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Detection and molecular characterization of tomato yellow leaf curl virus naturally infecting *Lycopersicon esculentum* in Egypt

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Summary. – Tomato yellow leaf curl virus (TYLCV) infections of tomato crops in Egypt were widely spread in 2014. Infected symptomatic tomato plants from different governorates were sampled. TYLCV strains Israel and Mild (TYLCV-IL, TYLCV-Mild) were identified by multiplex and real-time PCR. In addition, nucleotide sequence analysis of the V1 and V2 protein genes, revealed ten TYLCV Egyptian isolates (TYLCV from TY1 to 10). Phylogenetic analysis showed their high degree of relatedness with TYLCV-IL Jordan isolate (98%). Here we have showed the complete nucleotide sequence of the TYLCV Egyptian isolate TY10, sampled from El Beheira. A high degree of similarity to other previously reported Egyptian isolates and isolates from Jordan and Japan reflect the importance of phylogenetic analysis in monitoring virus genetic diversity and possibilities for divergence of more virulent strains or genotypes.

Keywords: multiplex PCR; real-time PCR; TYLCV-IL; TYLCV-Mild; phylogenetic analysis

Introduction

Tomato yellow leaf curl disease (TYLCD) is a serious threat to tomato production in many areas of the world. Yield losses near to 100% were in geographically diverse locations such as central Southeast Asia, the Far East Asia, USA, Caribbean islands, Tropical Africa and several Mediterranean countries (Czosnek and Laterrot, 1997; Nakhla and Maxwell, 1998; Moriones and Navas-Castillo, 2000; Ueda *et al*., 2004; Rojas *et al*., 2007; Brown and Idris, 2008). In Egypt, it was first reported in 1969 (Nour El-din *et al*., 1969) and caused several losses in tomato yields in 1991 (Nakhla *et al*., 1993). The disease is continuing to cause epidemics that are devastating tomato crops widely and rapidly. TYLCD is caused by a complex of several virus species, belonging to the genus *Begomovirus*, transmitted by the whitefly *Bemisia tabaci* in a circulative and persistent manner. *Begomovirus* is one of four genera in the family *Geminiviridae* comprising viruses with circular single stranded DNA genome and tomato yellow leaf curl virus (TYLCV) is the most wide spread species in tomato plants.

The genome of TYLCV contains six partially overlapping open reading frames (ORFs) bidirectionally arranged into two transcriptional units that are separated by an intergenic region of approximately 300 nucleotides, containing motifs for viral genome replication and transcription (Rybicki *et al*., 2000). V1 encoding a coat protein, and V2 encoding a pre-coat protein that acts as a suppressor of RNA silencing are partially overlapping genes (Glick *et al*., 2008; Jiang *et al*., 2012). The product of the complementary C1 ORF is the only virus-encoded protein replicase or replication initiator (Rep) which plays the key role in initiating the rolling circle replication by virtue of its nicking and ligation property (Arupratan *et al*., 2004). The C3 protein, the gemini viral replication enhancer (REn) protein is able to increase viral DNA accumulation and enhance infectivity and symptoms expression (Hormuzdi and Bisaro, 1995). The C2 and the C4 proteins were demonstrated to be suppressors of the RNA-silencing pathway (Abhary *et al*., 2006), while C4 induces virus-like symptoms in host plants (Selth *et al*., 2004).

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Abbreviations: TYLCD = tomato yellow leaf curl disease; TYLCV = tomato yellow leaf curl virus

Factors contributing to the emergence of new variants over the years within the genus *Begomovirus*, have been attributed to mutation, recombination and pseudo recombination among TYLCV virus strains (Czosnek and Ghanim, 2011). Full-length genomic sequencing allows for a more comprehensive analysis of virus variability and different forces driving the evolution of TYLCV. Two strains infect tomato crops in the Middle East, tomato yellow leaf curl virus-Israel (TYLCV-IL) and tomato yellow leaf curl virus-Mild (TYLCV-Mild) strains. More recently, the strain "Spain" of another *Begomovirus*, the tomato yellow leaf curl Sardinia virus (TYLCSV-ES), has been identified in Jordan and Israel (Anfoka *et al*., 2005). To develop effective control strategy against TYLCD, it is necessary to identify the virus species or strains causing the infection. The present study aims at the identification of TYLCV infection and the study of virus species by partial and complete genome sequencing of Egyptian isolates for identification of their phylogenetic relationships with the previously reported isolates.

Materials and Methods

Collection of TYLCD-symptomatic tomato plants. Ninety *Lycopersicon esculentum* (tomato) leaf samples from plants showing virus related symptoms and representing different tomato cultivars were collected. Samplings were from different cultivars of tomato from selected governorates in Egypt.

Total DNA extraction. Extraction of total DNA from symptomatic and healthy tomato plants was done as described by Zhou *et al*. (2001). Briefly, 200 mg of leaf tissue ground in 2 ml of grinding buffer containing 2% CTAB (cetyltrimethylammonium bromide), 100 mmol/l Tris-HCl, pH 8, 20 mmo/l EDTA, 1.4 mol/l NaCl, 1.0% sodium sulphite and 2% PVP-40. After incubation at 65°C for 15 min, an equal volume of chloroform/isoamyl alcohol (24:1) was added and centrifuged at maximum speed for 15 min for 2 times. Two hundred µl of 4 mol/l NaCl and 300 µl of iso-propanol was added to aqueous layer, mixed, and incubated at -20°C for 1 h. Samples were centrifuged at maximum speed for 30 min at 4°C to pellet the DNA, washed with 70% ethanol and resuspended in 100 µl of nuclease-free H_2O. Total DNA was stored at -20°C until use for PCR analysis.

Detection of TYLCV. The presence of TYLCV has been proved in DNA extracts of *L. esculentum* by using primers TYLCV-TY1F, TYLCV-TY2R (Accotto *et al*., 2000), that amplify a region of 579 nts from the coat protein (CP) gene (Table 1). PCR reaction was done in a total volume of 25 µl. Viral DNAs were amplified by PCR in a reaction mixture containing 5 µl of 5x Mg-free buffer, 3 µl $MgCl_2$ 25 mnol/l, 1 µl (5 nmol/ml) of each primer (TYLCV-TY1F, TYLCV -TY2R), 0.5 µl dNTP mix (10 mmol/l), 0.2µl of 5U/µl GoTaq® G2 DNA polymerase (Promega, USA) and 1µl of the extracted DNA. PCR cycle parameters are described in Table 1. Products were visualized in 1% agarose gel by electrophoresis.

