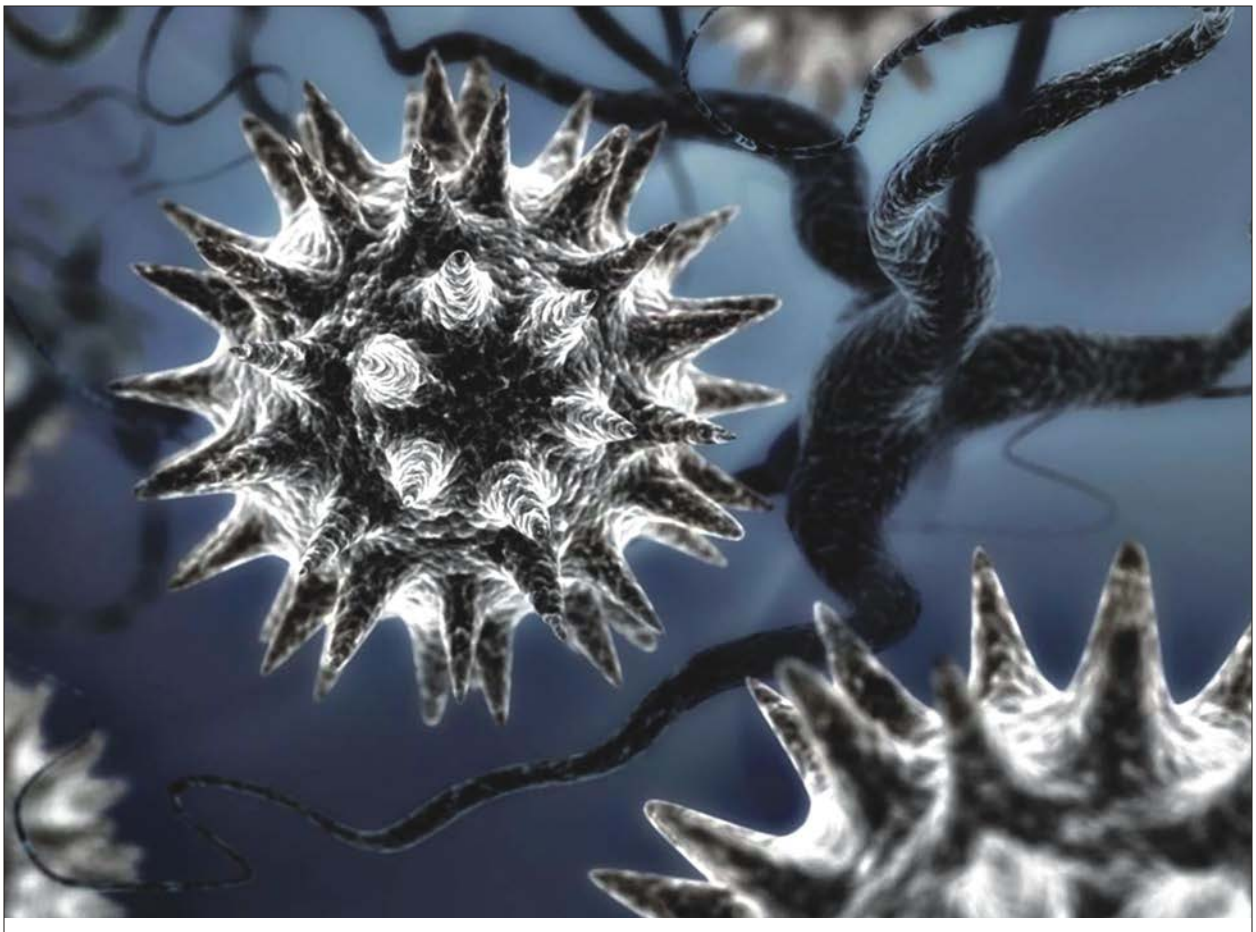

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Molecular Characterization of Tomato Yellow Leaf Curl Disease Associated Viruses in Saudi Arabia

