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Characterization, Genetic Diversity of *Tomato Yellow Leaf Curl Virus* Egyptian Isolate

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A B S T R A C T

Tomato yellow leaf curl Virus (TYLCV) threatens the production of tomatoes both in Egypt and the world. A virus was isolated from naturally infected tomato and pepper plants, grown in different areas in Egypt. The investigated isolate was characterized using electron microscopy, serology, biological tests, and polymerase chain reaction (PCR) using degenerate primers were used to amplify partial sequences (~530 bp) of the begomovirus coat protein gene (Cp) from samples of diseased plants. DNA sequence analyses of Egyptian isolate revealed high nucleotide sequence identities to isolates of TYLCV (99%) in GenBank. TYLCV-EG encodes to 176 amino acids residues with MW 19.651 KDa. Twenty amino acids were detected of TYLCV-EG starting with Alanine (A) and ending with Tyrosine (Y). Phylogenetic analysis of Cp gene sequence of TYLCV-EG suggesting that the sequence variations observed in this isolate and some of those identified and published on GenBank may be attributable to intra-specific recombination events involving some TYLCV isolates. The great variability of the TYLCV isolates worldwide should be considered when breeding programs for virus resistance are established. A tomato line tolerant/resistant to a particular TYLCV isolate may not be as effective against another distantly related virus isolate.

Key words: *Tomato, pepper, PCR, and sequencing analyses, statistical analysis*

Introduction

Tomato yellow leaf curl virus (TYLCV) is widely distributed in Egypt and has been reported as a main problem for tomato crop production in both open field and protected agricultures in different governorates, and it causes yield losses up to 100% Aref et al. (1994). Moustafa (1991) recorded that 100% of the fall- grown tomato plants are usually infected with TYLCV and production losses may reach 80%. TYLCV belongs to the family Geminiviridae, genus Begomovirus, and transmitted by the whitefly *Bemisia tabaci* (Hemiptera; Aleyrodidae) in a circulative and persistent manner (Huang et al. 2012; Götz et al. 2012). TYLCV has been found to be the causal agent of a novel disease of common bean (*Phaseolus vulgaris* L.) Campos et al. (1999) and also occurs in peppers (*Capsicum annuum*) (Reina et al. 1999; Góngora-Castillo et al. 2012). TYLCV consists of geminate, quasi-isometric particles which have been measured at 20 nm in diameter and 30 nm in length. The virus genome is composed of a single monopartite circular single-stranded DNA molecule of about 2.8 Kb nucleotides that encodes a replication-associated protein (Rep), the coat protein (CP), and proteins such as the replication enhancer protein (REn), and the transcription activator protein (TrAP), that participate in the control of replication and gene expression. Open reading frames (ORFs) are organized bi-directionally, separated by an intergenic region (IR) that contains key elements for the replication and transcription of the viral genome, including the origin of replication (Gronenborn 2007). Several techniques such as enzyme linked immuno-sorabent assay (ELISA) and dot-blotting immuno-binding assay (DBIA), as well as polymerase chain reaction (PCR) provide a sensitive and specific for detection and identification of whitefly transmitted geminiviruses (WTGV) in the infected plants (Tsai et al. 2006; Gorovits et al. 2013).

In this paper, a new strain of TYLCV was isolated and analysis it molecular relationship with the type strains of TYLCV in GenBank.

Materials and methods

Source of the virus isolate

Naturally infected tomato (*Solanum lycopersicum* cv. Castle Rock) and pepper (*Capsicum annuum* cv. Chilli) plants exhibiting typical symptoms of geminivirus were collected from El-Khataba, El-Behera governorate and Qaha and Faculty of Agriculture, University of Ain Shams farm, El-Qaluobiya governorate in April and May 2010. Twenty and sixteen tomato samples were collected from El-Khataba and Qaha, respectively. As well as, twenty and twenty-five pepper samples were collected from El-Khataba and University of Ain Shams farm, respectively.

Detection of TYLCV

The Indirect-ELISA technique was applied as described by Clark and Adams (1977) to detect of TYLCV using polyclonal antibodies obtained from virology lab, Faculty of Agriculture, University of Ain Shams.

Mode of transmission

Syringe injection:

Healthy tomato plants cv. Super Marmand were inoculated by syringes using infected tomato sap according to (Allam et al. 1994). The inoculated plants as well as uninoculated ones were kept under greenhouse conditions and symptoms were observed daily up to 60 days. Syringe transmission efficiency was recorded as a number of infected plants / total number of inoculated plants.

Insect transmission

Whiteflies *Bemisia tabaci* biotype B belongs to family Aleroididae were collected from tomato plants grown in open fields and identified by the Department of Plant Protection, Faculty of Agriculture, Ain Shams University. Virus-free whiteflies were used as vectors in transmission experiment and insect transmission was done as previously described by (Ghanem et al. 2001). About twenty insects allowed feed on infected tomato cv. Super Marmand plants in insect proof cages. After 24 hrs acquisition access period, the insect allow to feed for 72 hrs on healthy hosts then the whiteflies were removed by spraying the tomato plants by KZ oil 95% EC and left for symptoms development. Insect inoculated plants were observed daily for a period of about 60 days.

Differential hosts and symptomatology

Five plants (21 days age) of each different plant species and cultivars belonging to six families (Table 1) were inoculated by sap mechanically and whitefly with the studied virus isolate under greenhouse conditions at (25-28ºC). External symptoms were observed daily for 60 days and confirmed by indirect-ELISA as described by Clark and Adams (1977) and dot blot immunoassay (DBIA) as described by (Abdel-Salam 1999; Ghanem et al. 2003).

Virus purification

TYLCV was purified from 100 g of infected tomato plants according to the protocol of (Black et al. 1963) and visualized after negative staining of the virus preparation with (2% Uranyle acetate pH 7.0) (Noordam 1973), then was examined by electron microscope at the Electron Microscope Unit, University of Al-Azhar.