Molecular identification of TYLCV strains by multiplex PCR. Two sets of previously published primers (Anfoka *et al*., 2008) detect and differentiate TYLCSV-ES, TYLCV-IL, TYLCV-Mild and tomato yellow leaf curl Sardinia virus-Sicily (TYLCSV-Sic) in symptomatic tomato plants. The first set includes TYAlmv2516 - TYAlmc115 pair that specifically detects TYLCSV-ES (but not other strains of TYLCSV species) and TYv2337, TYc138, and TYv2664 primers that differentiate between TYLCV-IL and TYLCV-Mild (Anfoka *et al*., 2005). The second set includes RVC427, VP2715 and Sa2267 primers, designed to detect all strains of TYLCSV, including the Italian TYLCSV-IT, the Sicilian TYLCSV-Sic and the Spanish (TYLCSV-ES) strains, was used to detect TYLCV and TYLCSV (Anfoka *et al*., 2008). The sequence of each primer as well as the PCR cycle parameters are described in Table 1. In particular, PCR reactions were optimized in a final volume of 25 µl containing: 0.5 µl dNTP mix (10 mmol/l), 5µl of 5x Mg-free buffer, 3 µl $MgCl_2$ 25 mmol/l, 1 unit of GoTaq® G2 DNA polymerase (0.2 µl) (Promega), 1 µl of each primer (10 µmol/l) and 1 µl of DNA template.

Real-time RT-PCR for detection and differentiation of Israel and Mild TYLCV strains. In order to design a primer pair able to identify TYLCV in plant tissue and also differentiate the presence of IL and Mild strains of the virus, a multiple sequence alignment of TYLCV complete genomes available at the GenBank database using Geneious 7.0.6 software package was done. Primers TYLCV 2234 F and TYLCV 2537R (Table 1 and Fig. 1) targeting regions conserved within both two strains and flanking a 324 bp region (covering partial ORFs C1 and C4) that differs IL and Mild isolates. Real-time RT-PCR reaction was performed in a total volume of 20 µl containing: 10 µl of 2x GoTaq® qPCR master mix (Promega), 2 µl of 10 mmol/l dNTP mix (Promega), 0.17 µl of 30 µmol/l CXR (carboxy-X-rhodamine reference dye), 1 µl each of 10 µmol/l TYLCV 2234 F and TYLCV 2537R primers and 0.5 µl of DNA. The reaction was carried out in an ABI Prism 7000 System (Applied Biosystems) using program parameters described in Table 1.

*Sequence analysis of V1+V2 genes and full-length genome of TYLCV Egyptian isolates***.** Primers designed from gene sequences of different TYLCV isolates were obtained from the GenBank database. The Clustal W 1.8 (BCM search launcher) was applied for multiple sequence alignments of each of the coat protein (V1) gene and the movement protein (V2) gene. The V2 and V1 sequences were conserved in most aligned sequences. The primer pairs TYLCV V2 F/ TYLCV V1 R2 and TYLCV FL F/ TYLCV FL R were designed to amplify V2+V1 and full length sequences of TYLCV, respectively (Table 1 and Fig. 1). PCR reaction was performed in a total volume of 25 µl using 12.5 µl of GoTaq ® long PCR master mix (Promega), 1 µl of each primer (5 nmol/ml) and 1 µl of the DNA sample. PCR cycle parameters as described in Table 1 were used. To determine the sequence of the amplified fragments, DNA was purified from excised bands from agarose gel using the Wizard® SV gel PCR clean-up system kit (Promega), ligated into pGEM T- easy vector (Promega) and cloned in *Escherichia coli* MC1022 competent cells. Extraction of recombinant plasmid DNA was done with the Wizard® Plus SV minipreps DNA purification system (Promega) and the sequences of the

Table 1. Description of primers and PCR conditions used for the amplification of TYLCV genome

References	PCR cycling conditions	Target region	Expected product size (bp)	Nucleotide position	Sequence (5' to 3')	Primers	
Anfoka *et al.*, 2005	94°C/2 min, 30 x 94°C/1min, 62°C/45 s, 72°C/1min, 72°C/10 min	IR	432	122-150[a] 2496-2523[a]	ATATTGATGGTTTTT TCAAAACTTAGAAG TTTTATTTGTTGGTGTTTG TAGTTGAAG	**TYAlmc115** **TYAlmv 2516**	
		IR	633	2311-2337[b] 138-162[b]	ACGTAGGTCTTGACATCT GTTGAGCTC AAGTGGGTCCCACATATT GCAAGAC	**TYv2337** **TYc138**	
		IR	314	18-44[c] 307-331[c]	ATTGACCAAGATTTTTA CACTTATCCC AAGTGGGTCCCACATATT GCAAGAC	**TYv2664** **TYc138**	Set 1
Anfoka *et al.*, 2008	95°C/3 min, 40 x 94°C/3 min, 52°C/30 s, 72°C/1 min, 2°C/10 min	IR	927	2268-2293[d] 398-421[d]	TGGAAAGTACCCCATTCAA GAACATC TGCCTTGGACART GGGGRCAGCAG	**Sa2267** **RVC427**	
	95°C/3 min, 40 x 94°C/3 min, 52°C/30 s, 72°C/1 min, 72°C/10 min	IR	528	61-87[e] 565-588[e]	ATACTTGGACACCTAAT GGCTATTTGG TGCCTTGGACART GGGGRCAGCAG	**VP2715** **RVC427**	Set 2
Accotto *et al.*, 2000	94°C/3 min, 35 x 94°C/30 s, 60°C/30 s, 72°C/30 s, 72°C/7 min	CP	579	447-464[d] 1007-1025[d]	GCCCATGTAYCGRAAGCC GGRTTAGARGCATGMGTAC	**TYLCV-TY1F** **TYLCV-TY2R**	
This study	94°C/3 min, 35 x 94°C/1 min, 55°C/30 s, 72°C/1.5 min, 72°C/10 min	CP&MP	1065	101-123[f] 1143-1165[f]	TGATTTGTCTTTATATACTT GGT GAGCAGTTGATCATGTATT GTAT	**TYLCV V2 F** **TYLCV V1 R2**	
	50°C/2 min, 95°C/10 s, 40 x 95°C/15 s, 60°C/1min	REP	324	2037-2064[f] 2340-2360[f]	TCATCTGGAACTTGAT TAAAAGAWGAAG CCATCCYAACAT TCAGGSAGC	**TYLCV2234 F** **TYLCV2537 R**	
	94°C/5 min, 34 x 94°C/10 s, 54°C/10 s, 72°C/4 min, 72°C/8 min	*V1*,*V2*,*C1*,*C2*, *C3*,*C4*, IR	2773	1016-1030[f] 999-1015[f]	ATGCCTCTAATCCAG GCGTACATGCCATATAC	**TYLCV FL F** **TYLCV FL R**	