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ABSTRACT

Tomato production in Saudi Arabia and anywhere is under constant threat of whitefly-transmitted begomoviruses that cause tomato yellow leaf curl disease (TYLCD). *Tomato yellow leaf curl virus* (TYLCV) that has a large number of strains affects tomato all over the world. Tomato samples with typical symptoms of TYLCD were collected from open fields and greenhouses from different locations of Hail and Qasim in Saudi Arabia. PCR was used to identify the pathogen using degenerate primers of Geminiviruses (AVcore and ACcore) and other specific primers. To determine exactly the virus strain, multiplex PCR was used with sets of specific primers for Tomato yellow leaf curl Almeria strain (TYLCV-Alm), *Tomato yellow leaf curl virus* Israel strain (TYLCV-IL), *Tomato yellow leaf curl virus* Mild strain (TYLCV-Mld) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV). All tested samples showed negative with specific primers in multiplex-PCR, nevertheless, most of symptomatic tomato samples were positive with the use of degenerate primers. Notwithstanding, PCR products were cloned and sequenced as well as deposited in GenBank. Thus, the results showed that all obtained isolates were found to have >95% similarity with *Tomato leaf curl virus* isolates from Sudan and Yemen.

Key words: TYLCV, tomato, Saudi Arabia, multiplex-PCR

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the most popularly grown vegetable crops in the world. The worldwide high consumption of tomato is mostly due to its acceptable flavor and high nutritive value. Tomato yellow leaf curl disease is one of the most important virus affecting tomatoes and thus have induced severe damages to crops from tropical and subtropical regions, (Czosnek and Latterrot, 1997). The *Tomato yellow leaf curl virus* is not transmitted from plant to plant mechanically by handling but is spread from infected *Solanaceous* weed species to cultivated tomato plants by whiteflies (Ajlan *et al*., 2006; Al-Ani *et al*., 2011; Richardson, 2013). Tomato leaf curl virus disease is one of the most destructive diseases, of tomato crop (Saikia and Muniyappa, 1989). This disease is caused by a complex of several virus species in the genus *Begomovirus* of the Family Geminiviridae which altogether are referred to as "Tomato yellow leaf curls viruses" (TYLCV) (Abhary *et al*., 2007; Diaz-Pendon *et al*., 2010). Over the past years, whiteflies and begomoviruses have become serious threats to the cultivation of a variety of vegetable crops of great importance in different parts of the world, especially in the tropics and sub-tropics (Perefarres *et al*., 2012). Taxonomically they all belong to at least six species and 15 strains of viruses. Similarly, at least nine different virus species more or less related phylogenetically and

strains of them, have been associated with TYLCD, among others such as Tomato yellow leaf curl virus, TYLCV (Moriones and Navas-Castillo, 2000; Fauquet and Stanley, 2005; Stanley *et al*., 2005). TYLCV relates to virus species members of the genus *Begomovirus*, family Geminiviridae, the genome of which is strictly monopartite (Kheyr-Pour *et al*., 1991; Navot *et al*., 1991) and thus encodes six open reading frames (ORFs), four on the complementary (-) strand (C1, C2, C3 and C4) and two on the viral (+) strand (V1 and V2) (Navot *et al*., 1991). So far no satellite DNA molecules associated with TYLCV-like viruses have been identified or isolated (Abhary *et al*., 2007). Nevertheless, different virus species have been associated with TYLCVD in many countries across the world: *Tomato Yellow leaf curl Israel* (TYLCV-IS), *Tomato Yellow leaf curl Mild* (TYLCV-Mld), *Tomato Yellow leaf curl Sardinia virus* (TYLCV-Sar), *Tomato Yellow leaf curl Malaga virus* (TYLCMalV) *Tomato yellow leaf curl Sudan virus* (TYLCSDV), *Tomato yellow leaf curl Axarquia virus* (TYLCAxV) and *Tomato yellow leaf curl Mali virus* (TYLCMLV) (Abhary *et al*., 2007; Fauquet *et al*., 2008; Lefeuvre *et al*., 2010). TYLCV is the name given to a complex of genetically different *Geminiviruses* family Geminiviridae, genus *Begomovirus* affecting tomato production world-wide (Czosnek and Latterrot, 1997). These viruses are particularly important in the Mediterranean areas, South America, Africa and South-East Asia (Rey *et al*., 2012). In Sudan, Tomato leaf curl disease is a serious disease in tomato. It is highly endemic throughout the country, with epidemic often reaching 100% (Yassin and Nour, 1965). Since 1989, *Geminiviruses* have been reported from tomato in several African and Asian countries including Burkina Faso, Tunisia, Egypt, Ivory Coast, Malawi, Mali, Niger, Nigeria, Gambia, Sudan and Tanzania (Cohen and Harpaz, 1964; Yassin and Nour, 1965; Cherif and Russo, 1983; D'Hondt and Russo, 1985; AVRDC, 1994). The high degrees of TYLCV diversity considered in the Middle East and amount of inter-strain and species recombination that has been identified between TYLCV and in different Middle Eastern *Begomovirus* species (Bananej *et al*., 2004; Khan *et al*., 2008). TYLCV was observed in Saudi Arabia since 1978, hence the serological and PCR were used to detect the virus by Al-Shahwan *et al*. (2002) and Ajlan *et al*. (2006). Thereafter, no available data referred exactly to the strain of TYLCV in Saudi Arabia. Thus, the study was conducted to characterize and identify Begomovirus affecting tomato in different location of Hail and Qasim in Saudi Arabia as well as compared with other Begomovirus sequences available in Gene Bank.