Extraction of total DNA

DNA was extracted from one gram of TYLCV infected leaves of *Lycopersicon esculentum* cv. Super Marmande, *Capsicum annuum* cv. Chilli, Pelargonium sp., *Datura metel* and *Chrysanthemum morifolium*, as well as grafted tomato plants using cetyl tri-methyl Ammonium Bromide (CTAB) method as described by Gibbs and Mackenize (1997). The yield DNA was measured by spectrophotometer and electrophoresis on agarose gel.

PCR amplification

A pair of degenerate oligonucleotide primers Brown et al. (2001) was to prime the amplification of DNA fragments of the TYLCV strains. V324 (+) primer corresponding to (5'-GCC YAT RTA YAG RAA GCC MAG-3') and C889 (-) primer corresponding to (5'-GGR TTD GAR GCA TGH GTA CAT G-3'). PCR reaction mixture consists of 2.5 µl of extracted DNA, 10 mM of each dNTPs (0.5 µl), 1 µl of 25 pmole from amplification primer V324 (+), 2.5 µl of 10X PCR buffer with 1.5 mM $MgCl_2$ and 0.5 µl Taq DNA polymerase (Roche). The amplification reaction was mixed with 18 µl of deionized H_2O to a total volume of 25 µl using PCR thermal cycler, UNOII from Biometra and using 0.2 ml micro Amp PCR tubes. Denaturation of the DNA was performed at 95ºC for 2 min, followed by 35 cycles of amplification with denaturation at 94ºC for 30 sec, annealing at 50ºC for 45 sec, and extension at 72ºC for 1 min. A single tailing cycle of long extension at 72ºC for 7 min was carried out in order to ensure flush ends on the DNA molecules. Finally, the amplification reactions were hold at 4°C. 5 µl of each PCR product was analyzed by electrophoresis in 1.5% agarose gel in 1X TAE buffer (0.04 M Tris acetate, 0.001 M EDTA, pH 8.0).

Purification of DNA amplicons

PCR products of TYLCV were purified by using GFX column and gel band purification kit (Amersham pharmaia Biotech, GmbH, Germany).The DNA samples were left in elution buffer at room temperature for 1 min and centrifuged at full speed for 1 min to recover the purified DNA.

Sequencing analysis

The partial nucleotide sequence of the Egyptian TYLCV isolate (TYLCV-EG) was aligned with TYLCV sequences available in the GenBank database using BLAST algorithm (http://www.ncbi.nlm.nih.gov) (Köklü et al. 2006). Multiple alignments and phylogenetic analysis of TYLCV sequencing were carried out using the programs (CLC sequence viewer V 6.8.1, Denmark) and (DNAMAN V 5.2.9 package, Madison, Wisconsin, USA), respectively. Homology trees were set up with the distance matrix using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (Sneath and Sokal 1973).

Results

Biological characterization of TYLCV isolate

Field inspection of TYLCV was determined firstly according to geminivirus symptoms. Tomato and pepper plants naturally infected with TYLCV showed systemic viral symptoms in the form of severe leaf curling, leaf crinkle with marginal yellowing, stem upright, twisted and stunted (Fig. 1).TYLCV was detected in naturally infected tomato and pepper plants exhibited geminivirus symptoms by indirect–ELISA using specific polyclonal antibody.

Host range and symptomatology

TYLCV was tested on nineteen plant species and cultivars belonging to eight families (Figs. 2 & 3 and Table 1). TYLCV showed different susceptibility to the tested plants when inoculated by syringe and whitefly. From nineteen tested plant species and cultivars, fourteen plants belonging to five families reacted systemically with TYLCV. These plants were *Beta vulgaris*, *Cucurbita pepo* cv. Eskandrani, *C. maxima*, *Cucumis sativus* cv. Alpha, *Glycine max*, *Chrysanthemum Morifolium*, *Phaseolus vulgaris*, *Capsicum annuum* cv. Chili, *Datura stramonium*, *D. metel*, *L. esculentum* cv. Super Marmande, *Nicotiana glutinosa*, *N. tabacum* cv. Samson and *N. tabacum* cv. White Burley. Seven plants showed no symptoms and not reacted systemically with TYLCV. These plants were Chenopodium amaranticolor, *Lactuca sativa*, *Pisum sativum*, *Pelargonium* sp., *Vicia faba*, *Zea mays* and *N. rustica*. The results were confirmed by indirect-ELISA and DBIA.

Morphology of Virus Particle

Electron microscopic examination of partially purified preparation of TYLCV revealed the presence of isometric particles, with single and paired geminivirus (monomers and dimmers), when negatively stained with 2% Uranyl acetate pH 7.0. The dimension of single particle is 22 nm and paired particle ranged from 20X30 nm to 24X30 nm (Fig . 4).

Identification of TYLCV by PCR

The total prepared DNA was amplified by PCR using degenerate oligonucleotides V324 (+) and C889 (-) as PCR primers. The size of the PCR product was estimated by comparing its electrophoretic mobility with those of standard DNA marker as shown in (Fig. 5).The amplified DNAs were in the expected size calculated (~530 bp).

Sequence data and phylogenetic Analysis

Automated DNA sequencing:

The partial nucleotide sequence of the TYLCV-EG isolate from *L. esculentum* cv. Super Marmande has been submitted to GenBank under the following accession number (JX901286). The TYLCV-CP-EG gene of isolate under study was aligned with the sequences from different geographical locations using (CLC sequence viewer multiple alignments programme) as shown in (Fig. 6). The Egyptian isolate had highest identity of 99% with Egyptian isolate (AY594174.1) available in GenBank. In this paper, we want compare between Egyptian isolate under study and geographically different TYLCV isolates in GenBank. 94.5 % with AJ867487.1 (TYLCV Mugla 2, Turkey) isolate, followed by 93.7% with EF429312.1 (TYLCV Fexgxian, China) and had lowest homology of 93.2% with FJ030876 (TYLCV-H11, Egypt) (Table 6). A phylogenetic tree was carried, including the partial nucleotide sequence of the *Cp* core region to the Egyptian isolate (TYLCV-EG) and other TYLCV isolates from other regions of the world. The analysis showed a close relationship between TYLCV-EG and TYLCV-Turkey (bootstrap value 94.5%) (Fig. 7).