[a]Referred to TYLCSV Acc. No. L27708; [b]Referred to TYLCV-IL Acc. No. AM409201; [c]Referred to TYLCV-Mild Acc. No. X76319; [d]Referred to TYLCSV Acc. No. X61153; [e]Referred to TYLCV-IL Acc. No. X15656; [f]Referred to TYLCV-IL Acc. No. KT921303.

plasmid inserts were submitted to GenBank. CLUSTAL W method in MEGA 6 software (Tamura *et al.* 2013) generated a multiple alignments and phylogenetic trees using Bootstrap test of phylogeny (1000 replicates) under the Neighbor-Joining method. Reference sequences downloaded from GenBank database were included as representatives of TYLCV isolates (Supplementary Table S1).

Results

Detection and differentiation of TYLCD-associated viruses by conventional and real-time PCR

Using primers TYLCV-TY1F and TYLCV-TY2R we have detected 62 TYLCV infected samples. When tested by multiplex PCR reactions, only amplicons of the expected sizes for TYLCV-Mild (314 bp) and TYLCV-IL (633 bp) were obtained using primer set 1 (Fig. 2a) while exclusively PCR products of 528 bp (TYLCV-IL) were observed when primer set 2 was used for amplification (Fig. 2b). DNA extracted from healthy tomato plants was negative.

When primer pair TYLCV 2234 F/TYLCV 2537 R was used in real-time PCR, a fluorescent signal was detected only from TYLCV-infected samples while no signal was obtained from healthy samples (Fig. 3a). Dissociation analysis of amplified products revealed two main peaks, the first one at 83.0–83.4°C and the second one at 85.6°C. The reaction conducted on healthy tomato plants (Fig. 3b) showed no peaks. Sequence analysis of TYLCV 2234 F/TYLCV 2537 R amplicons (324 bp) from samples showing single or mixed

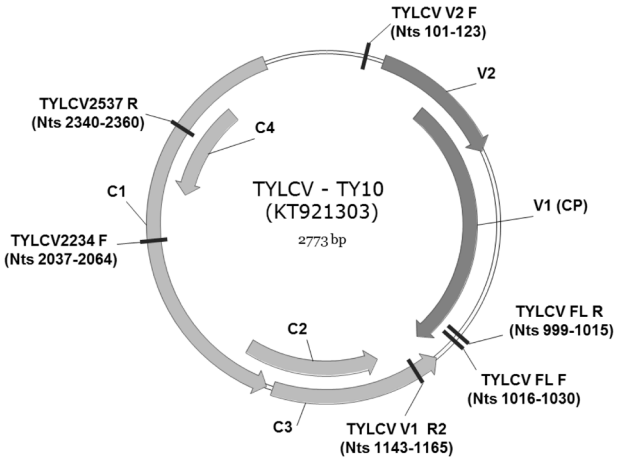

Fig. 1

Genome organization of the TYLCV TY10 Egyptian isolate (2,773 nts, Acc. No. KT921303) showing complementary (C1 to C4) and viral (V1 and V2) ORFs

Localization of primers designed and used in this study. Details of primers are in Table 1.

infection allowed to assign the peak at lower temperature (83.0–83.4°C) to PCR products amplified form TYLCV-IL strain and the peak at higher temperature (85.6°C) to amplicons from the TYLCV-Mild strain as shown in Fig. 3b. Additional small peaks at 75.5–76.2°C, appearing on infected samples, were not associated with any strain differentiation. Probably these are amplicons originating from some conserved region of the TYLCV genome but do not affect the results of the analysis. Using the real-time PCR assay, 65 out of 90 samples collected, were confirmed as positive for TYLCV.

Incidence of TYLCD-associated viruses in Egypt

Obtained results, revealed that the overall incidence of TYLCV infection in all governorates was 68.8% (62/90) by conventional PCR and 72.2% (65/90) by real-time PCR. In particular, PCR assays detected the virus in samples collected from El Sharkeya, El Qalyoubeya, Kafr El Sheikh, Giza, El Gharbeya and El Beheira governorates and not in samples from Alexandria, Damietta, El Dakahlia and El Fayoum governorates (Table 2). Results obtained using the real-time PCR assay were in complete agreement with those obtained by PCR analyses except for detection of TYLCV-IL in three samples from Kafr El Sheikh, Alexandria and El Beheira governorates that were negative in PCR reactions.

TYLCV-IL caused infection in all positive TYLCV samples. However, TYLCV-Mild was detected only in samples with mixed infection by both strains. One sample from El Sharkeya governorate, two samples from El Gharbeya and three samples from EL Giza governorate had mixed infection. Single infections with TYLCV-IL (56 samples) were prominent (62.2%), when compared with mixed infected (TYLCV-IL + TYLCV-Mild) samples (6.6%). There was no