MATERIALS AND METHODS

Samples collection: Tomato samples showing leaf curling, yellowing, stunting and leaf thickening symptoms typical of begomoviruses were collected from Hail and Qasim area in Saudi Arabia during years 2011 and 2012, including samples from open field and green houses. In Hail a total of 160 leaf samples from tomato plants showing TYLCD symptoms were collected from four fields in various locations. In addition, 96 leaf samples were collected from two fields in various locations in Qasim. Samples were either processed immediately or kept at -20°C prior to analysis.

DNA extraction: Total nucleic acids were extracted from infected and healthy tomato plants through the methods adapted from Rezk *et al*. (2006); 50 mg leaf tissue grinded in 600 µL extraction buffer (50 mM EDTA, 100 mMTris HCl, 500 mM NaCl and 10 mM β-mercaptoethanol) and incubated at 65°C for 10 min. The mixture was kept on ice for 10 min followed by centrifugation at 14000 rpm for 10 min. The supernatant was transferred to a new vials and diluted 1:10 with distilled water before using in Polymerase Chain Rraction (PCR) reaction.

Detection of TYLCD- associated viruses using multiplex PCR: Multiplex PCR using set of primers as shown in Table 1 of TYAlmc115/TYAlmv2516 to detect TYLCV-Alm, TYv2664/TYc138 to detect TYLCV-IL, TYv2337/TYc138 to detect TYLCV-MLd as well as other set of primers Sa2267/RVC427 were used to detect *Tomato yellow leaf curl Sardinia virus* (TYLCSV) as described by Anfoka *et al.* (2008). The second set of primers, AVcore/ACcore were used for all begomoviruses in separate PCR reaction and the sequences of all the used primers are shown in Table 1. PCR reactions were optimized for 50 µL reaction and the final concentration of the components were 25 µM of each deoxynucleotidetriphsphate (dNTPs), 1×PCR buffer, 2.5 µM MgCl2, 3 units *Taq* DNA polymerase, 0,2 µM of each complementary and viral sense primer and 5 µL of extracted nucleic acid as template. As negative controls, PCR was carried out in tubes pre-incubated with leaf extracts from healthy tomato plants and tomato samples infected with tomato leaf curl virus obtained from Egypt (TYLCV-EG) were used as positive control. For multiplex PCR cycle parameters were as follows; 94°C for 2 min, 30 cycles at 94°C for 1 min, 62°C for 90 sec, 72°C for 1 min and final extension cycle at 72°C for 10 min^{-1}. The other PCR cycle parameter using the degenerate primers AvCore/AcCore were as follows ; 94°C for 2 min^{-1} and 35 cycles at 94°C for 1 min, 55°C for 2 min, as well as 72°C for 2 min and final extension cycle at 72°C for 10 min^{-1}.