Statistical analysis of DNA alignment sequence

Statistical analysis of alignment sequence for TYLCV-EG with other TYLCV isolates available from GenBank (Table 2) showed that molecular characters TYLCV-EG, the molecular weight (١٦٢,٢٧٧ kDa), base composition (A=

١٤٧, C=٩٩ , G=١٤٠ and T=1٤4), (C+G)= 239 and (A+T)= 291 and Frequencies nucleotides (A= 0.277, C= 0.١٨٧, G= 0.2٦٤ and T= 0.2٧2).

Table 1. Host range of TYLCV as determined by syringe injection and whitefly (B.tabaci biotype B) transmission. Existence of virus was confirmed by indirect-ELISA and DBIA.

Host plants	Syringe injection				Whitefly inoculation			
	Symptoms	I.P (weeks)	O.D.	DBIA	Symptoms	I.P (weeks)	O.D.	DBIA
Chenopodiaceae								
B. vulgaris	LC,E	3-4	+	+	LC,E	3-4	+	+
Ch. amaranticcolor	--	4	-	-	-	4	-	-
Compositae								
L. sativa	--	4	0.254	-	--	4	-	-
Cucurbitaceae								
Cucurbita maxima	LK	4	+	+	--	4	-	-
C. pepo cv. Eskandrani.	--	3-4	-	-	LM, LK	4	+	+
Cucumis sativus	LK,B	4-5	+	++	LK	4	+	+
C. lanatus	--	5	-	-	--	4	-	-
Fabaceae								
Glysine max	LC,R	3-5	+	++	LC,R	2-5	+	++
Phaseolus vulgaris	LK,Mal	3-5	+	++	LK,N,Mal	2-5	+	++
Pisum sativum	--	5	-	-	--	4	-	-
Vicia faba	--	5	-	-	--	4	-	-
Geraniaceae								
Pelargonium sp.	--	5	-	-	--	4	-	-
Asteracea								
Chrysanthemum morifolium	LK,B	5	+	+	LK,B	4	+	+
Graminea								
Zea mays	--	4	-	-	--	4	-	-
Solanaceae								
Capsicum annuum cv.Chilli	LK,LC,LN	4-5	+	+++	Lk,B,S,Mal	3-5	+	++
Datura stramonium								
D. metel	LK,E,S, LK,Mal	2-4	+	++	LK,E,S,Mal	2-4	+	++
L. esculentum	LK,LC,MY,S	4	+	+	Mild LK	4	+	+
cv. Super Marmande		2-4	+	+++	LK,LC,MY,	3-6	+	++
Nicotiana glutinosa	LC,R,B,S,Mal				SU, ST,S			
N. rustica	--	2-5	+	++	B	2-5	+	++
N. tabacum		5	-	-	--	5	-	-
cv. White Burley	VC							
cv .Samson	VC	4	+	+	VC	4	+	+
		4	+	+	VC	4	+	+

I.P.=incubation period, O.D. =optical density, DBIA=dot blot immunoassay, LC=leaf curling, LM=leaf malformation, LN=leaf narrowing,
E= epinasty LK=leaf crinkle, R=rugosity, Mal=malformation, N= necrosis, B=blistering, S=stunting, MY=marginal yellowing, SU, stem upright, ST=stem twisting, VC=vein clearing. Negative control = 0.135, (++) strong positive reaction, (+) weak positive reaction, (-) negative reaction, (--) symptomless.

Table 2. Statistical analysis of alignment sequence and proteins available of GenBank.

Comparison	TYLCV-isolates						
	EF429311.1	EF028239.1	EF429312.1	FJ030876.1	AJ867487.1	JX901286	EU625369.1
Nucleic acid (N.A)	DNA	DNA	DNA	DNA	DNA	DNA	DNA
Length of N.A (nt)	553	545	551	567	579	530	545
M.W (kDa)	169,075	166,767	166,567	173,397	177,071	162,277	165,022
No. of nucleotides base composition							
Adenine(A)	164	159	162	168	174	147	161
Cytosine(C)	89	89	136	96	96	99	140
Guanine(G)	134	137	87	138	140	140	92
Thymine(T)	166	160	166	164	169	144	152
C + G	223	226	223	234	236	239	232
A + T	330	319	328	332	343	291	313
Frequencies nucleotides							
A	0,297	0,292	0,294	0,296	0,301	0,277	0,295
C	0,161	0,163	0,247	0,169	0,166	0,187	0,257
G	0,242	0,251	0,158	0,243	0,242	0,264	0,169
T	0,300	0,294	0,301	0,289	0,292	0,272	0,279
statistical analysis for protein							
Length of amino acid	184 aa	181 aa	183 aa	189 aa	193 aa	176 aa	181 aa
M.W (kDa)	21,312	21,255	20,494	21,942	22,411	19,651	20,295
Isoelectric point	6,64	10,06	9,62	10,08	6,76	9,05	10,33

Table 3. Amino acids counts of TYLCV-EG with those of the other TYLCV proteins.

