (Fars and Kerman provinces) of country plus one isolate from Neyshabur, Razavi Khorasan province **(Table 1).** The sequences of the non-coding intergenic region (IR) of seven other isolates have been included in our analysis as well (27) **(Table 1)**.

Table 1. *Publicly available Iranian TYLCV-IL sequences in GenBank; their GenBank accession numbers, origins, genome sizes and published year*

Virus Isolates	Original host	Geographical origin (province)	Year	GenBank accession number	Genomic region*/Size (nt)
TYLCV-IL[IR:Boj:A1]	Tomato	North Khorasan	2012	KC106648	Full genome/2781
TYLCV-IL[IR:Boj:A2]	Tomato	North Khorasan	2012	KC106649	Full genome/2781
TYLCV-IL[IR:BoJ:C3]	Tomato	North Khorasan	2012	KC106650	Full genome/2779
TYLCV-IL[IR:BoJ:C5]	Tomato	North Khorasan	2012	KC106651	Full genome/2779
TYLCV-IL[IR:BoJ:C6]	Tomato	North Khorasan	2012	KC106652	Full genome/2779
TYLCV-IL[IR:Sh47:07]	Tomato	Fars	2009	GU076447	Full genome/2781
TYLCV-IL[IR:Or28:07]	Tomato	Kerman	2009	GU076445	Full genome/2781
TYLCV-IL[IR:Sh46:07]	Tomato	Fars	2009	GU076446	Full genome/2781
TYLCV-IL[IR:Sh40:07]	Tomato	Fars	2009	GU076444	Full genome/2781
TYLCV-IL[IR:Ta30:06]	Tomato	Kerman	2009	GU076440	Full genome/2781
TYLCV-IL[IR:Abadeh]	Tomato	Fars	2008	FJ355946	Full genome/2782
TYLCV-IL[IR: Neyshabur]	Tomato	Razavi Khorasan	2012	JQ414025	Full genome/2780
TY-Baghan	Tomato	Boushehr	2006	DQ855468;EF197895	CP; C1-IR-V2/558,643
TY-Mashad	Tomato	Razavi Khorasan	2006	DQ855469;EF197896	CP; C1-IR-V2/558,643
TY- Dargaz	Tomato	Razavi Khorasan	2006	DQ855470;EF197897	CP; C1-IR-V2/558,643
TY- Ashkzar	Tomato	Yazd	2006	DQ855471;EF197898	CP; C1-IR-V2/558,643
TY- Sarkhan	Tomato	Hormozgan	2006	DQ855472;EF197899	CP; C1-IR-V2/558,643
TY- Delijan	Tomato	Markazi	2006	EF199813;EF197901	CP; C1-IR-V2/558,643
TY- Behbahan	Tomato	Khozestan	2006	EF199814;EF197902	CP; C1-IR-V2/558,643

* Coat protein (CP) (558 nt partial sequences); Replication- associated protein gene (C1); Intergenic region (IR); Movement protein (V2)

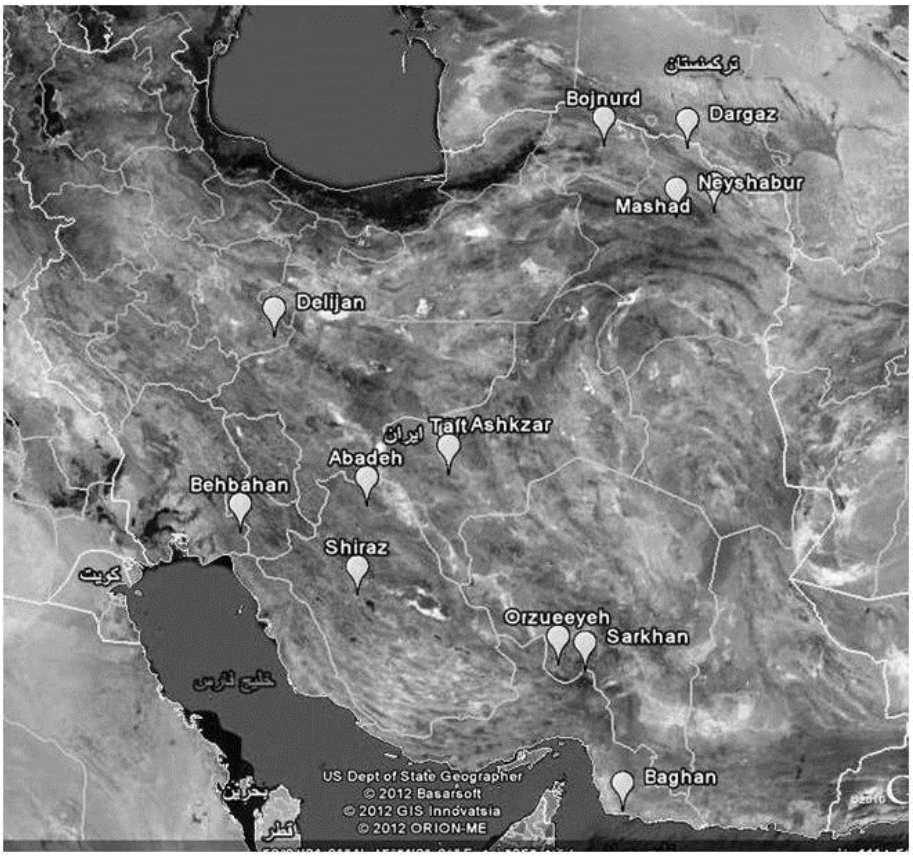

Figure 1. Distribution map of Iranian isolates of TYLCV-IL strain; balloons indicate points for which the virus sequences are available in GenBank

MATERIAL AND METHODS

-Viral sources and detection

Leaf samples from three symptomatic tomato plants (A, B and C) displaying typical TYLCD symptoms (leaf curling, discoloration, distortion and stunting) were collected in a tomato production greenhouse (GPS coordinates: 37°26'36.08"N 57°18'53.97"E**)** in North Khorasan province, Bojnurd,Iran in 2010. Leaves from asymptomatic tomato plants (H1and H2) were collected to serve as negative healthy controls in our lab experiments as well. Total DNA was extracted from the both fresh and frozen leave tissues according to the modified CTAB method (29, 30).