Cloning of DNA and sequencing: To confirm the identity of the amplified fragments, PCR products of TYLCV DNA that were obtained with samples collected from Hail and Qasim were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), cloned into pGEM-T Easy Vector (Promega,USA) according to manufacturer's instructions and transformed into DH5a high library-efficiency competent *Eschrichia coli* (Invitrogen Corporation, USA). DNA sequences were determined by the didexynucleotidemethod using Thermo Sequence dye terminator cycle sequencing kit, on ABI 377 DNA sequencer (Perkin-Elmer, Applied Biosystems, USA). Six partial clones with two isolates from Qasim (TYLCV-q25 and TYLCV-q26) and four isolates from Hail (TYLCV-h1, TYLCV-h3, TYLCV-h11, TYLCV-h13) were sequenced.

Sequence analysis: Alignment analysis was performed on partial nucleotide sequences of the Saudi Arabian TYLCV isolates using the online BLAST service of the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/). Phylogenic tree was constructed from the multiple alignments with begomovirus sequences available in GenBank database (Table 2) using the DNAMAN software (Lynnon, Canada) with the neighbor-joining method (Saitou and Nei, 1987) and the Jukes-Cantor distance-correction method (Jukes and Cantor, 1969). Also homology

Table 1: The primer names, sequences and references

Primer name	Primer sequence 5' ----------3'	Reference
AVcore	GCCHATRTAYAG RAAGCCNAGRAT	Brown *et al.* (2001)
ACcore	GGRTTDGARGCATGHGTACANGCC	Brown *et al.* (2001)
TYAlmc115	ATATTGATGGTTTTTTCAAAACTTAGAAG	Anfoka *et al.* (2005)
TYAlmv2516	TTTTATTTGTTGGTGTTTGTAGTTGAAG	Anfoka *et al.* (2005)
TYc138	AAGTGGGTCCCACATATTGCAAGAC	Anfoka *et al.* (2005)
TYv2337	ACGTAGGTCTTGACATCTGTTGAGCTC	Anfoka *et al.* (2005)
TYv2664	ATTGACCAAGATTTTTACACTTATCCC	Anfoka *et al.* (2005)
Sa2267	TGGAAAGTACCCCATTCAAGAACATC	Anfoka *et al.* (2008)
RVC427	TGCCTTGGACA(A/G)TGGGG(A/G)CAGCAG	Anfoka *et al.* (2008)

Table 2: Virus acronyms and GenBank accession No. for TYLCV isolates used in sequence analysis

Accession No.	Acronym	Virus	References
KC428387	TYLCV-HL1	TYLCV-[Hail 1]	This study
KC428388	TYLCV-HL3	TYLCV-[Hail 3]	This study
KC432613	TYLCV-HL11	TYLCV-[Hail 11]	This study
KC432614	TYLCV-HL13	TYLCV-[Hail 13]	This study
KC479016	TYLCV-Qas25	TYLCV-[Qasim 25]	This study
KC479017	TYLCV- Qas26	TYLCV-[Qasim 26]	This study
HE819244	TYLCV- (Man-SD)	TYLCV-(Sudan)	Khan *et al.* (2013)
AY 044138	TYLCV-SD	TYLCV-(Sudan)	Idris and Brown (2005)
AY 044137	TYLCV-(Jez-SD)	TYLCV-[Jizera-Sudan]	Idris *et al.* (2006)
JF919733	TYLCV-(Had-Sud)	TYLCV-(Sudan)	Idris *et al.* (2012)
EF110891	TYLCV-Yam	TYLCV-(Yamen)	Isakeit *et al.* (2005)
AY594174	TYLCV-Egy	TYLCV-(Egypt)	Abhary *et al.* (2006)
AY134494	TYLCV-PR	TYLCV-[Puerto Rico]	Bird *et al.* (2001)
X15656	TYLCV-IL	TYLCV-(Israel)	Navot *et al.* (1991)
X76319	TYLCV-Mld	TYLCV-(Israel)	Antignus and Cohen (1994)
GU126513	TYLCV-Kor	TYLCV-(Korea)	Kim *et al.* (2011)
GU076448	TYLCV-Ker23	TYLCV-(Iran)	Lefeuvre *et al.* (2010)
GU076449	TYLCV-Ker21	TYLCV-(Iran)	Lefeuvre *et al.* (2010)
GU076454	TYLCV-Gen	TYLCV-(Iran)	Lefeuvre *et al.* (2010)
L27708	TYLCV-Alm	*Tomato yellow leaf curl Almeria virus*	Reina *et al.* (2003)
AF271234	TYLCV-Mal	*Tomato yellow leaf curl Malaga virus*	Monci *et al.* (2002)
X 61153	TYLCV-Sar	*Tomato yellow leaf curl Sardinia virus*	Kheyr-Pour *et al.* (1991)
X63015	TYLCV-Th	*Tomato yellow leaf curl Thailand virus*	Rochester *et al.* (1994)

tree was performed using the protein among the core region of coat protein of isolated TYLCV isolates (h1, h3, h11, h13, q25 and q26) and the DNAMAN software (Lynnon, Canada).