No.	Amino acid and symbol	TYLCV-isolates						
		EF429312.1	EF028239.1	EF429311.1	FJ030876.1	AJ867487.1	JX901286.1	EU625369.1
1	Alanine (A)	7	6	3	3	9	8	6
2	Cysteine (C)	6	5	4	6	8	5	4
3	Aspartic Acid (D)	3	10	3	10	3	5	4
4	Glutamic Acid (E)	10	7	4	6	11	9	0
5	Phenylalanine (F)	6	12	8	11	7	7	10
6	Glycine (G)	13	12	10	14	12	11	8
7	Histidine (H)	2	7	11	4	3	3	2
8	Isoleucine (I)	8	7	24	8	7	14	8
9	Lysine (K)	5	14	7	14	6	6	6
10	Leucine (L)	12	4	21	7	10	21	27
11	Methionine (M)	2	7	4	7	3	9	4
12	Asparagine (N)	8	9	15	7	8	3	6
13	Proline (P)	6	10	9	10	8	6	11
14	Glutamine (Q)	6	9	4	8	8	3	3
15	Arginine (R)	7	17	6	19	7	11	12
16	Serine (S)	22	10	19	13	20	20	18
17	Threonine (T)	3	8	15	7	3	9	20
18	Valine (V)	9	17	8	20	11	15	11
19	Tryptophan (W)	9	3	2	1	9	4	1
20	Tyrosine (Y)	13	7	3	8	13	2	6

Table 4. Amino acids Frequencies of TYLCV-EG with those of the other TYLCV proteins.

No.	Amino acid and symbol	TYLCV-isolates EF429312.1	EF028239.1	EF429311.1	FJ030876.1	AJ867487.1	JX901286.1	EU625369.1
1	Alanine (A)	0,038	0,033	0,016	0,016	0,047	0,045	0,033
2	Cysteine (C)	0,033	0,028	0,022	0,032	0,041	0,028	0,022
3	Aspartic Acid (D)	0,016	0,055	0,016	0,053	0,016	0,028	0,022
4	Glutamic Acid (E)	0,054	0,039	0,022	0,032	0,057	0,051	0,000
5	Phenylalanine (F)	0,033	0,066	0,044	0,058	0,036	0,040	0,055
6	Glycine (G)	0,071	0,066	0,055	0,074	0,062	0,062	0,044
7	Histidine (H)	0,011	0,039	0,060	0,021	0,016	0,017	0,011
8	Isoleucine (I)	0,043	0,039	0,131	0,042	0,036	0,080	0,044
9	Lysine (K)	0,027	0,077	0,038	0,074	0,031	0,034	0,033
10	Leucine (L)	0,065	0,022	0,115	0,037	0,052	0,119	0,149
11	Methionine (M)	0,011	0,039	0,022	0,037	0,016	0,051	0,022
12	Asparagine (N)	0,043	0,050	0,082	0,037	0,041	0,017	0,033
13	Proline (P)	0,033	0,055	0,049	0,053	0,041	0,034	0,061
14	Glutamine (Q)	0,033	0,050	0,022	0,042	0,041	0,017	0,017
15	Arginine (R)	0,038	0,094	0,033	0,101	0,036	0,062	0,066
16	Serine (S)	0,120	0,055	0,104	0,069	0,104	0,114	0,099
17	Threonine (T)	0,016	0,044	0,082	0,037	0,016	0,051	0,110
18	Valine (V)	0,049	0,094	0,044	0,106	0,057	0,085	0,061
19	Tryptophan (W)	0,049	0,017	0,011	0,005	0,047	0,023	0,006
20	Tyrosine (Y)	0,071	0,039	0,016	0,042	0,067	0,011	0,033

Table 5. Nucleic acid sequence homology between TYLCV-EG and different TYLCV isolates in GenBank.

Accession number	FJ030876.1	◌EF429312.1	AJ867487.1	JX901286.1	EF028239.1	EF429311.1	EU625369.1
FJ030876.1	100%						
◌EF429312.1	97.7%	100%					
AJ867487.1	97.7%	98.3%	100%				
JX901286.1	93.2%	93.7%	94.5%	100%			
EF028239.1	97.1%	96.7%	97.3%	92.6%	100%		
EF429311.1	55.4%	56.8%	55.3%	44.9%	55.2%	100%	
EU625369.1	52.3%	53.1%	51.5%	44.5%	51.8%	62.5%	100%

A B

Figure 1. Naturally infected tomato cv. Castle Rock (A) and pepper cv. Chilli (B) showing severe leaf curling, leaf crinkle with marginal yellowing, stem upright, twisted and stunting.

Table6.

1. *V. faba* (Syringe injection) (-)	2. *C. annuum* cv. Chilli (Syringe injection) (+)	3. *L. esculentum* cv. Super Marmande (Syringe injection) (+)	4. *D. metel* (Syringe injection) (+)	5. *D. stramonium* (Syringe injection) (+)	6. *Chrysanthemu-m morifolium* (Syringe injection) (+)
12 .*V. faba* (Whitefly transmission) (-)	11. *C. annuum* cv. Chilli (Whitefly transmission) (+)	10.*L. esculentum* Super Marmande (Whitefly transmission) (+)	9. *D. metel* (Whitefly transmission) (+)	8. *D. stramonium* (Whitefly transmission) (+)	7. *Chrysanthemu-m morifolium* (Whitefly transmission) (+)

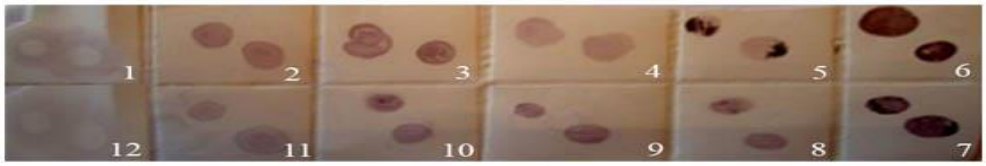

Figure 2. Dot blot immunoassay of TYLCV-syringe and whitefly inoculated plants.
(+) = positive reaction (-) = negative reaction.