Begomoviral DNA was detected in the infected leaves by polymerase chain reaction (PCR) using the universal primer pair AVcore and ACcore (Unpublished) which amplify a 576 nucleotide fragment corresponding to the core region of coat protein gene of almost all begomoviruses. Subsequently, the TYLCV DNA was tracked in the infected samples by using the primer pair TYC1F (5ó-GGG/CCT/AGA/GAC/CTG/GCC/CAC-3ó, nucleotides 2087–2107) and TYC1R (5ó-CCG/GTA/ATA/TTA/TAC/GGA/TGG/C-3ó, nucleotides 171–150), amplifying a 856 nucleotides fragment corresponding to the 5ó half of the C1 gene of TYLCV(31). Total DNA extracted from an infected tomato with TYLCV-Ir2 used as positive control in the PCR detections. The possible presence of DNA-B and DNA- β in the sampled leaves were tested by using the primer pairs PBL1v2040/PCRc1 (32), and Beta01/Beta02 (33).

-Rolling circle amplification (RCA) of total DNA

The circular viral genomes were amplified from the total DNA of infected plant leaves using phi29 DNA polymerase (34) (TempliPhi Amplification kit, GE Healthcare, USA) as follows: first 1 µl of the leaf total DNA extraction was added to the 5µl of the sample buffer, then heated to 95 ◦C for 3min to denature the DNA, chilled on ice, and finally mixed with 5µl of reaction buffer plus 0.2µl of enzyme mix (containing Phi29 DNA polymerase and random hexamers in 50%glycerol). The reaction mixtures were incubated for 22h at 30 ◦C, followed by inactivation of the enzyme at 65 ◦C for 10 min. At the end, the RCA product quality and an estimated quantity were analyzed by agarose gel electrophoresis.

-Isolation, cloning and sequencing of viral full length genomes

The total DNA extractions of infected samples were subjected to RCA in order to amplify concatemers of viral circular genomes. Subsequently, the amplified concatemers of viral genomes were digested with *SphI* to yield linearised monomers of full length genomes. The linearised DNA monomers were sticky-end ligated to the Calf Intestine Alkaline Phosphatase (CIAP) treated/*SphI* digested pGEM 3ZF+ (Promega, USA). Heat shock transformation of the monomer-inserted plasmids into the E.coli DH5α was performed. Positive bacterial clones were selected through the blue and white screening and their plasmids were purified using the Miniprep kit (QIAGEN, USA). Finally the viral full length genomes (A1, A2, C3, C5 and C6) originated from infected tomato plants A and C were commercially sequenced on both strands by the primer walking at BIONEER, South Korea.

-Viral sequence analysis and phylogenetic reconstruction

In adition to the five new and fully edited sequences of the viral full length genome isolated from Bojnurd, Iran, the complete and partial sequences (each seven sequences) of Iranian TYLCV-IL isolates used for comparison were retrieved from GenBank (http://www.ncbi.nlm.nih.gov) **(Table 1).** The sequences of Iranian isolates were aligned at the full length genome(12 sequences), intergenic region(IR) (19 sequences), and six ORFs(each 12 sequences) level with the nine selected begomoviruses sequences available in GenBnak **(Table 2)** using CLUSTALW implemented with MEGA5 (35) software. The sequence comparisons and pairwise sequence identities were calculated for each alignment by BioEdit (36). Maximum likelihood phylogenetic tree of full length genomes were reconstructed using MEGA5 with the selected best-fit evolutionary model, T92, and 1000 bootstrap iterations option. The GenBank-retrieved partial sequences of coat protein gene (V1) of seven Iranian TYLCV-IL isolates were excluded from our sequence data analysis.

Table 2. *Selected begomoviral full genomes retrieved from GenBank; their accession numbers, acronyms and sizes*

Virus Isolates	Acronym	GenBank accession no.	Genome size (nt)
Tomato yellow leaf curl virus-Iran2	TYLCV-Ir2	EU085423	2776
Tomato yellow leaf curl virus-Oman	TYLCV-OM	DQ644565	2765
Tomato yellow leaf curl virus-Iran	TYLCV-IR	AJ132711	2771
Tomato yellow leaf curl virus-Mild [Israel]	TYLCV-Mld	X76319	2790
Tomato yellow leaf curl virus-Israel [Israel:rehovot]	TYLCV-IL	X15656	2787
Tomato yellow leaf curl virus-Israel[Spain:Almeria]	TYLCV-IL[Almeria]	AJ489258	2781
Tomato yellow leaf curl virus-Gezira[Sudan]	TYLCV-Gez	AY044138	2780
Tomato yellow leaf curl Sardinia virus[Spain]	TYLCSV	X61153	2773
Tomato leaf curl Iran virus[Iran]	TLCIV	NC005842	2763

RESULTS

The completely sequenced genomes of the five PCR-verified isolates **(Figure 2)** of Iranian TYLCV-IL originated from Bojnurd, North-eastern Iran were determined and deposited in Genebank. PCR with with primer pairs PBL1v2040/PCRc1 and Beta01/Beta02 that are able to detect DNA-B and DNA-β showed no amplification, hence this indicates the monopartite nature of virus (es) in the the infected tomatoes.

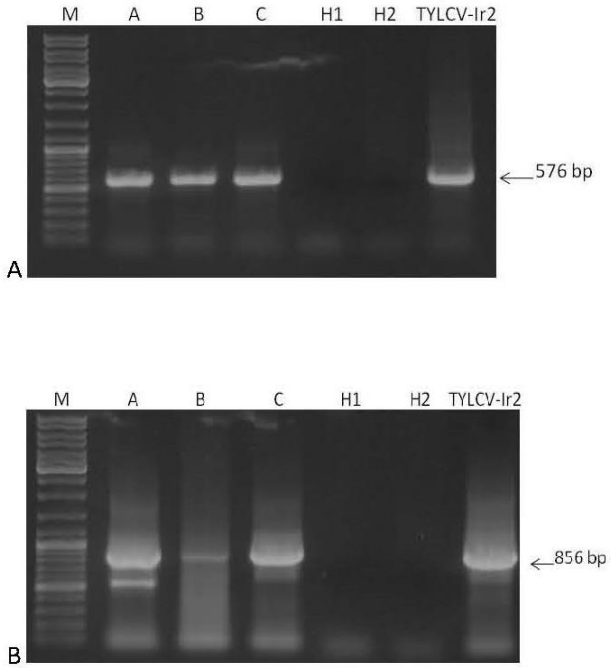

Figure 2. Agarose gel electrophoresis of PCR-amplified products of the total DNA extracted from the leaf tissue of the sampled tomato plants, using primers AVcore, ACcore (A) and primers TYC1F, TYC1R (B). M, 1kb molecular size marker (GeneRuler DNA Ladder Mix, ready-to-use, Fermentas); A,B and C indicate symptomatic tomato plants sampled from the tomato production greenhouse in Bojnurd (37°26'36.08"N 57°18'53.97"E); H1 and H2, healthy tomato plants used as negative control; TYLCV-IR2, total DNA extracted from an infected tomato with TYLCV-Ir2 used as positive control.