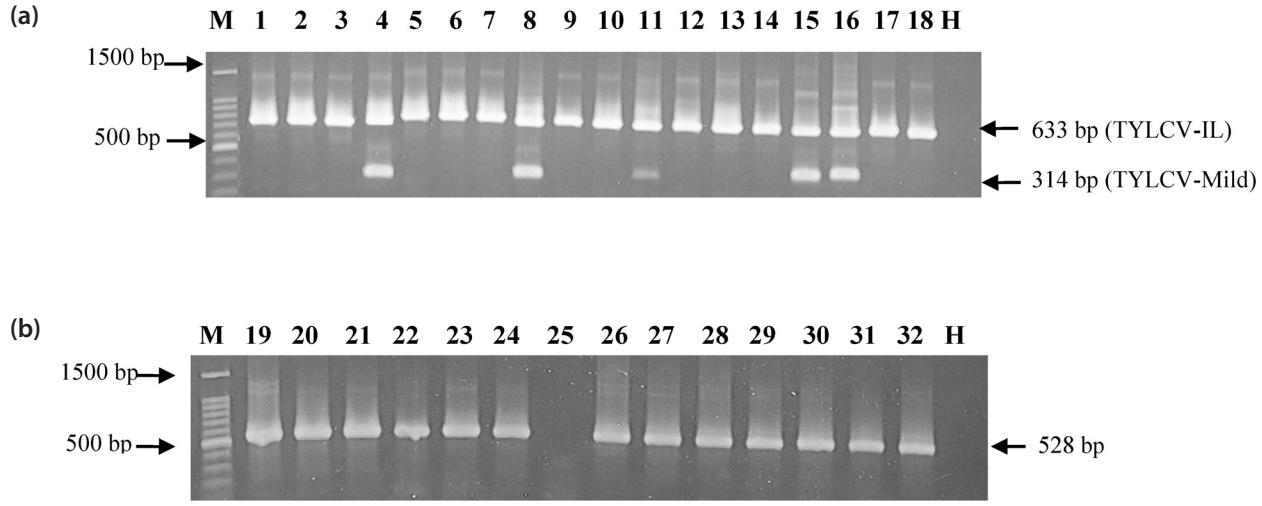

Fig. 2

Identification of TYLCD-associated viruses in symptomatic tomato plants collected from different governorates in Egypt

The agarose gels stained by ethidium bromide show PCR products (TYLCV-Mid 314 bp; TYLCV-IL 633 bp) obtained using **(a)** primer set 1 (TYv2337, TYc138, TYv2664, TYAlmv2516, 440 and TYAlmc115) and **(b)** primer set 2 (Sa2267, VP2715 and RVC427). Lane H: DNA extracted from healthy tomato plants. Lane M: 100 bp DNA marker (Promega).

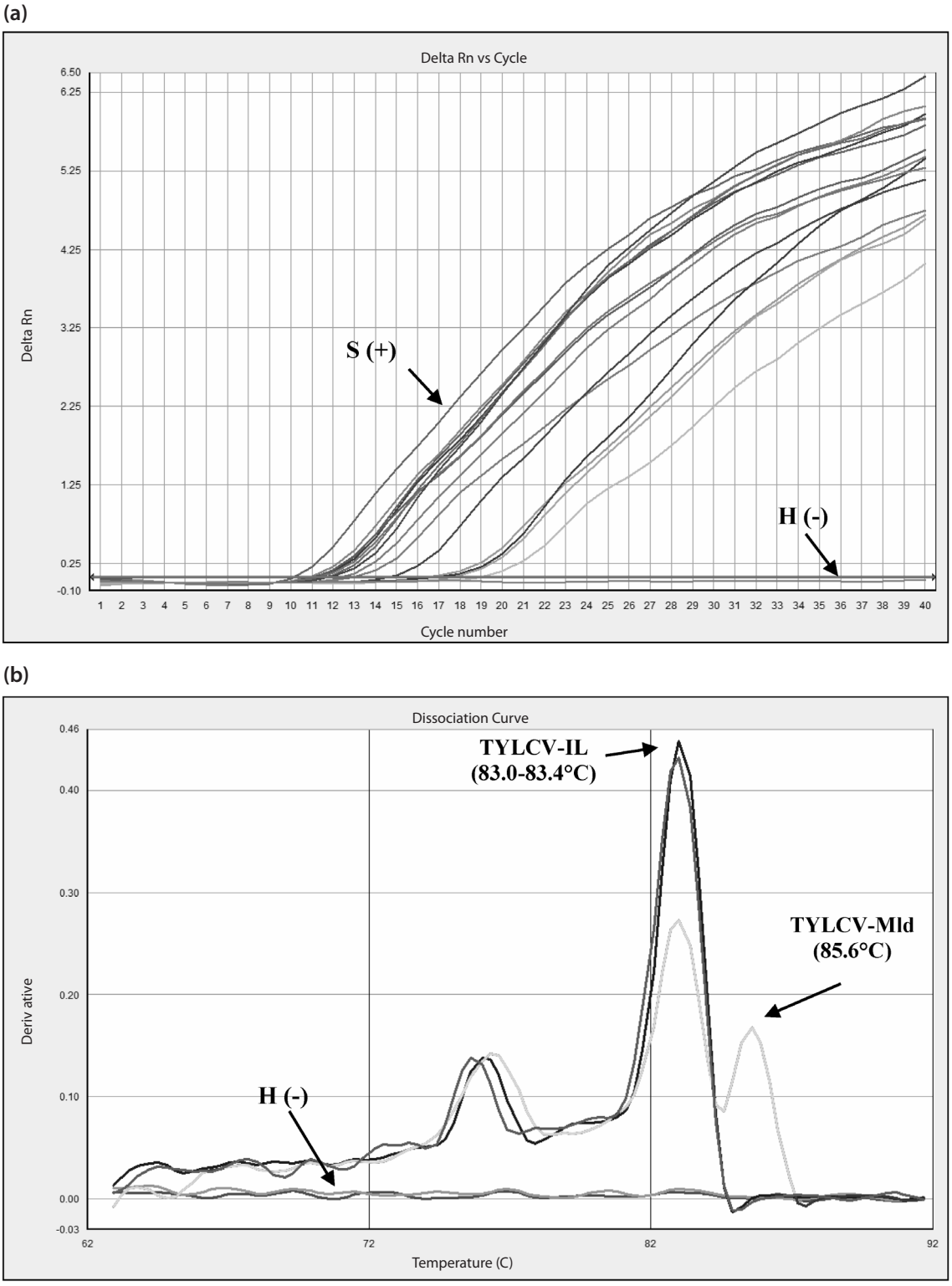

Fig. 3

Molecular detection and characterization of TYLCV-IL and TYLCV-Mild by real-time RT-PCR

(a) Amplification plot of DNA extracted from positive samples [S (+)] and healthy tomato plant [H(-)]. **(b)** Dissociation analysis of amplified products allows to identify TYLCV-IL strain by a single peak (Tm = 83.0–83.4°C) while TYLCV-IL + TYLCV-Mild mixed infection by an additional peak with Tm = 85.6°C. No peaks were observed from reaction conducted on healthy tomato plants [H (-)].

Table 2. Survey for the detection of TYLCV in selected governorates in Egypt tested by conventional and multiplex PCR

Location	Infected/tested	Single infection				Mixed infection
		TYLCV-IL	TYLCV-MILD	TYLCV-ES	TYLCV-SIC	IL+MILD
El Sharkeya	11/11	10	1	0	0	1
El Qalyoubeya	9/9	9	0	0	0	0
Kafr El Sheikh	8/9	8	0	0	0	0
El Giza	10/10	8	3	0	0	3
El Gharbeya	9/9	7	2	0	0	2
El Beheira	15/17	14	0	0	0	0
Alexandria	0/10	0	0	0	0	0
Damietta	0/5	0	0	0	0	0
El Dakahlia	0/6	0	0	0	0	0
El Fayoum	0/4	0	0	0	0	0
Total	62/90	56/90	6	0	0	6

detection of Italian, Sicilian or Spanish strains of TYLCSV in the surveyed Egyptian governorates.