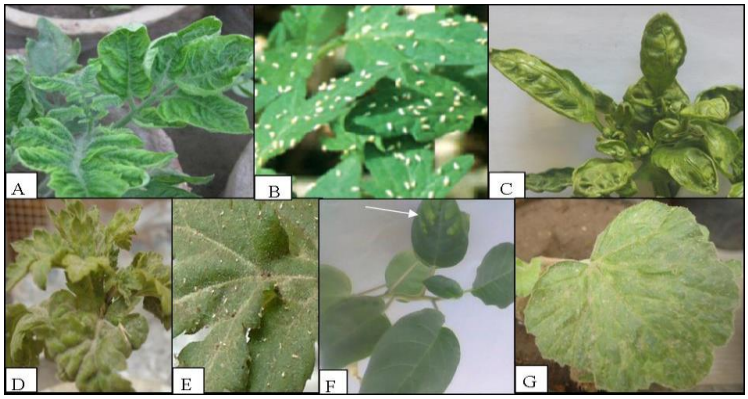

Figure 3. Differential plants inoculated with TYLCV infected tomato plants by whiteflies and showing geminivirus symptoms on (A) *L. esculentum* showing: leaf crinkle, marginal yellowing, stem upright, enations and leaf curling (Cup shape leaves), (B) Whiteflies inoculate TYLCV in *L. esculentum*,(C) *Capsicum annuum* showing: Leaf crinkle, blistering, stunting and malformation, (D) *Chrysanthemum morifolium* showing: Leaf crinkle and blisters, (E) Whiteflies inoculate TYLCV in *Ch. morifolium*, (F) *D. metel* showing: Vein yellowing and mild leaf crinkle and (G) *Cucurbita pepo* showing: Leaf crinkle and blistering.

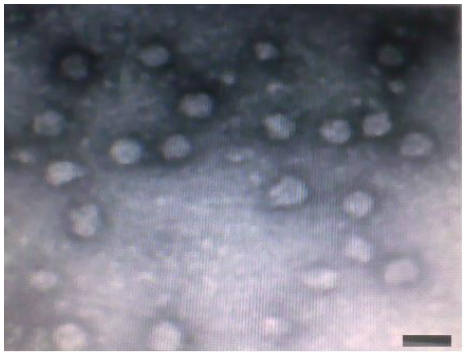

Figure 4. Electron micrographs showing: partially purified TYLCV negatively stained with 2% Uranyl acetate, bar represents 100 nm.

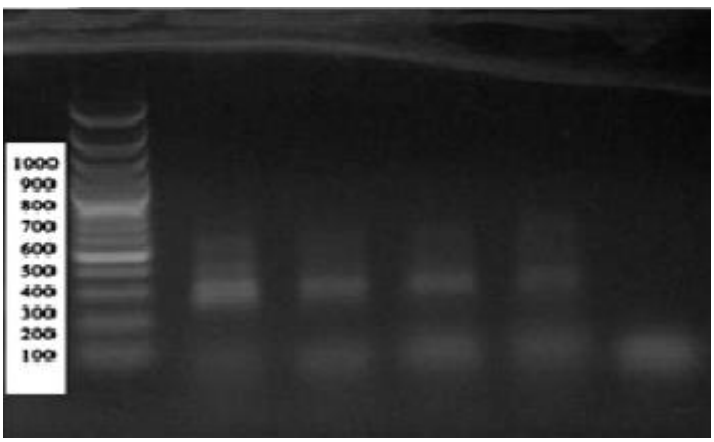

Figure 5. 1.5% agarose gel electrophoresis showed PCR products (~530 bp) of Cp isolated from TYLCV naturally infected plants using degenerate primer. Lane 1: *Lycopersicon esculentum* cv Super Marmande, lane 2: *Capsicum annuum* cv. Chili, lane 3: *Chrysanthemum morifolium* and lane 4: *Datura metel*. Lane 5: Control sample and lane M: 100 bp DNA ladder size marker.