Pairwise comparisons of percent sequence identity performed from the alignment of these new sequences with both the Iranian closest relatives (seven isolates) of BLASTn results in GenBank **(Table 3)** and the selected begomoviruses **(Table 4)** at eight different genomic level comprising the full-genome, intergenic region(IR) and six ORFs indicates that all Iranian TYLCV-IL isolates except for TYLCV-IL[IR:Sh40:07] share 97.3-99.9% identity with each other at full genome level. TYLCV-IL [IR:Sh40:07] shares 94.8-95.1% identity with other Iranian isolates. The comparisons with selected begomoviruses show all Iranian TYLCV-IL full genomes are more similar to that of TYLCV-IL with 94.6-96.7% identity. TYLCV-IL [IR:Abadeh] full genome represents lowest identity while that of TYLCV-IL[IR:Sh40:07] display highest identity with TYLCV-IL genome. Both isolates come from close distances geographically (Fars province). Furthermore, TYLCV-IL [IR: Boj: C5] complete sequence from northeast Iran share 94.8% identity with those of both TYLCV-IL and TYLCV-IL[Almeria]. Also shared 94.9 identity with both TYLCV-IL and TYLCV-IL[Almeria] full genome was observed among TYLCV-IL[IR:Sh46:07], TYLCV-IL[IR:Sh47:07] and TYLCV-IL[IR:Ta30:06] genomes.

The comparisons of percent nucleotide identity of C1 alignment indicate all Iranian isolates except TYLCV-IL [IR:Sh40:07] possess identity of 98-100% to each other. Isolate TYLCV-IL [IR: Sh40:07] share 91.2-91.6% identity with other Iran-originated TYLCV-ILs. The comparisons of C1 sequence identity percentage of Iran's isolates with those of selected viruses denote that all isolates except TYLCV-IL [IR: Sh40:07] have maximum identity of 91.3-91.7% to TYLCV-IL [Almeria] C1 gene. C1 gene of TYLCV-IL [IR: Sh40:07] shares highest identity (96.4%) with the corresponding gene of TYLCV-IL. Percent sequence identity compared at the C2 gene level reveals that all isolates of TYLCV-IL from Iran share 98.2-100% identity with one another. The comparisons of C2 gene of all Iranian isolates with that of selected begomoviruses indicate maximum identity of (98.0-98.7%) with TYLCV-IL. In addition to TYLCV-IL, C2 region of TYLCV-IL [IR: BoJ: C3] and TYLCV-IL[IR:BoJ:C5] isolates share identities of 98% and 98.2%, respectively, with TYLCV-IR and TYLCV-Mld isolates. Besides, TYLCV-IL [IR: Sh40:07] shows 98.7% identity with both TYLCV-IL and TYLCV-IL [Almeria]. Results of C3 and C4 nucelotide identity percentage from the pairwise comparisons among all Iran-originated TYLCV-IL isolates indicate identities of 98.2-100% (with the exception of TYLCV-IL[IR:Abadeh]) and 94.8-100% except for TYLCV-IL[IR:Sh:40:07], respectively. Strikingly, The C3 gene of TYLCV-IL [IR:Abadeh] is 92.1-93.3% identical to other TYLCV-IL sequences isolated in Iran. The isolate TYLCV-IL [IR: Sh: 40: 07] has 89.9-94% identity at the level of C4 sequence to the rest of Iranian isolates. The nucleotide-comparison outcomes of C3 and C4 open reading frames of Iranian isolates with those of the chosen begomoviral genomes display in a row 91.6-98.7% and 87.3-95.9% maximum identity with the TYLCV-IL isolate. Isolates TYLCV-IL [IR: BoJ: C3] and TYLCV-IL [IR:BoJ:C5] share the highest identity of 98.7 for the C3 region with both TYLCV-IL and TYLCV-Mld. Percent sequence identity values of V1 and V2 genes in pairwise comparisons of the viral sequences isolated from Iran represent 97.2-100% and 97.7-100% identities respectively for the given regions among all isolates in Iran. In these comparisons, TYLCV-IL [IR: Sh: 40: 07] with 97.9-98.5% identity with the rest of Iranian isolates could separate southern part isolates of Iran from those of north-eastern part. Comparisons at the V1/V2 sequence level of Iranian isolates with the selected viruses respectively show the highest sequence identities of 98.1-99.6% and 98.8-99.7% for the given regions with TYLCV-Mld and TYLCV-Mld/TYLCV-IL[Almeria].

The comparisons of the intergenic region sequence identitiy of nineteen Iranian isolates (Data not shown) with each other show that TYLCV-IL [IR: Sh: 40: 07] isolate with 89.1-93.9% identity with other Iranian isolates clearly differentiates the south-central isolates from the northeastern ones. North-eastern isolates except Mashad isolate shares 91.7-94.5% identities with south-central isolates at the non-coding region sequence level. Moreover, among the northeastern isolates the TYLCV-IL [IR: Boj: A1] and TYLCV-IL [IR: Boj: A2] share 92.3-92.9% with the rest of the isolates of this geographical region. Iranian isolates of TYLCV-IL share maximum identity of 91.7-96.4% with TYLCV-IL [Almeria] isolate at the level of the non-coding sequences. Full genomes of Iranian isolates indicate the lowest identities of 72.7-73.4% and 75.3-75.7% with TLCIV and TYLCSV isolates, respectively.