RESULTS

Detection and identification of TYLCVD- associated viruses PCR: In the majority of the fields vested for collection from Hail and Qasim almost all fields with plants showed more or less symptoms of TYLCD. The sampled plants however, showed severe symptoms of TYLCD such as: yellowing, stunting and curling of leaves (Fig. 1).

Moreover, the results obtained from multiplex PCRs showed that the isolates TYLCSV, TYLCV-IL, TYLCV-mld and TYLCV-Alm were not detected in all the collected samples. However through analysis with PCR using degenerate primers for Begomoviruses AvCore/AcCore products of the expected size (~580 bp) were obtained for most samples with TYLCD symptoms but no bands were obtained with healthy samples (Fig. 2) thus indicating begomovirses infection. The percentage of infection in the tested samples were 92.5% (148/160) of samples collected with symptoms from Hail and 75% (72/96) of samples collected with symptoms from Qasim.

Nucleotide sequence data for TYLCV: The PCR products obtained with the leaf samples were cloned and sequenced to identify the suspected TYLCV strain. The core region of the CP, which was amplified with the primers AvCore/AcCore were sequenced and submitted in GenBank under accession numbers KC428387 (Hail: h1), KC428388 (Hail: h3), KC432613 (Hail: h11), KC432614 (Hail: h13), KC479016 (Qasim: q25) and KC479017 (Qasim: q26). Phylogenetic analysis was carried out for the partial sequences (CP core region) of

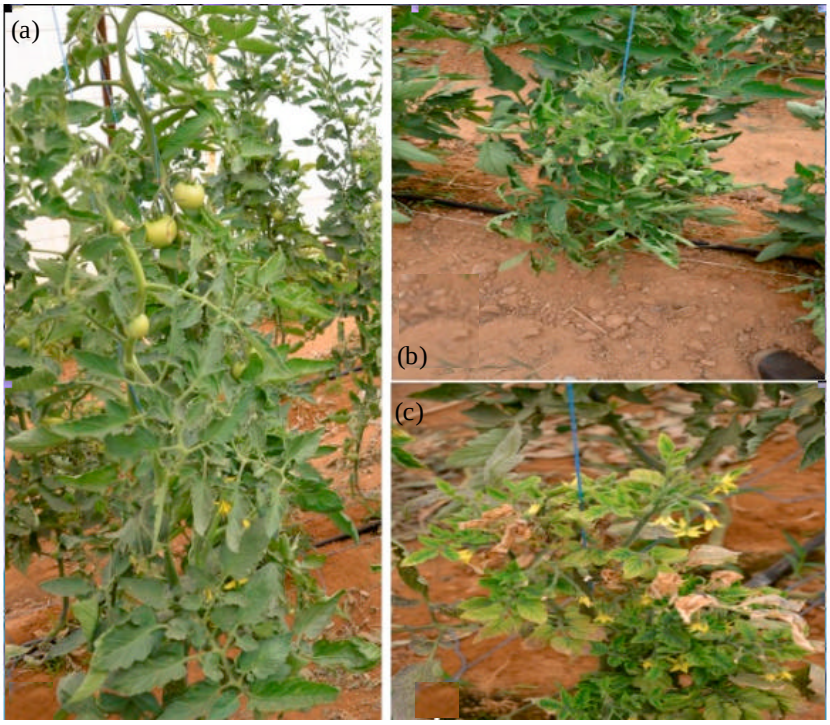

Fig. 1(a-c): Natural TYLCD symptoms (leaf curling, yellowing, stunting and thickening) appearing on tomato plants, (a) In green houses in Saudi Arabia and (b and c) Comparable with other healthy looking plants