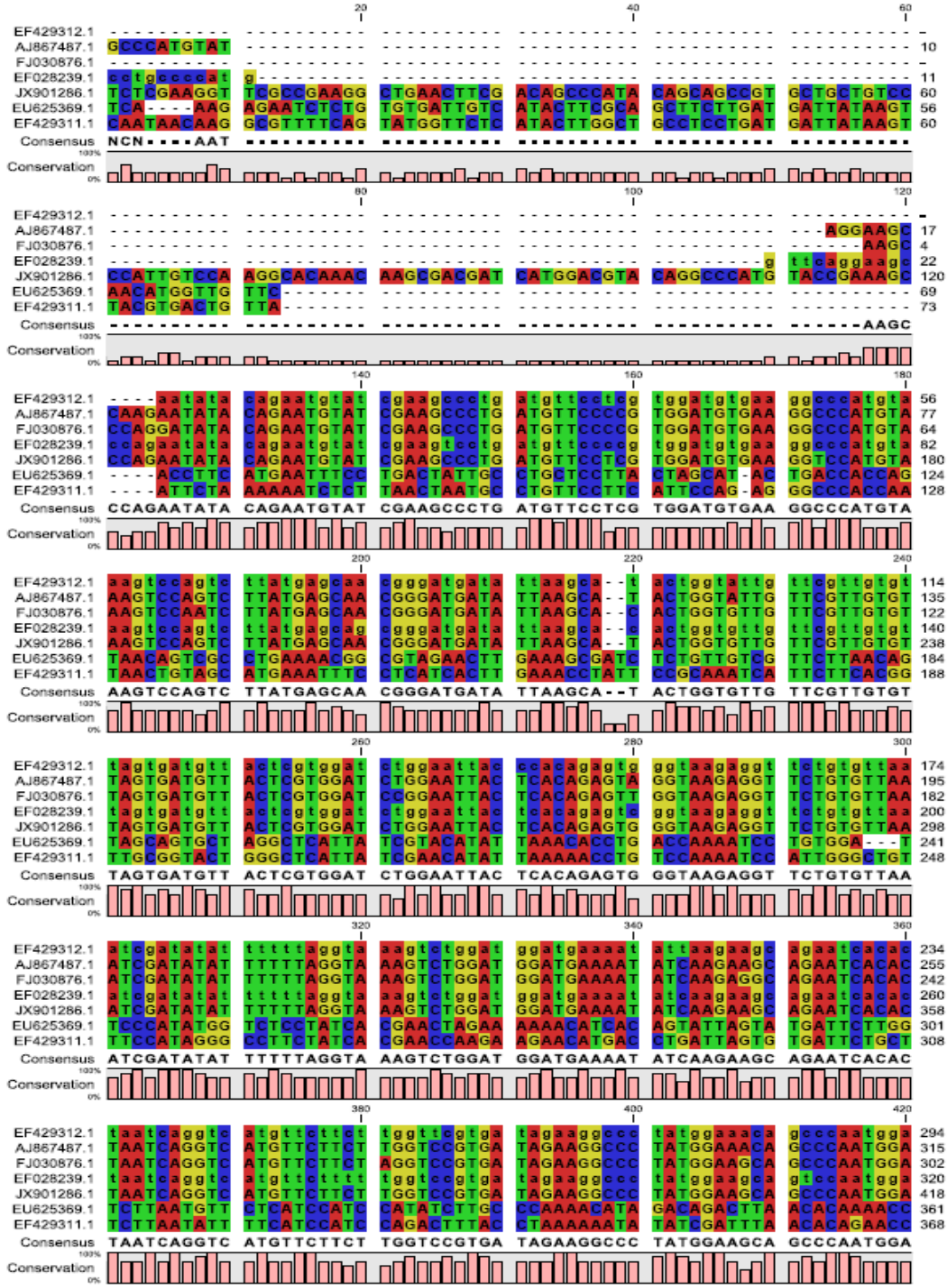

Figure 6 . Cluster multiple sequence alignment of TYLCV isolates based on the nucleotide sequences of the *Cp* gene. Isolates indicated above were as following: TYLCV-current study, JX901286 {(coat protein (*Tomato yellow leaf curl virus*-EG)}, EU625369.1 {(coat protein (*Tomato yellow leaf curl virus*)}, EF429312.1 {(coat protein [*Tomato yellow leaf curl virus* – Fengxian)} , AJ867487.1 {(coat protein (*Tomato yellow leaf curl virus* – Mugla-2)}, EF028239.1 {(Coat protein [*Tomato yellow leaf curl virus*-[SJC]} , FJ030876{(coat protein [*Tomato yellow leaf curl*-[H11]} and EF429311.1{(coat protein (*Tomato yellow leaf curl virus* - Sunqiao)}.

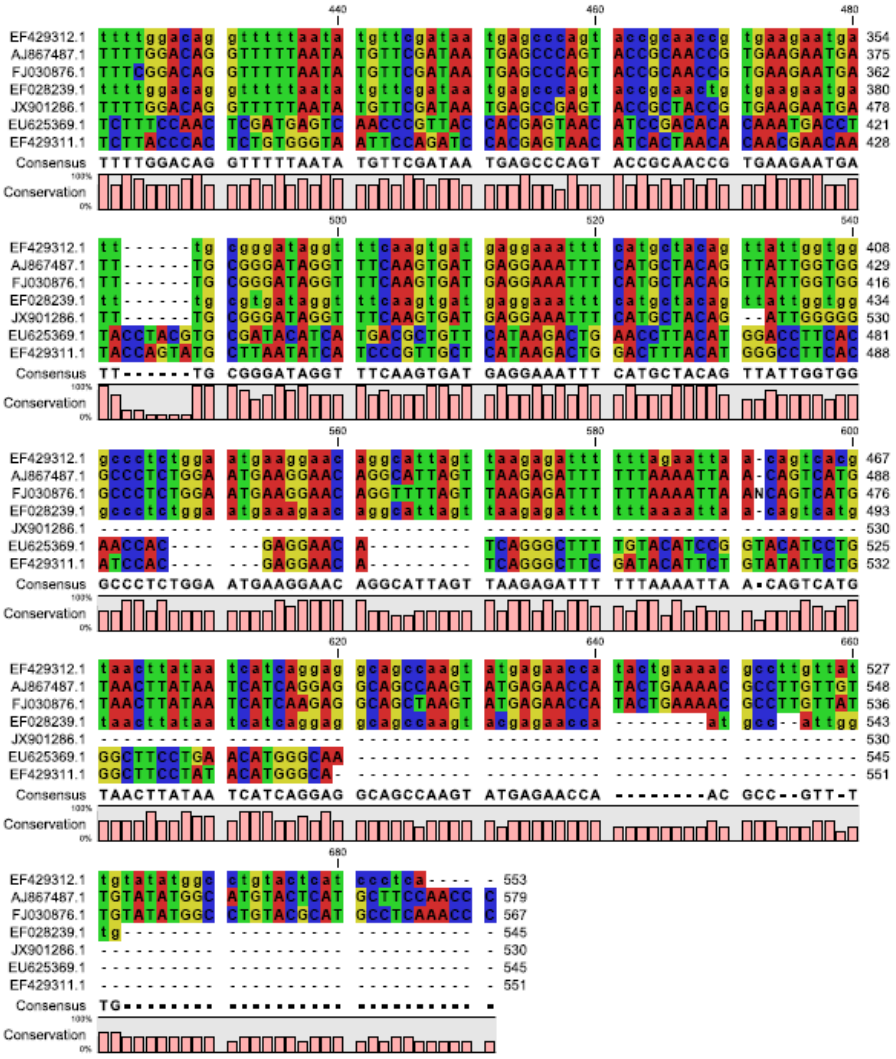

Figure 7. Cluster multiple sequence alignment of TYLCV isolates based on the nucleotide sequences of the *Cp* gene. Isolates indicated above were as following: TYLCV-current study, JX901286 {(coat protein (*Tomato yellow leaf curl virus*-EG)}, EU625369.1 {(coat protein (*Tomato yellow leaf curl virus*)}, EF429312.1 {(coat protein [*Tomato yellow leaf curl virus* – Fengxian)} , AJ867487.1 {(coat protein (*Tomato yellow leaf curl virus* – Mugla-2)}, EF028239.1 {(Coat protein [*Tomato yellow leaf curl virus*-[SJC]} , FJ030876{(coat protein [*Tomato yellow leaf curl*-[H11]} and EF429311.1{(coat protein (*Tomato yellow leaf curl virus* - Sunqiao)}.