Molecular characterization of TYLCV isolates in Egypt

TYLCV genome region containing V1 and V2 genes of 10 Egyptian isolates from different governorates, were subjected to amplification, sequencing and comparison against sequences of isolates available in the GenBank database. Moreover, the full genome of TYLCV-TY10 isolate from Wady El-Natron in El Beheira governorate was amplified and sequenced.

Phylogenetic analysis of V1 and V2 genes from 10 Egyptian TYLCV isolates

Nucleotide sequences of Egyptian TYLCV isolates TY1 to TY10 containing V1 and V2 overlapped genes (1,065 bp) received GenBank Acc. Nos. KT921304 to KT921313, respectively.

A phylogenetic tree was generated by MEGA6 software from alignment based on the nucleotide sequence of the TYLCV Egyptian isolates and sequences of 22 selected isolates available in GenBank (Fig. 4). Egyptian isolates were grouped together in the same branch, except TY3 and TY10 isolates, and showed higher degree of identity (~98%) in comparison with the isolate TYLCV-IL from Jordan (GQ861426). A higher phylogenetic distance (identity ~96%) was observed in comparison with CR isolate from Costa Rica (KF533855).

Within the same branch, the TY2 and TY7 isolates from El Qalyoubeya and El Gharbeya governorates, respectively, showed around 98% of identity with the other Egyptian isolates (TY1, TY4, TY6, TY7, TY9 and TY10) that were grouped in a different sub-cluster. Moreover, isolates TY2 (El Qalyoubeya) and TY6 (Giza) showed lower identity

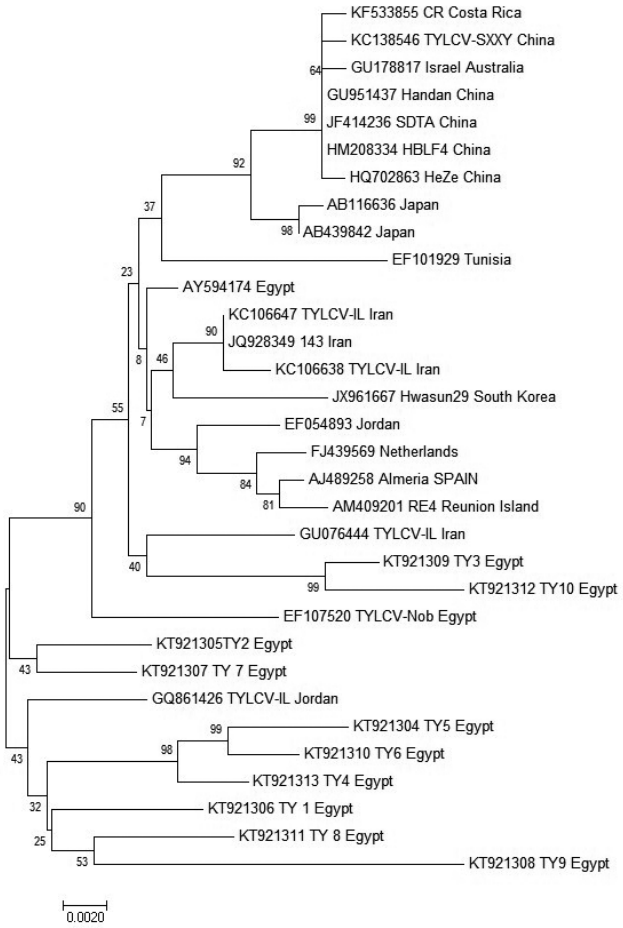

Fig. 4

Phylogenetic analysis of the 10 Egyptian TYLCV isolates based on V1+V2 sequences

Phylogenetic analysis of the 10 Egyptian TYLCV isolates (TY1 to TY10) compared to previously reported TYLCV isolates, according to nucleotide sequences of V1+V2 ORFs. The tree was constructed using the NJ method under the MEGA6 software (Tamura *et al.*, 2013). Bootstrap analysis performed with 1000 replicates. The scale bar shows the number of substitutions per nucleotide.