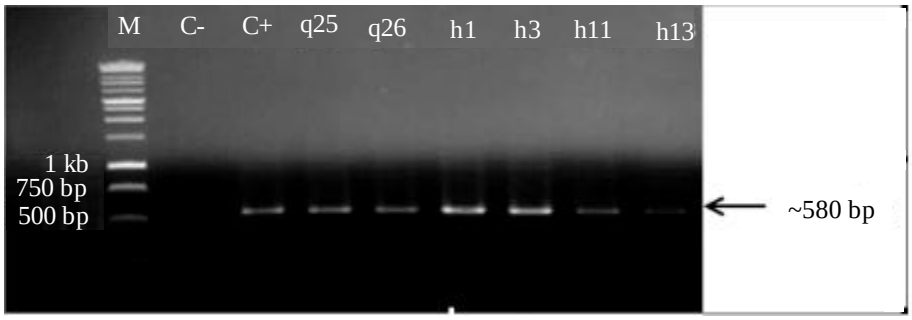

Fig. 2: Gel electrophoresis for PCR product using the degenerate primers AvCore and AcCore; Lanes q25 and q26 samples from Qasim and h1, h3, h11, h13 samples from Hail as shown are positive, C^+ = positive control and C^- = Negative control

the obtained sequences for Saudi Arabian isolates (h1, h3, h11, h13, q25 and q26) and representative sequences of TYLCV isolates from Iran, Yemen, Sudan, Israel, Egypt, Korea and others that are available in GeneBank as shown in Table 2. The phylogenetic tree showed that all TYLCV isolates in this study are in same cluster with the isolates from Sudan and Yemen. The

Table 3: Showing the different amino acids between the different isolates of TYLCV (h1, h3, h11, h13, q25 and q26) and its location

Isolate code						
q26	q25	h13	h11	h3	h1	Amino-acid possession
T	T	T	T	S	T	14
Y	Y	S	Y	Y	Y	143
C	C	C	R	C	C	149
Y	Y	Y	S	Y	Y	158
N	N	N	S	N	N	159
G	G	R	R	R	G	169
H	H	L	L	H	H	174
E	D	E	E	M	E	176
A	A	A	A	V	A	178
L	L	V	V	L	L	179

T: Therionine, S: Serine, Y: Tyrosine, C: Cysteine, R: Arginine, N: Asparagine, G: Glysine, H: Histidine, L: Leucine, E: Glutamic, M: Methionine, D: Aspartic, A: Alanine and V: Valine

analysis also showed that the highest nucleotide identities were 98 and 95% obtained with members of the species, TYLCV from Sudan: *Tomato leaf curl Sudan virus* [TYLCV-Man-SD] (HE819244) and [TYLCV-Had-SD] (JF919733) respectively, but was more than 97% among the isolates in this study. While it was 94% with *Tomato leaf curl Gezera virus* [TYLCV-Jez-SD] (AY044137) and 92% with *Tomato leaf curl virus* from Yamen [TYLCV-Yem] (EF110891). Nevertheless, the identity was less than 74% with TYLCV isolate from Egypt, Israel, Korea, Iran and others (Fig. 3). Hence, the Alignment identity of the protein sequences for obtained protein from the sequences of those isolates were between 97-100%. On this note, Fig. 4 and 5 and Table 3, showed that the identity was 100% between h1 and q26 but no amino acid different was 99% with q25 using only one amino acid different which is D (Aspartic) instead of E (-Glutamic)on the possession No 176. The identity between h3 and q25, q26 and h1 was 98% with 4 amino acids deferent in the possession No. 14, 169, 176 and 178. Finally the identity between h11 and h13 (which were more related together) with previous isolates was 97%.