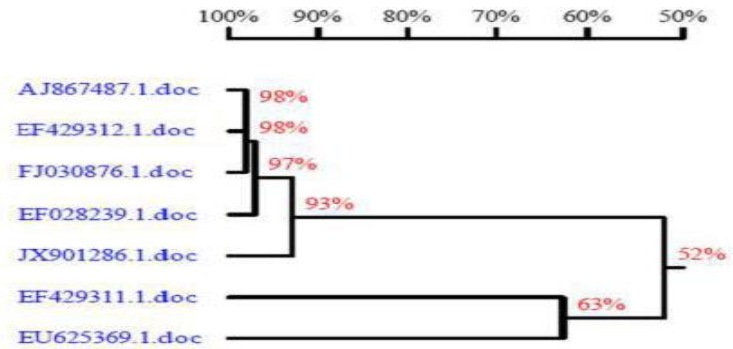

Figure 8. Phylogenetic tree constructed using UPGMA analyses showing predicted relationships between TYLCV isolates based on the nucleotide sequences of the *Cp* gene. Isolates indicated above were as following: TYLCV-current study, JX901286 {(coat protein (*Tomato yellow leaf curl virus*-EG)}, EU625369.1 {(coat protein (*Tomato yellow leaf curl virus*)}, EF429312.1 {(coat protein [*Tomato yellow leaf curl virus* – Fengxian)} , AJ867487.1 {(coat protein (*Tomato yellow leaf curl virus* – Mugla-2)}, EF028239.1 {(Coat protein [*Tomato yellow leaf curl virus*-[SJC]} , FJ030876{(coat protein [*Tomato yellow leaf curl*-[H11]} and EF429311.1{(coat protein (*Tomato yellow leaf curl virus* - Sunqiao)}. Scale represents percent homology.

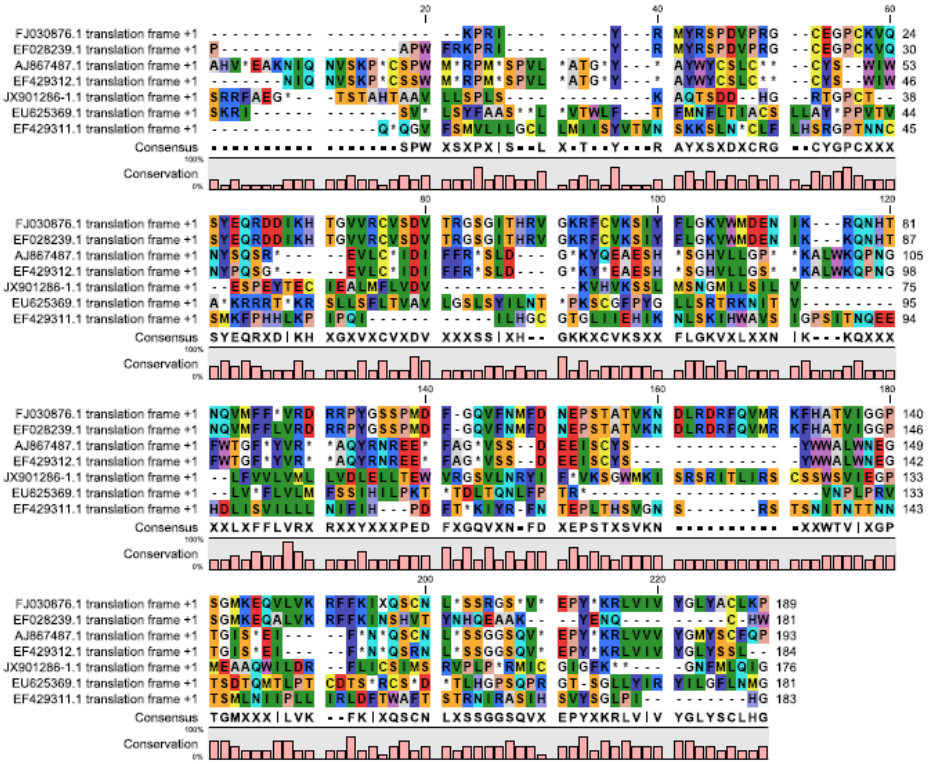

Figure 9. Cluster multiple sequence alignment of TYLCV isolates based on the amino acid sequences of the CP gene. Isolates indicated above were as following: TYLCV-current study, JX901286 {(coat protein (*Tomato yellow leaf curl virus*-EG)}, EU625369.1 {(coat protein (*Tomato yellow leaf curl virus*)}, EF429312.1 {(coat protein [*Tomato yellow leaf curl virus* – Fengxian)} , AJ867487.1 {(coat protein (*Tomato yellow leaf curl virus* – Mugla-2)}, EF028239.1 {(Coat protein [*Tomato yellow leaf curl virus*-[SJC]} FJ030876{(coat protein [*Tomato yellow leaf curl*-[H11]} and EF429311.1{(coat protein (*Tomato yellow leaf curl virus* - Sunqiao).

Protein statistics of amino acids alignment

TYLCV-EG encodes to 176 amino acids residues with MW 19.651 KDa and point isoelectric (PI) 9.05 (Fig. 8 and Table 2). The amino acid composition data of coat protein gene showed different compositions and frequencies of amino acids in different isolates (Fig. 8 and Tables 3 & 4). Twenty amino acids were detected of TYLCV-EG starting with Alanine (A) and ending with Tyrosine (Y). Leucine (L) was found to be the major amino acid in TYLCV-EG (21) with frequencies of 0.119, followed by Serine (S) with frequencies of 0.114. However, Tyrosine (Y) was the lowest count of amino acids (2) of TYLCV-EG with frequencies of 0.011.