Table 3. *Percentage nucleotide sequence identities for the full genome (FG), intergenic region (IR) and ORFs (V1,V2,C1,C2,C3,C4) of the Iranian TYLCV-IL isolates; comparisons are shown in four pairs: FG/ IR; V2 / V1; C1/C4; C2 /C3*

Virus Isolates	TYLCV-IL [IR_Boj_C3]	TYLCV-IL [IR_Boj_C5]	TYLCV-IL [IR_Boj_C6]	TYLCV-IL [Neyshabur]	TYLCV-IL [IR_Boj_A1]	TYLCV-IL [IR_Boj_A2]	TYLCV-IL [Orzuieyeh]	TYLCV-IL [Shiraz46]	TYLCV-IL [Shiraz40]	TYLCV-IL [Shiraz47]	TYLCV-IL[Taft]	TYLCV-IL [Abadeh]
TYLCV-IL [IR_Boj_C3]	100.0											
TYLCV-IL [IR_Boj_C5]	99.8/100.0 100.0/99.7 99.8/100.0 99.7/100.0	100.0										
TYLCV-IL [IR_Boj_C6]	99.5/100.0 99.7/99.4 99.5/99.6 99.2/99.5	99.7/100.0 99.7/99.7 99.7/99.6 99.5/99.5	100.0									
TYLCV-IL [Neyshabur]	99.2/98.3 99.4/99.2 99.6/99.6 99.5/99.2	99.4/98.3 99.4/99.4 99.8/99.6 99.7/99.2	99.4/98.3 99.7/99.7 99.7/100.0 99.7/92.2	100.0								
TYLCV-IL [IR_Boj_A1]	98.3/92.9 98.8/99.2 98.7/98.7 99.2/99.0	98.4/92.9 98.8/99.4 98.9/98.7 99.5/99.0	98.5/92.9 99.1/99.7 98.8/99.0 99.5/99.0	98.3/92.3 98.8/99.4 98.9/99.0 99.7/98.7	100.0							
TYLCV-IL [IR_Boj_A2]	98.3/92.9 99.1/99.0 98.7/98.7 99.2/99.0	98.4/92.9 99.1/99.3 98.9/98.7 99.5/99.0	98.5/92.9 99.4/99.6 98.8/99.0 99.5/99.0	98.3/92.3 99.1/99.3 98.9/99.0 99.7/98.7	99.9/100.0 99.7/99.8 100.0/100.0 100.0/100.0	100.0						
TYLCV-IL [Orzuieyeh]	97.9/94.5 98.2/97.5 98.6/98.3 99.0/99.0	98.0/94.5 98.2/97.8 98.8/98.3 99.2/99.0	98.1/94.5 98.5/98.0 98.7/98.7 99.2/99.0	97.9/93.9 98.2/97.8 98.8/98.7 99.5/98.7	97.6/91.7 98.2/98.0 98.7/99.0 99.2/98.5	97.6/91.7 98.5/97.9 98.7/99.0 99.2/98.5	100.0					
TYLCV-IL [Shiraz46]	97.8/94.5 98.5/97.8 98.3/94.8 98.7/98.7	97.9/94.5 98.5/98.0 98.5/94.8 99.0/98.7	98.0/94.5 98.8/98.3 98.4/95.1 99.0/98.7	97.8/93.9 98.5/98.0 98.5/95.1 99.2/98.5	97.7/92.3 99.1/98.5 98.4/95.4 99.0/98.2	97.7/92.3 99.4/98.4 98.4/95.4 99.0/98.2	98.7/96.8 99.1/98.7 99.2/96.4 99.2/98.7	100.0				
TYLCV-IL [Shiraz40]	94.8/91.0 98.2/98.3 91.2/89.9 98.2/99.2	95.0/91.0 98.2/98.5 91.4/89.9 98.5/99.2	95.0/91.0 98.5/98.8 91.3/90.2 98.5/99.2	94.8/90.4 98.2/98.5 91.4/90.2 98.7/99.0	94.8/89.1 98.8/99.0 91.4/90.6 98.5/98.7	94.8/89.1 99.1/98.9 91.4/90.6 98.5/98.7	95.2/92.6 98.2/98.1 91.9/91.2 99.2/98.7	95.1/93.2 99.1/98.4 91.5/93.3 98.5/98.5	100.0			
TYLCV-IL [Shiraz47]	97.8/94.2 98.5/97.8 98.4/94.8 98.7/98.7	97.9/94.2 98.5/98.0 98.6/94.8 99.0/98.7	98.0/94.2 98.8/98.3 98.5/95.1 99.0/98.7	97.8/93.6 98.5/98.0 98.6/95.1 99.2/98.5	97.7/92.0 99.1/98.5 98.5/95.4 99.0/98.2	97.7/92.0 99.4/98.4 98.5/95.4 99.0/98.2	98.7/96.4 99.1/98.7 99.3/96.4 99.2/98.7	99.9/99.6 100.0/100.0 99.9/100.0 100.0/100.0	95.1/92.9 99.1/98.4 91.6/93.3 98.5/98.5	100.0		
TYLCV-IL[Taft]	97.8/95.2 97.7/97.4 98.4/94.8 99.0/99.0	97.9/95.2 97.7/97.6 98.6/94.8 99.2/99.0	98.0/95.2 98.0/97.9 98.5/95.1 99.2/99.0	97.8/94.5 97.7/97.6 98.6/95.1 99.5/98.7	97.7/92.9 98.2/98.1 98.5/95.4 99.2/98.5	97.7/92.9 98.5/98.0 98.5/95.4 99.2/98.5	98.7/97.4 98.8/98.3 99.1/96.4 99.5/99.0	99.4/98.7 99.1/99.6 99.5/100.0 99.7/99.7	95.1/93.9 98.2/98.0 91.6/93.3 98.7/98.7	99.4/98.4 99.1/99.6 99.6/100.0 99.7/99.7	100.0	
TYLCV-IL [Abadeh]	97.4/94.5 98.2/97.2 98.0/95.1 98.7/92.3	97.5/94.5 98.2/97.5 98.2/95.1 99.0/92.3	97.6/94.5 98.5/97.8 98.1/95.4 99.0/92.3	97.6/94.5 98.2/97.8 98.2/95.4 99.2/92.1	97.3/92.3 98.8/98.0 98.1/95.7 99.0/92.1	97.3/92.3 99.1/97.9 98.1/95.7 99.0/92.1	98.3/96.8 98.8/98.1 98.7/96.7 99.2/92.3	98.8/98.0 99.7/99.2 98.7/99.0 99.5/93.1	94.8/93.2 98.8/97.9 91.4/94.0 98.5/92.1	98.8/97.7 99.7/99.2 98.8/99.0 99.5/93.1	98.8/98.7 98.8/98.8 98.8/99.0 99.7/93.3	100.0

Table 4. *Percentage nucleotide sequence identities[1] for the full genome (**FG**), intergenic region (**IR**) and ORFs (**V1, V2, C1, C2, C3, C4**) of Iranian TYLCV-IL isolates and selected begomoviruses[2]; Comparisons are shown in four pairs: FG/ IR; V2 / V1; C1/C4; C2 /C3*