DISCUSSION

The name TYLC has been used before to characterize viral diseases affecting tomato crops occurring within a broad tropical and subtropical belt, worldwide (Green and Kalloo, 1994). The result in this study has shown that the multiplex PCR with virus-specific primers for the Mediterranean TYLCV isolates used by Anfoka *et al.* (2008), did not succeed in detecting the TYLCD-associated viruses in symptomatic tomato plants collected from infected fields in Saudi Arabia. However, the degenerate primers of AVcore and ACcore designed by Brown *et al.* (2001) for Geminiviruses succeeded in amplifying the expected band size of Geminiviruses of the tested infected samples. The percentage of infections were 72 and 92.5% for collected samples from Qasim and Hail, respectively, with typical TYLCV symptoms as described by (Gafni, 2003; Crescenzi *et al.*, 2004; Ajlan *et al.*, 2006; Zambrano*et al.*, 2007; Abd El-Monem *et al.*, 2011). Hence, this result is referred to as the collected symptomatic tomato samples infected with Geminiviruses. And so, the nucleotide sequences were carried out to characterize the virus name and strain. Nevertheless, the results of nucleotide sequences of the amplified fragments indicated that all tested samples relate to Tomato leaf curl virus. Thus, the highest nucleotide identities (94, 95 and 98%)

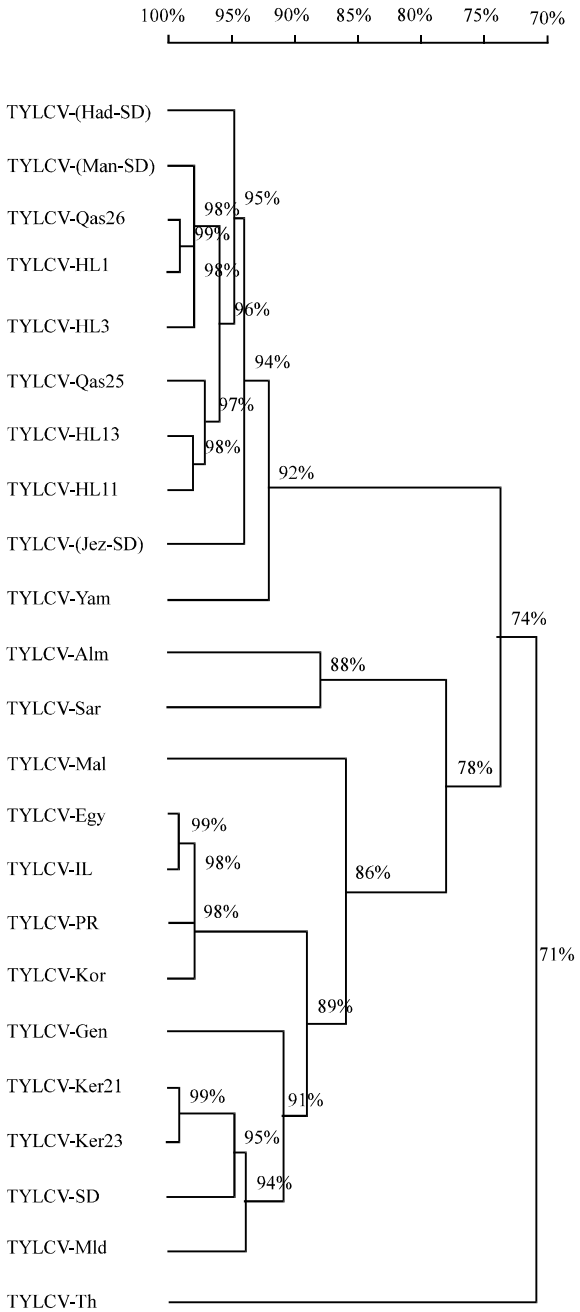

Fig. 3: Neighbor joining tree shows the genetic distance among the core region of the coat protein gene of TYLCV genomes. The sequences were either obtained in this study or selected from GenBank and were aligned with the Optimal Alignment method of DNAMAN. Notwithstanding, the tree was set up with a Jukes and Cantor distance matrix using the Neighbor Joining method of DNAMAN

were obtained among members of the species Tomato leaf curl Sudan virus from Sudan: Tomato leaf curl Sudan virus (HE819244) identify by Khan *et al.* (2012), JF919733 identified by Idris *et al.* (2012) and AY044137 that was identified by Idris and Brown (2005). Although the