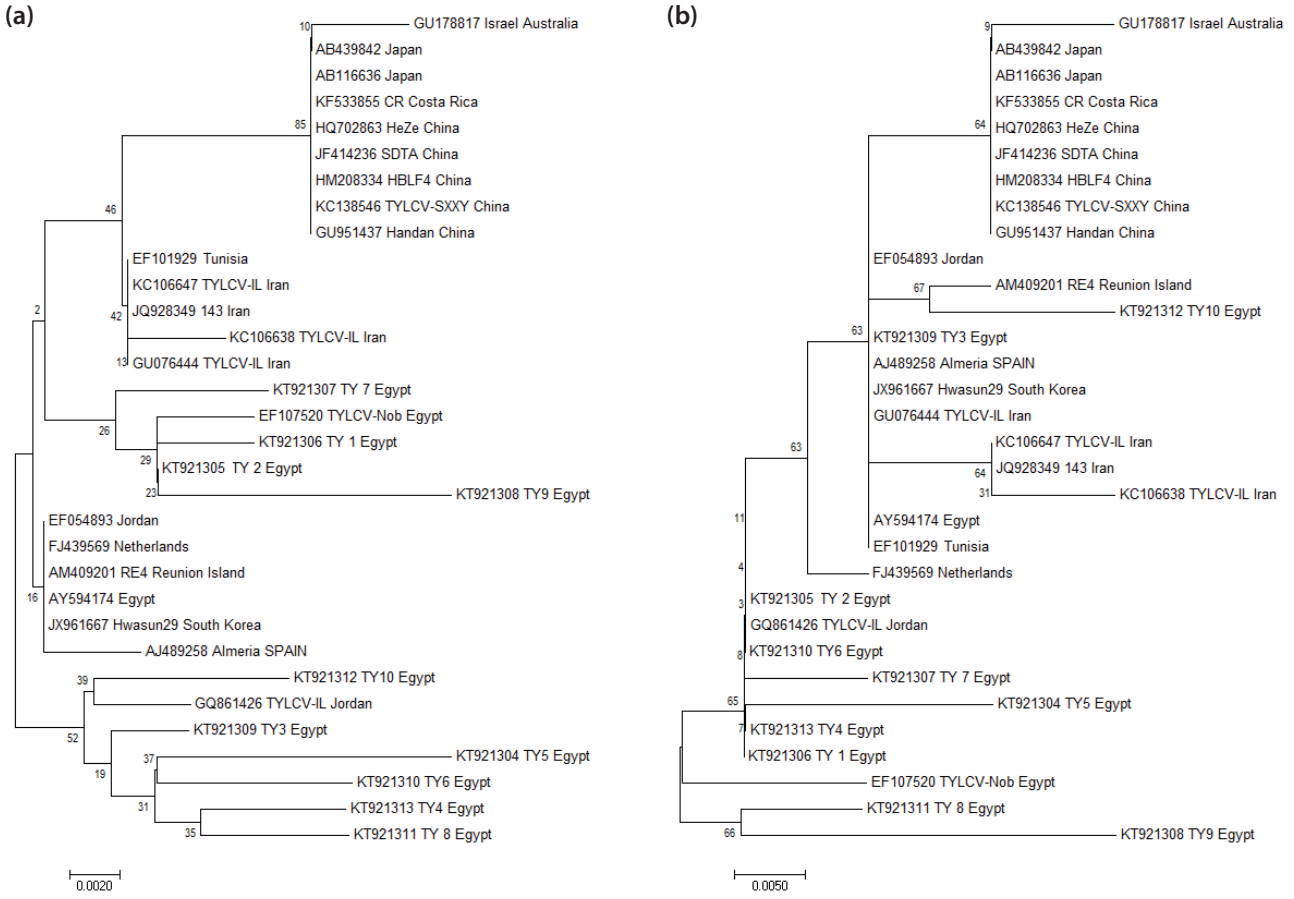

Fig. 5

Phylogenetic analysis of the 10 Egyptian TYLCV isolates based on V1 and V2 sequences

Phylogenetic analysis of the 10 Egyptian TYLCV isolates (TY1 to TY10), compared with previously reported TYLCV isolates, based on amino acid sequences of V1 **(a)** and V2 **(b)** ORFs. The tree was constructed using the NJ method under the MEGA6 software (Tamura *et al*., 2013). Bootstrap analysis performed with 1000 replicates. The scale bar shows the number of substitutions per nucleotide.

(~96%) within the same branch. The two isolates TY3 and TY10 from Kafr El Sheikh and El Beheira, respectively, grouped together in a different cluster showing the best identity (98%) in comparison with the isolate TYLCV-IL from Iran (GU076444).

Phylogenetic dendrograms, based on alignment of the deduced amino acid sequences of V2 and V1 genes, were also constructed. For the V1 gene, the Egyptian isolates separated in two distinct clusters (Fig 5a). The first one includes TY1, TY2, TY7, TY9 and the TYLCV-Nob (Egypt, EF107520) isolates that share identity from 97.1 to 98.3%. In the second cluster TY3 to TY6, TY8 and TY10 grouped together with the isolate TYLCV-IL form Jordan (GQ861426) showing identity from 97.5 to 98.5%. When the V2 gene was analyzed all 10 Egyptian isolates, with the exception of TY3 and TY10, clustered together with the Jordanian (GQ861426) and the Egyptian (EF107520) isolates showing degree of relatedness from 98.0 to 99.7% (Fig. 5b). The TY3 isolate showed higher identity (99.4%) with the isolate EF054893 from Jordan while the TY10 isolate was in 98.8 % similar to the RE4 isolate from the Reunion Island (AM409201).

Phylogenetic analysis of full-length genome of TYLCV TY10 isolate

Full-length genome of TYLCV-TY10 Egyptian isolate from a tomato sample collected from Wady El-Natron in El Beheira governorate (hybrid cultivar 1077) was amplified using specific primers (Table 1). The complete genome of the TY10 isolate comprised 2,773 nts (Acc. No. KT921303) showing the typical genome organization of the viral species (Fig. 1). V2 gene (147–497 nts) encodes movement protein, V1 gene (301–1,083 nts) encodes capsid protein while C3