Virus Isolates	TYLCV-IL [IR_Boj_A1]	TYLCV-IL [IR_Boj_A2]	TYLCV-IL [IR_Boj_C3]	TYLCV-IL [IR_Boj_C5]	TYLCV-IL [IR_Boj_C6]	TYLCV-IL [Neyshabur]	TYLCV-IL [Shiraz46]	TYLCV-IL [Shiraz47]	TYLCV-IL [Shiraz40]	TYLCV-IL[Taft]	TYLCV-IL [Abadeh]	TYLCV-IL [Orzuieyeh]
TYLCV-Ir2	85.6/62.8	85.6/62.8	85.5/62.1	85.6/62.1	85.7/62.1	85.6/62.1	86.1/62.6	86.1/62.3	85.6/63.6	86.1/63.3	85.6/62.6	85.9/62.0
	95.1/97.2	95.4/97.1	94.5/96.5	94.5/96.7	94.8/97.0	94.5/96.7	95.4/97.8	95.4/97.8	95.1/97.1	95.1/97.6	95.1/97.2	94.5/96.9
	80.1/66.0	80.1/66.0	80.3/66.6	80.4/66.6	80.4/66.9	80.4/66.9	80.7/68.2	80.8/68.2	79.5/71.9	80.6/68.2	80.1/68.2	80.8/66.6
	86.5/41.9	86.5/41.9	86.5/42.1	86.7/42.1	86.7/42.1	86.7/42.1	86.5/41.7	86.5/41.7	87.5/72.1	86.7/41.9	87.0/43.6	87.2/42.1
TYLCV-OM	88.6/69.9	88.6/69.9	88.6/70.7	88.7/70.7	88.8/70.7	88.7/70.1	88.8/71.2	88.8/70.8	87.9/71.8	88.9/71.2	88.3/70.2	88.9/70.8
	95.4/97.2	95.7/97.1	94.8/96.5	94.8/96.7	95.1/97.0	94.8/96.7	95.7/96.9	95.7/96.9	95.4/96.9	95.4/96.7	95.4/96.1	94.8/96.3
	85.1/79.6	85.1/79.6	85.3/80.2	85.4/80.2	85.4/80.5	85.4/80.5	85.6/82.6	85.7/82.6	82.3/81.9	85.9/82.6	85.3/83.3	85.9/80.5
	87.9/92.8	87.9/92.8	87.9/93.5	88.2/93.5	88.2/93.3	88.2/93.5	87.9/93.0	87.9/93.0	88.9/93.5	88.2/93.3	88.4/86.6	88.7/94.3
TYLCV-IR	91.1/88.5	91.2/88.5	90.9/91.0	91.0/91.0	91.0/91.0	90.9/90.7	91.4/92.6	91.4/92.3	90.2/91.0	91.4/93.2	91.0/92.9	91.4/92.6
	98.0/97.5	98.2/97.6	98.0/97.0	98.0/97.2	98.2/97.5	98.0/97.2	98.8/97.1	98.8/97.1	98.0/97.2	98.0/96.7	98.5/96.6	98.5/96.9
	86.6/80.5	86.6/80.5	86.6/81.2	86.7/81.2	86.6/81.5	86.7/81.5	87.1/83.6	87.2/83.6	83.9/80.0	87.2/83.6	86.9/84.3	87.2/81.5
	97.7/46.2	97.7/46.2	**98.0**/46.6	**98.2**/46.6	97.7/46.4	98.0/46.4	97.7/46.1	97.7/46.1	98.2/46.4	98.0/46.2	97.7/48.1	98.5/46.4
TYLCV-Mid	91.2/77.6	91.2/77.6	91.0/77.0	91.1/77.0	91.1/77.0	91.0/76.7	91.2/77.8	91.2/77.5	91.4/78.1	91.3/79.1	90.6/77.8	91.0/77.5
	99.4/99.6	**99.7/99.4**	**98.8/99.0**	**98.8/99.0**	**99.1/99.3**	**98.8/99.0**	**99.7/98.7**	**99.7/98.7**	**99.4/99.2**	**98.8/98.3**	**99.4/98.1**	**98.8/98.1**
	85.0/66.0	85.0/66.0	84.7/66.0	84.9/66.0	84.8/66.3	84.9/66.3	85.5/68.9	85.6/68.9	85.4/72.2	85.6/68.9	84.8/68.2	85.3/66.6
	98.5/98.5	98.5/98.5	**98.0/98.7**	**98.2/98.7**	98.0/98.5	98.2/98.2	98.0/97.7	98.0/97.7	98.5/98.2	98.2/98.0	98.0/91.3	98.7/98.5
TYLCV-IL	**94.7**/88.5	**94.7**/88.5	**94.7**/91.0	**94.8**/91.0	**94.9**/91.0	**94.7**/90.7	**94.9**/92.6	**94.9**/92.3	**96.7**/91.0	**94.9**/93.2	**94.6**/92.9	**95.0**/92.6
	99.1/98.5	99.4/98.4	98.5/97.8	98.5/98.0	98.8/98.3	98.5/98.0	99.4/97.7	99.4/97.7	99.1/98.2	98.5/97.3	99.1/97.1	98.5/97.1
	91.4/**88.0**	91.4/**88.0**	91.2/**87.3**	91.4/**87.3**	91.3/**87.7**	91.4/**87.7**	91.5/**90.6**	91.6/**90.6**	**96.4/95.9**	91.6/**90.6**	91.4/**91.3**	**92.1/88.6**
	98.7/98.7	**98.7/98.7**	**98.0/98.7**	**98.2/98.7**	**98.2/98.7**	**98.5/98.5**	**98.2/98.0**	**98.2/98.0**	**98.7/98.5**	**98.5/98.2**	**98.2/91.6**	**99.0/98.7**
TYLCV-IL [Almeria]	94.6/**91.7**	94.6/**91.7**	94.6/**93.6**	**94.8/93.6**	94.8/**93.6**	94.6/**92.9**	**94.9/95.8**	**94.9/95.5**	96.6/**93.9**	**94.9/96.4**	94.5/**95.8**	94.9/**95.5**
	99.4/98.8	**99.7**/98.7	**98.8**/98.3	**98.8**/98.5	**99.1**/98.5	**98.8**/98.3	**99.7**/98.1	**99.7**/98.1	**99.4**/98.4	**98.8**/97.8	**99.4**/97.6	**98.8**/97.6
	91.5/87.7	**91.5**/87.7	**91.3**/87.0	**91.5**/87.0	**91.4**/87.3	**91.5**/87.3	**91.6**/90.3	**91.7**/90.3	96.2/95.5	**91.7**/90.3	**91.5**/91.0	92.0/88.3
	98.2/98.5	98.2/98.5	97.5/98.5	97.7/98.5	97.7/98.5	98.0/98.2	97.7/97.7	97.7/97.7	**98.7**/98.2	98.0/98.0	97.7/91.3	98.5/98.5
TYLCV-Gez	88.2/71.2	88.2/71.2	88.1/70.3	88.2/70.3	88.3/70.3	88.3/70.9	88.4/70.5	88.4/70.2	88.6/72.4	88.5/71.7	88.1/71.7	88.3/70.2
	96.8/96.2	97.1/96.1	96.2/95.4	96.2/95.7	96.5/96.0	96.2/95.7	97.1/96.1	97.1/96.1	96.8/96.1	96.2/95.7	97.4/95.8	96.8/95.6
	83.6/65.4	83.6/65.4	83.7/66.0	83.8/66.0	83.7/66.3	83.8/66.3	84.0/67.3	84.1/67.3	83.7/71.2	84.1/67.3	83.5/67.3	84.0/65.7
	96.0/94.5	96.0/94.5	95.8/94.8	96.0/94.8	96.0/94.8	96.3/95.0	96.0/94.3	96.0/94.3	97.0/95.0	96.3/94.5	96.0/88.6	96.8/95.5
TYLCSV	75.3/58.0	75.3/58.0	75.4/58.8	75.5/58.8	75.5/58.8	75.5/58.5	75.5/57.7	75.5/57.4	75.7/58.6	75.5/58.0	75.3/57.7	75.6/57.4
	81.4/81.3	81.7/81.2	81.4/81.2	81.4/81.3	81.7/81.3	81.4/81.2	81.7/81.5	81.7/81.5	82.0/81.2	81.4/81.7	81.4/81.2	81.4/81.8
	75.8/70.8	75.8/70.8	75.9/71.5	76.1/71.5	75.9/71.5	76.2/71.5	76.0/72.6	76.1/72.6	76.2/71.3	76.0/72.6	76.1/73.3	76.2/70.8
	76.2/77.5	76.2/77.5	76.2/78.0	76.4/78.0	76.2/77.7	76.4/78.0	76.9/78.0	76.9/78.0	77.4/78.5	76.9/78.0	76.7/72.4	76.9/78.5
TLCIV	72.9/58.3	72.9/58.3	72.7/57.5	72.8/57.5	72.8/57.5	72.7/57.1	73.1/57.0	73.1/58.3	73.4/59.2	73.1/58.3	73.1/58.6	73.3/57.6
	76.9/72.3	77.2/72.2	76.6/71.8	76.6/71.9	76.6/72.0	76.3/71.8	77.7/72.4	77.7/72.4	78.0/72.5	76.9/72.0	77.4/72.0	77.4/72.8
	76.5/80.9	76.5/80.9	76.4/81.5	76.6/81.5	76.5/81.8	76.6/81.8	76.8/83.6	76.9/83.6	76.3/80.3	76.9/83.6	76.9/84.6	77.0/81.8
	76.7/77.2	76.7/77.2	76.7/77.7	76.9/77.7	76.7/77.7	76.9/77.7	77.2/77.5	77.2/77.5	77.9/78.2	77.4/77.7	77.2/73.1	77.4/77.7