Discussion

TYLCV is a whitefly-transmitted geminivirus that causes devastating damage considered as the most serious virus in Egypt (El-Dougdoug et al. 1996; Farag et al. 2005). TYLCV has a wide distribution, so it is important to study the field inspection. The identification of virus isolate and comparing with the other isolates recorded from different parts of the world, in database of GenBank to control the distribution of this virus disease. Early diagnosis of TYLCV was essentially based on distinct geminivirus symptoms observation. Incidence of TYLCV-infection was done on tomato and pepper plants cultivated in the fields and under greenhouse conditions. In the present study field inspection of diseased plants collected from El-Khataba, El-Behera governorate and Qaha and Faculty of Agriculture, University of Ain Shams farm, El-Qaluobiya governorate was carried out using symptamology and indirect-ELISA detection. This study indicated that tomato and pepper plants infected with TYLCV exhibited systemic geminivirus symptoms of severe leaf curling, leaf crinkle with marginal yellowing, stem upright, twisted and stunted as reported by many investigators (El-Dougdoug et al. 1996; Ajlan et al. 2006; Gorovits et al. 2013). Indirect-ELISA using TYLCV specific polyclonal antibodies were confirmed the identity of the isolated TYLCV from tomato and pepper plants. These results were in an agreement with that obtained by (Abouzid et al. 2002). Indirect-ELISA has proved to be very efficient for detection of many plant viruses as reported by Clark and Adams (1977) because of their sensitivity, specificity and speed. All collected tomato samples gave positive reaction with percentage 100%, while 65% of collected pepper samples gave positive reaction. This results indicated that the presence of high population of whiteflies that transmitted TYLCV efficiently. TYLCV isolate infected some of tested members of family Chenopodiaceae, Cucurbitaceae, Fabaceae and Solanaceae. Furthermore, no symptoms were observed on tested members of family Compositae and Graminae. These results agreed with Singh and Reddy (1993) and Ajlan et al. (2006). TYLCV showed variation in symptoms and latent period on different hosts showed in (Table 1). The production of new begomovirus strains as result of high rate of recombination between species, as well as

within and across genera (Ribeiro et al. 2003; Varma and Malathi 2003). The examination with the electron microscope of the isolated virus particles revealed the presence of isometric particles with single and paired geminivirus (monomers and dimmers) with dimension of 22 nm and 20 X 30 nm to 24 X 30 nm, respectively when negatively stained with 2% Uranyl acetate pH 7.0. These results were in the range obtained by (El-Dougdoug et al. 1996; Harrison and Robinson 1999; Varma and Malathi 2003; Ajlan et al. 2006). The most consistent amplification of DNA fragment was obtained using degenerate oligonucleotide broad spectrum primers V324 (+) and C889 (-) as reported by (Brown et al. 2001; Aref et al. 1993 & 1994; Farouk et al., 2011). The results showed that degenerate PCR primers for amplification of portions of the DNA components of whitefly transmitted geminiviruses were designed from highly conserved regions of the viral genome identified from nucleotide sequence alignment. These primers should have general application for the amplification of DNA fragments from a wide range of whitefly-transmitted geminiviruses. Such results indicate that PCR technique as an effective diagnostic tool and greatly facilitate studies of geminiviruses epidemiology and etiology. Simon et al. (2003); Tsai et al. (2006) reported that PCR is an extremely sensitive and specific technique for the detection and determination of genetic diversity among geminiviruses. The size of the PCR product of coat protein gene (Cp) amplified from both naturally and artificially infected tomato was (~530 bp). These results were in an agreement with Brown et al. (2001) who used PCR to detect and establish provisional identity of begomoviruses through amplification of a (~530 bp) fragment of the begomovirus coat protein gene (Cp), referred to as the 'core' region of the Cp gene (core Cp). The core Cp fragment contains conserved and unique regions, and was hypothesized to constitute a sequence useful for begomovirus classification. Rybicki (1994) reported that the core CP gene sequence has been accepted by the ICTV as a desirable marker for virus identity when a full-length genomic sequence is not available. Partial nucleotide sequence (~530 nt) of TYLCV-CP-EG (JX901286) of the current study was aligned with other published Cp sequences of TYLCV. The homology tree of TYLCV-EG revealed high degree of similarity (99%) TYLCV isolates available in GenBank. Abd El-Monem (2011) found that similarity between Egyptian isolate with other isolates in GenBank was 98%. Development of molecular tools for virus detection, strain identification and genetic engineering of plants for virus resistance (Dunez 1988). The cloning and sequencing of PCR fragments has contributed to the classification and phylogeny of geminiviruses (Rojas 1992). Delatte et al. (2005) proposed that, according to the ICTV criteria for begomovirus species demarcation using DNA complete sequence (Fauquet et al. 2003) is considered new species as their nucleotide identities with other begomovirus are below 89%. For the future, it will be important to sequence more isolates of TYLCV-causing viruses of Egypt to monitor the viral genotypes, and to be able to follow possible changes in the virus population structure. In addition, the great variability of the TYLCV isolates worldwide should be considered when breeding programs for virus resistance are established. A tomato line tolerant/resistant to a particular TYLCV isolate may not be as effective against another distantly related virus isolate.

Conclusion

TYLCV has a wide distribution, so it is important to study the field inspection. The identification of virus isolate and comparing with the other isolates recorded from different parts of the world, in database of GenBank to control the distribution of this virus disease. Partial nucleotide sequence (~530 nt) of TYLCV-CP-EG (JX901286) of the current study was aligned with other published Cp sequences of TYLCV. The homology tree of TYLCV-EG revealed high degree of similarity (99%) TYLCV isolates available in GenBank.

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