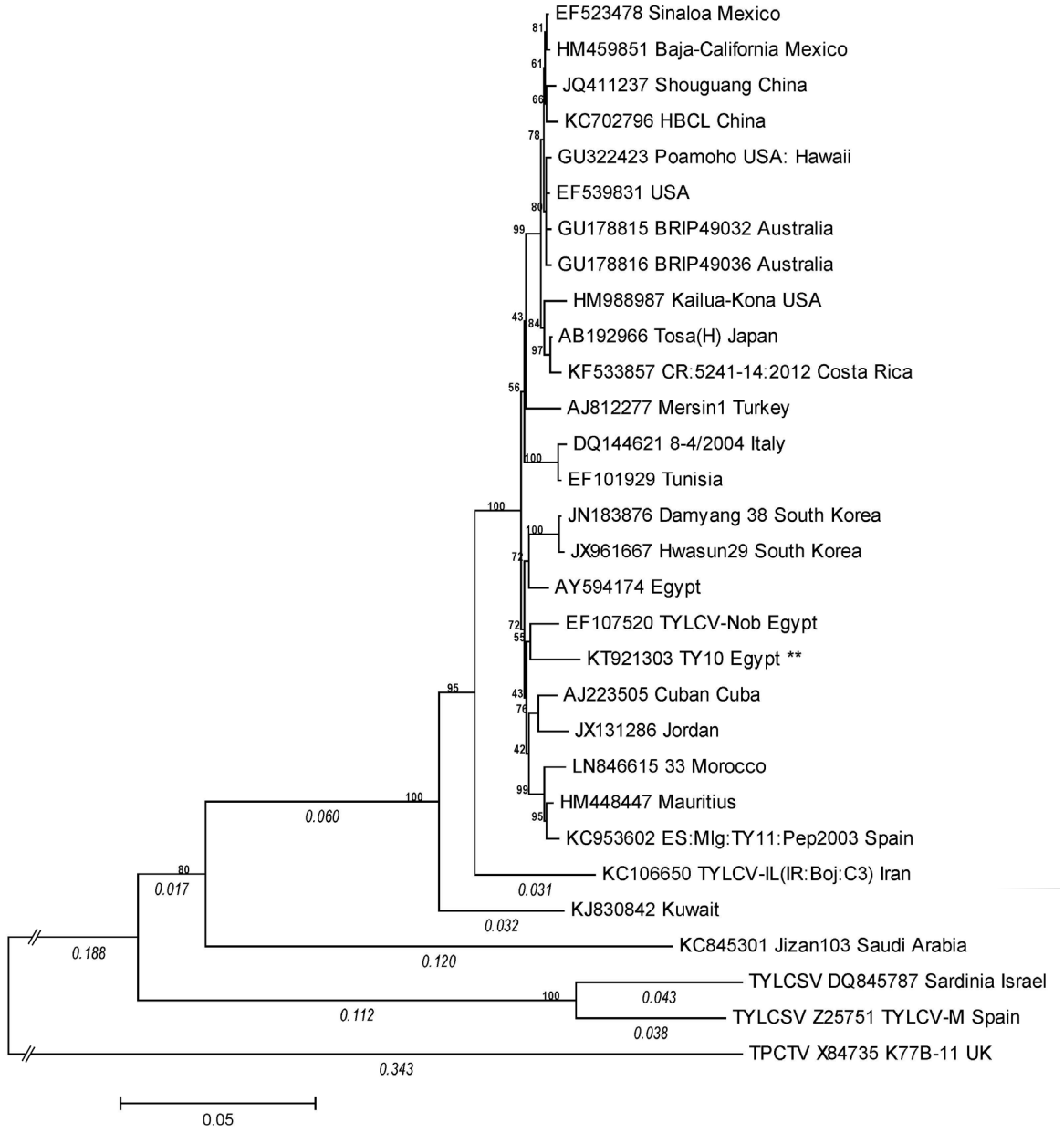

Fig. 6

Phylogenetic analysis of the full-length genome of TYLCV-TY10 Egyptian isolate

Phylogenetic analysis of the full-length genome of TYLCV-TY10 Egyptian isolate compared with previously reported TYLCV isolates based on nucleotide sequence. The tree produced using the NJ method under the MEGA6 package (Tamura *et al.*, 2013). Bootstrap analysis performed with 1000 replicates. The scale bar shows the number of substitutions per nucleotide. Tomato pseudo-curly top virus (TPCTV) was used as outgroup.

(1,080–1,523 nts), C2 (1,225–1,632 nts), C1 (1,541–2,614 nts) and C4 (2,170–2,463 nts) genes encode C1, C2, C3 and C4 proteins, respectively.

The phylogenetic tree generated from alignment based on the nucleotide sequences of full-length genome of TYLCV-TY10 isolate and 26 selected isolates available in the GenBank database was obtained using Neighbor-Joining method and Bootstrap test under the MEGA6 package (Fig. 6). The analysis revealed that the isolate TY10 shares higher degree of relatedness with the Egyptian isolates EF107520 and AY594174 (97.8 and 97.4%, respectively). Also, phylogenetic identity of 97.6% was observed between TY10 isolates and

JX131286 and Tosa (H) (AB192966) isolates from Jordan and Japan, respectively. Among the other TYLCV GenBank sources considered, the TY10 isolate showed a nucleotide identity ranging from 80.0 to 97.4% comparing with the Jizan103 isolate from Saudi Arabia (KC845301) and the isolate KJ830842 from Kuwait, respectively. The tomato pseudocurly top virus (TPCTV) sequence was used as outgroup.

Discussion

TYLCD is a disease complex of increasing concern for tomato growers in many regions of the world (Guindon and Gascuel, 2003; Khan *et al.*, 2008; Fazeli *et al.*, 2009; Garcı´a-Andre´s *et al.*, 2007). TYLCV is known to infect tomato in Egypt and in the last three years, the incidence of infection was very high. During the survey visual estimates of infection by TYLCV was up to 100% in some areas. Symptoms of leaf curling, yellowing and stunted growth are very characteristic for the infection. It is known that the virus exists in the Mediterranean area in several strains also. Characterization of TYLCV potential strains causing infection was by strain specific primer sets in PCR

Under natural conditions, single and mixed infection occurs and each may lead to the production of new viral genetic variation in the population (Rojas *et al.*, 1993; Padidam *et al.*, 1999; Khan *et al.*, 2013). Our results reported an overall detection percentage of TYLCV higher than 72%. TYLCV-IL was more predominant strain, while TYLCV-Mild was present in the 6.6% of the samples. Mixed infections were detected in several areas confirming the genetic variability of the population as previously reported in several studies (Anfoka *et al.*, 2005; Anfoka *et al.*, 2008; Hajimorad *et al.*, 1996; Owor *et al.*, 2007). This may lead to a synergistic rather than a cross protection effect (Morilla *et al.*, 2004). The Mediterranean basin has been identified as the main launch pad of global TYLCV movements (Lefeuvre *et al.*, 2010; Fauquet *et al.*, 2005). As high diversity degree of the virus has been reported also in the Middle East (Bananej *et al.*, 2004; Khan *et al.*, 2008), we decided to develop and use in our analyses a more sensitive detection method based on real-time PCR. Results confirmed higher sensitivity of real-time PCR technique that identified 72.2% of infected samples in comparison with 68.8% of those detected by conventional PCR. More interestingly, the real-time PCR assay was able to differentiate, simply by dissociation analysis, between the two most abundant TYLCV-IL and TYLCV-Mild strains and therefore demonstrate to be a reliable tool to investigate TYLCD etiology. Both strains have the broadest geographical ranges stretching in the Old World from Japan in the east (Sugiyama *et al.*, 2008), Spain in the west (Navas-Castillo *et al.*, 1999), the Indian Ocean island of Reunion (Peterschmitt *et al.*, 1999) and Australia (Stonor *et al.*, 2003) in the south. According to Lefeuvre *et al.* (2010) TYLCV has jumped at least twice between the Old and New world (McGlashan *et al.*, 1994; Duffy and Holmes, 2007) and is currently in North and South America (Zambrano *et al.*, 2007; Czosnek and Laterrot, 1997). TYLCV-IL is therefore considered to have an unusually large geographical range.

Our results indicate a high infection rate of the virus in Egypt. It points to a significant degree of diversity at both the nucleotide and the amino acid levels of V1 and V2 genes within the ten Egyptian TYLCV-IL isolates. V1 and V2 genes are less affected by amino acid changes than other genes (Yang *et al.*, 2014) but at the nucleotide level TY3 and TY10 were more similar to TYLCV-IL (GUO76444) isolate from Iran than to the other Egyptian isolates analyzed. Moreover, amino acid analysis of V1 gene suggests the presence of two main TYLCV clusters in Egypt while V2 gene analysis confirms the distance of TY3 and TY10 from the other Egyptian isolates.