[1] Values in bold denote the highest nucleotide sequence identities with Iranian TYLCV-IL isolates for each genomic region

[2] Tomato yellow leaf curl virus-Iran2 (TYLCV-Ir2); Tomato yellow leaf curl virus-Iran (TYLCV-IR); Tomato yellow leaf curl virus-Oman (TYLCV-OM); Tomato yellow leaf curl virus-Mid (TYLCV-Mid); Tomato yellow leaf curl virus-Israel (TYLCV-IL); Tomato yellow leaf curl virus-Gezira (TYLCV-Gez); Tomato yellow leaf curl virus-IL[Almeria] (TYLCV-IL[Almeria]); Tomato yellow leaf curl Sardinia virus (TYLCSV); Tomato leaf curl Iran virus(TLCIV)

Maximum likelihood phylogenetic tree of the full length genome of the Iranian isolates reconstructed using MEGA5 with the 1000 iterated bootstrap support values of the individual nodes represents a clear clustering of the isolates according their origin of geography **(Figure 3).** This geophylogenetic analysis depicts two clusters A and B of Iranian TYLCV-IL variants separated clearly, with the exception of TYLCV-IL [IR: Sh40:07] which is grouped phylogenetically with TYLCV-IL and TYLCV-IL [Almeria]. This finding might be able to explain that this Shiraz-originated isolate may play a possible role of as a bridge between Mediterranean basin isolates of the TYLCV-IL and Iranian variants. The full genome geophygenetic tree represents the clustering of Iranian TYLCV-IL isolates into the two groups of variants including a first-time reported group of variants from the northeastern Iran in this paper.

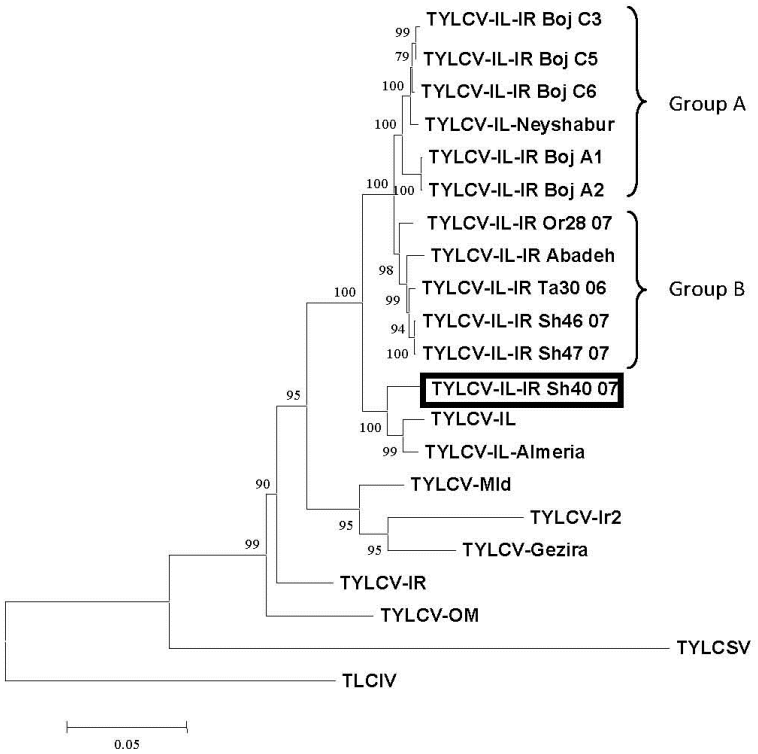

Figure 3. Phylogenetic tree of the full genome of the Iranian TYLCV-IL isolates with nine selected begomoviruses (Table 1 and 2) was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The tree is rooted by TLCIV and the percentage values of the bootstrap support (1000 iterations) for the individual nodes are indicated. The scale bar shows the number of substitutions per site.