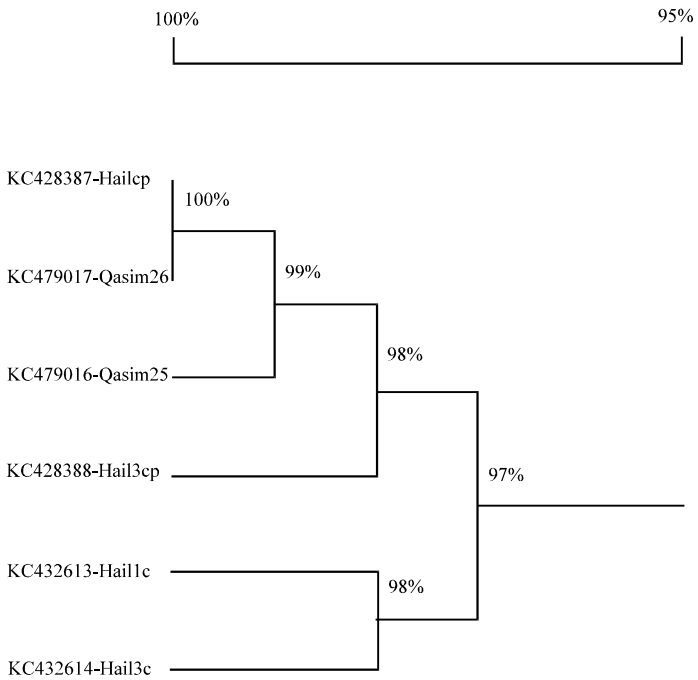

Fig. 4: Homology tree showing the protein homology among the core region of the coat protein of the TYLCV isolates (h1, h3, h11, h13, q25 and q26)

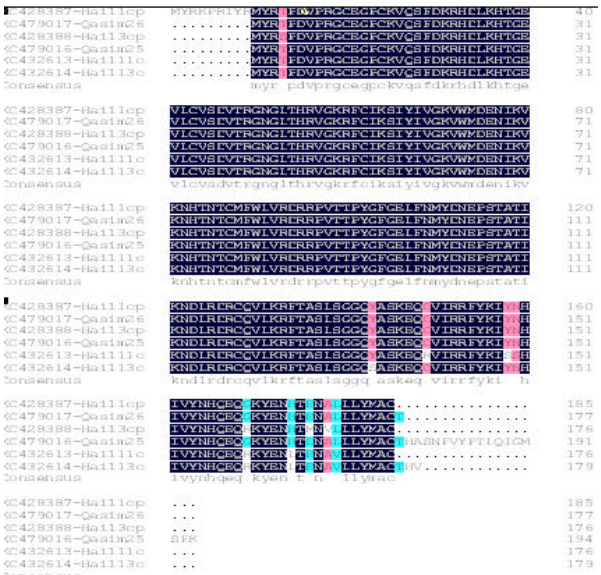

Fig. 5: Protein alignment of the core region of coat protein for TYLCV isolates (h1, h3, h11, h13, q25 and q26)

identity was 92% with Tomato leaf curl virus from Yemen (EF110891) that identified by Isakeit *et al.* (2005). Notwithstanding, the identity was less than 74% with TYLCV isolates from

Korea, Iran and all the Mediterranean TYLCV isolates such as Egypt, Jordan and Israel. In like manner, this result is in line with Idris *et al.* (2012) who sequential analysis of the complete genome of TYLCV strains from Sudan (ToLCSDV) and Yemen (ToLCYEB) and found that those strains shared 86-91% identity with the previously described ToLCSDV from the Nile Basin. Despite that this is the first study of the nucleotide sequences for the TYLCV-Saudi Arabian isolates; it's result referred to the Saudi Arabian isolates closely near to Sudan and Yemen isolates and far away from Mediterranean TYLCV isolates. In addition, some previous studies for the incidence of TYLCV were conceded but all the studies were based on symptoms observation and serological testes. Moreover, TYLCV was detected by Ajlan *et al.* (2006) in Saudi Arabia using ELISA tests and PCR with degenerate primers but they do not determine actually what strain that are found. Therefore, this the first study on genome sequence of this virus in Saudi Arabia and in GenBank as well as the first record of sequences if TYLCV isolates from this expanded country. On this note, we can conclude from this study that there is begomovirus associated with tomato in Saudi Arabia showed high CP gene sequence similarity with TYLCV-Sudan and Yemen. To identify exactly the viral species or strain of the obtained begomovirus the full length of nucleotide sequences will carried out in the next studies. However, as a result of the expanded area and diversity of climate of the country, there are many expected strains that can be found in Saudi Arabia which will however be our interest in next studies.

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