DISCUSSION

The newly emerging and re-emerging geminiviruses even in the regions previously free from these viruses cause frequent epidemics. Evolution of new variants in geminivuses is one of the major contributory factors for the emergence and spread of new geminivirus diseases (37). Nucleotide substitutions or complicated recombination events are considered as the major culprit for the genetic variation within the genome of TYLCV-like viruses (8, 38). Presence of the world most devastating strain of TYLCV, TYLCV-IL, in different parts of Iran depicts an inflow rout of the virus variants from the south to the north-eastern parts of country **(Figure 1)**. Since the regions around Iran is considered as the center of recent TYLCV diversity and its ongoing intensive evolution, the presence of five TYLCV stains including the most notorious stain, TYLCV-IL, inside country suggests that Iran is more likely close to the global center of TYLCV diversity (17). From this point of view, The results of full genome and other non-coding and coding regions of Iranain TYLCV-IL isolates most probable raise this idea that TYLCV-IL[IR:Sh:40:07](red highlighted in **Figure 3**) originated from Shiraz, South of Iran might be a possible linkage between Mediterranean region and Iranian TYLCV-IL isolates. The phlogenetic analysis here represents two isolates of TYLCV-Mld and

TYLCV-IR most likely could be considered as the probable ancestors of Shiraz isolate which in turn play a ancestral role for the best well-known TYLCV-IL[IL:Reo:86]-X15656) isolate first reported from Israel in1990s. This is a new finding here; albeit required to be more explored with more isolates from the Shiraz region.

Seven isolates of TYLCV-IL from southern (Khozestan, Boushehr and Hormozgan provinces), central (Markazi and Yazd provinces) and southeastern regions (Razavi Khorasan province) of Iran have been previously clustered in one variant group based on the intergenic region (IR) (27) **(Table 1).** Application of these seven non-coding sequences with other twelve IR sequences derived from the full length genome data shows five phyogenetic groups including groups 1-5 **(Figure 4).** The intergenic region sequence analysis displays more divergent grouping of the variants so that new isolates from northeastern parts of Iran (five isolates from Bojnurd, North Khorasan province and one isolate from Neyshabur, Razavi Khorasan province) forms two distinct groups of variants including groups 3 and 4.

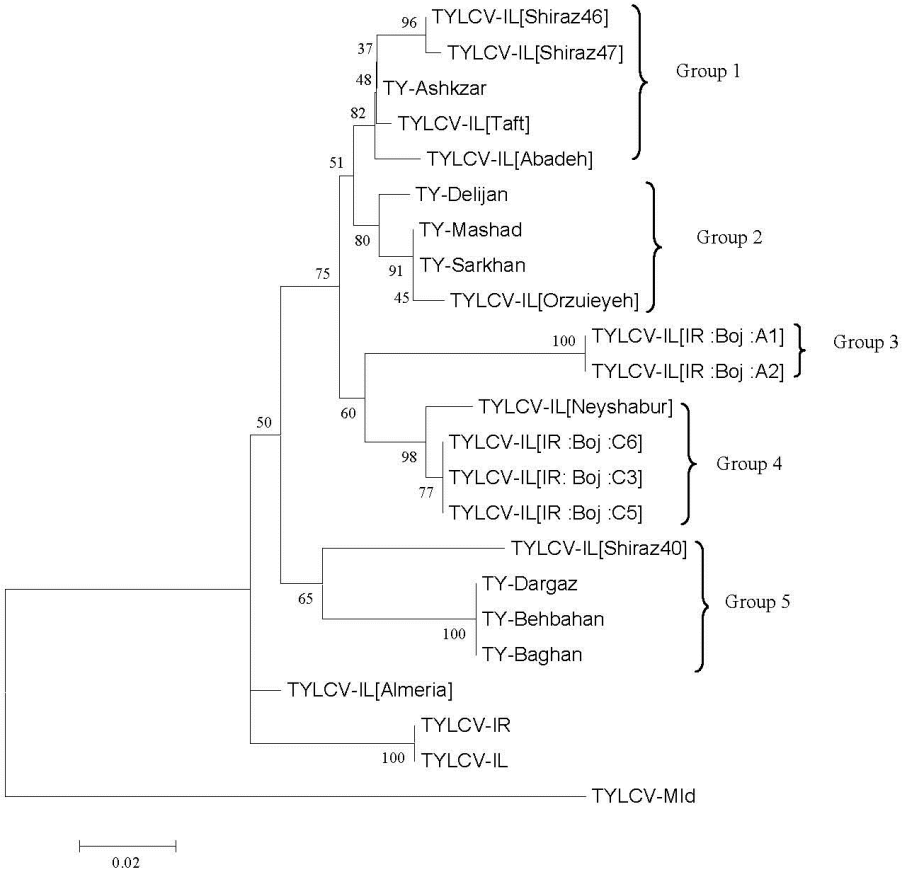

Figure 4. Phylogenetic tree of the intergenic region (IR) of the nineteen Iranian TYLCV-IL isolates (grouped 1-5) with four selected begomoviruses (Table 1 and 2) was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The tree is midpoint rooted for clarity and the percentage values of the bootstrap support (1000 iterations) for the individual nodes are indicated. The scale bar shows the number of substitutions per site.

The intergenic region of geminiviruses possesses the origin of DNA replication sequences (*ori)* including the conserved nonanucelotide (TAATATT/AC) within the loop in the stem-loop structure and short repeated nucleotide sequence units known as iterons which are considered as the Rep-binding motifs (39). The iterated sequences of TYLCV-IL, predicted to be involved in TYLCV-IL Rep binding, consist of one iteron (5′-GGTGT-3′) located between nucleotide coordinates 2627-2631 and two directly repeated units with a 3 nt spacer (5′-GGTGT**ATC**GGTGT-3′) located in the virus-sense strand between nucleotide coordinates 2645-2657, just upstream of the TATA box of the Rep promoter. These repetitive sequences are postulated to act as specific binding sites (the 5′ and the 3′ Rep-binding motifs, respectively) for Rep

protein during replication. The Rep of each geminivirus specifically binds to its own Rep-binding motifs and it has been shown that even one nucleotide change in each of the Rep binding motifs will result in independently replicating variants (40, 41). Interestingly, the common region (CR) sequence alignment of Iranian TYLCV-IL isolates displays that the TYLCV-IL isolates from northeastern Iran including five Bojnurd isolates plus one Neyshabur isolate share a 4 nt spacer(ATCC) at the 3′ Rep binding motif while isolates from south of Iran contain 3 nt spacer, ATC. Besides, the 5′ Rep binding motifs of two isolates originated from Bojnurd, i.e. TYLCV-IL[IR:Boj:A1] and TYLCV-IL[IR:Boj:A1], share a nucleotide replacement of A to T at the beginning of 5' Rep motif; AATC **(Figure 5).** It is, therefore, possible that one nucleotide difference in the 3′ Rep-binding motif sequence of northeastern isolates of Iranian TYLCV-IL results in independent replication of these variants by their own Rep.

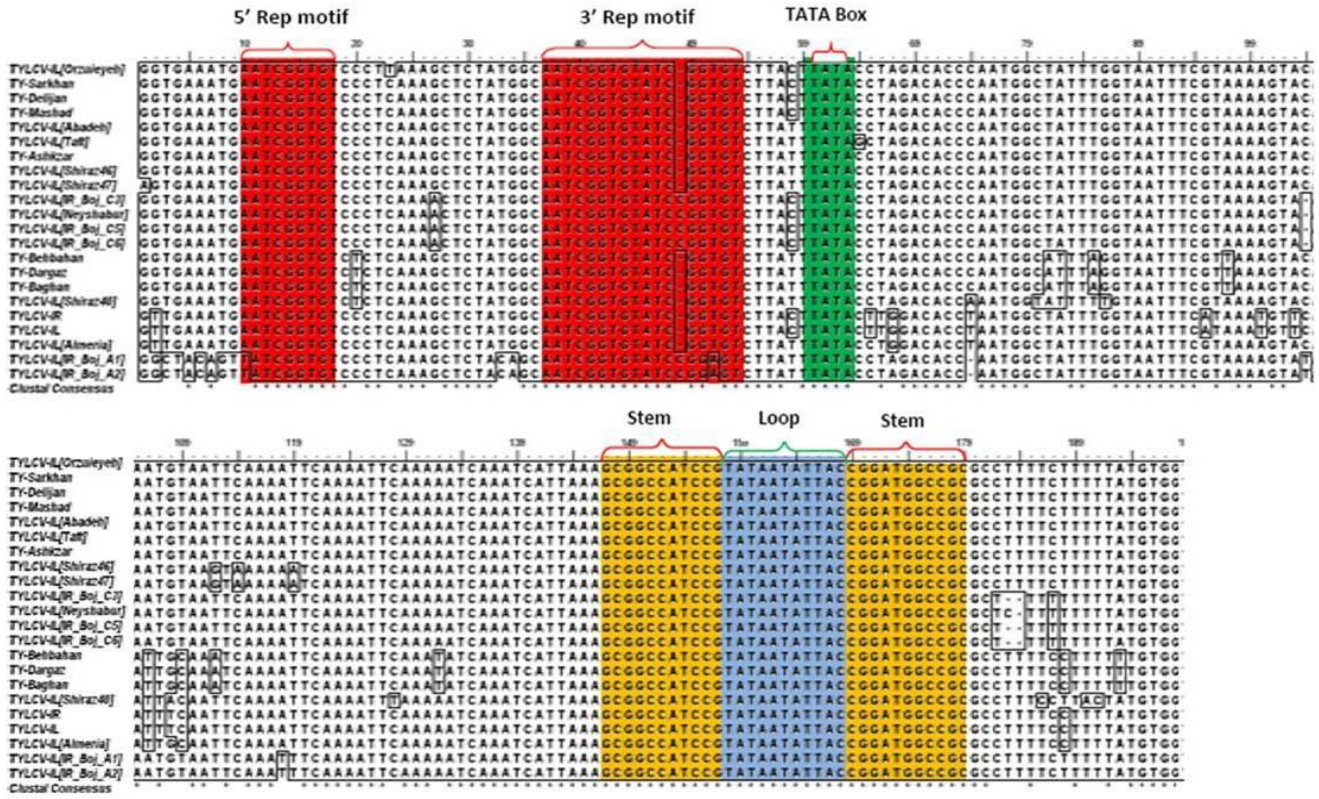

Figure 5- The alignment of common region (CR) sequences of 19 Iranian TYLCV-IL isolates and selected TYLCVs (TYLCV-IL; TYLCV-IL[Almeria]; TYLCV-IR) (Table 1 and 2) ; stem and loop sequences of the predicted stem-loop structure, the position of 5' and 3' section of Rep binding motifs (**Iterons**) and TATA box of the Rep promoter are highlighted**.**
in GenBank; their GenBank accession numbers, origins, genome sizes and published year

CONCLUSION

This is the first occurrence report of TYLCV-IL variants in northeastern region of Iran, North Khorasan province. In general, five new complete sequences of TYLCV-IL variants from Bojnurd area, North Khorasan, Iran plus an Isolate recently published in GenBnak from Neyshabur, Razavi Khorasan, Iran (TYLCV-IL[IR:Neyshabur:2012]; JQ414025) shape a distinct group of TYLCV-IL variants which is phylogenetically clustered in the Group A. This group together with another group (Group B) of variants from southern parts (Fars and Kerman provinces) of Iran creates a more general picture of TYLCV-IL population dynamic in country. Group A and B of variants which are first-time reported here represent two distinct emerging variant groups in Iran. Epidemiologically, the TYLCV-IL variants available in the Iran geography pose a potential great threat and large limitation to the production of tomato and other host crops in this country and the surrounding neighbors. The presence of new variants in the previously virus-free regions and also ongoing variation inside the population require constant monitoring of the TLCV-IL variant populations in Iran.

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