In order to improve knowledge about genetic assessment of TYLCV population in Egypt, the complete genome of TY10 isolate (2,373 nts) has been sequenced and compared to previously reported sequences of Egyptian isolates (Abdallah *et al.*, 2000; Abhary *et al.*, 2006). Phylogenetic analysis of the sequences has shown that TY10 has a close degree of relatedness to the other Egyptian isolates but also to Jordanian and Japanese isolates.

Phylogenetic analysis conducted on V1+V2 genes from ten isolates and on the complete genome of TY10 isolate may suggest a multiple introduction of TYLCV to Egypt due to the increasing international exchange of plant material. Moreover, high variability among *Geminivirus* genomes in progeny populations was reported over a short period of time (Yang *et al.*, 2014). The East African cassava mosaic virus (EACMV) (*Geminivirus* genus) has shown mean rates of 1.60×10^{-3} and 1.53×10^{-4} nucleotide substitutions per site per year for DNA-A and DNA-B components respectively (Duffy and Holmes, 2009). Considering the continuous genetic variations of the virus our results suggest that also in Egypt the mean substitution rate of the virus could be 2.88 x 10^{-4}nucleotide per site per year as estimated in previous studies independently by the recombination frequency of the virus (Duffy and Holmes, 2008). This may lead to the production of isolates acquiring traits for being more virulent to plant infection or allows co-transmission of more than one isolate by the whitefly vector (Ohnishi *et al.*, 2011).

Continuous monitoring of TYLCV isolate sequences is therefore necessary to the understanding of this virus complex epidemiology and spread. This also is fundamental to the proper application of management strategies of the disease. In fact, the dissemination of tolerant cultivars in case of TYLCD has more safety impact than the release of genetically modified plants (Morilla *et al.*, 2004) due to the genetic diverging of TYLCV isolates.

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Supplementary information is available in the online version of the paper.

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Supplementary information

Detection and molecular characterization of tomato yellow leaf curl virus naturally infecting *Lycopersicon esculentum* in Egypt

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Supplementary Table S1. Reference TYLCV sequences and their DDBJ/EMBL/GenBank Acc. Nos. used in phylogenetic analysis

Sequence reference	Original host	Geographic origin	Isolate	Acc. No.
V1 and V2 gene sequences of TYLCV isolates				
NCBI	*Solanum lycopersicum*	Jordan	TYLCV-IL	GQ861426
Abhary *et al.*(2006)	not stated	Egypt	–	AY594174
Lefeuvre *et al.* (2010)	*Solanum lycopersicum*	Iran	TYLCV-IL	GU076444
Botermans *et al.* (2009)	*Solanum lycopersicum*	Netherlands	3181291	FJ439569
NCBI	not stated	Jordan	–	EF054893
Morilla *et al.* (2005)	*Capsicum anuum*	Spain	Almeria	AJ489258
Hosseinzadeh *et al.* (2014)	*Datura stramonium*	Iran	TYLCV-IL	KC106647
NCBI	*Solanum lycopersicum*	Iran	143-Bojnord	JQ928349
NCBI	*Solanum lycopersicum*	China	HeZe	HQ702863
Delatte *et al.*(2005)	*Solanum lycopersicum*	Reunion	RE4	AM409201
Barboza *et al.* (2014)	*Solanum lycopersicum*	Costa Rica	CR:5240-16:2012	KF533855
Hosseinzadeh *et al.* (2014)	*Datura stramonium*	Iran: Bojnurd	TYLCV-IL	KC106638
NCBI	*Solanum lycopersicum*	China	SDTA	JF414236
NCBI	–	China	HBLF4	HM208334
NCBI	*Solanum lycopersicum*	Australia	BRIP49041	GU178817
NCBI	*Solanum lycopersicum*	China	Handan	GU951437
Ueda *et al.* (2004)	*Solanum lycopersicum*	Japan	–	AB116636
NCBI	*Solanum lycopersicum*	China	TYLCV-SXXY	KC138546
NCBI	Tomato	South Korea	Hwasun29	JX961667
NCBI	not stated	Japan	–	AB439842
Gharsallah Chouchane *et al.* (2007)	not stated	Tunisia	–	EF101929
Abdallah *et al.* (2000)	not stated	Egypt	TYLCV-Nob	EF107520
Full length genome sequences of TYLCV isolates				
Ueda *et al.* (2005)	*Lycopersicon esculentum*	Japan	Tosa	AB192965
NCBI	not stated	Cuba	Cuban	AJ223505
Koklu *et al.* (2006)	*Lycopersicon esculentum*	Turkey	Mersin1	AJ812277
Abhary *et al.*(2006)	not stated	Egypt	–	AY594174
Accotto *et al.* (2003)	*Lycopersicon esculentum*	Italy: Sicily	8-4/2004	DQ144621

Sequence reference	Original host	Geographic origin	Isolate	Acc. No.
NCBI	not stated	Tunisia	–	EF101929
Abdallah *et al.* (2000)	Tomato	Egypt	TYLCV-Nob	EF107520
NCBI	not stated	Mexico	Sinaloa	EF523478
Rojas *et al.* (2007)	not stated	USA	–	EF539831
NCBI	*Solanum lycopersicum* cv. Temptation	Australia	BRIP49032	GU178815
NCBI	*Solanum lycopersicum*	Australia	BRIP49036	GU178816
Melzer *et al.* (2010)	*Lycopersicon esculentum*	USA: Hawaii	Poamoho	GU322423
Lobin *et al.* (2010)	Tomato	Mauritius	Mauritius	HM448447
Cardenas-Conejo *et al.* (2010)	*Capsicum annuum*	Mexico	Baja-California	HM459851
NCBI	*Solanum lycopersicum*	USA	Kailua-Kona	HM988987
NCBI	Tomato	South Korea	Damyang 38	JN183876
NCBI	*Capsicum annuum*	China	Shouguang	JQ411237
NCBI	*Sinapisarvensis*	Jordan	–	JX131286
NCBI	Tomato	South Korea	Hwasun29	JX961667
Hosseinzadeh *et al.* (2014)	tomato	Iran	TYLCV-IL(IR:Boj:C3)	KC106650
NCBI	*Lycopersicon esculentum*	China	HBCL	KC702796
NCBI	*Lycopersicon lycopersicum*	Saudi Arabia	Jizan103	KC845301
NCBI	*Capsicum annuum*	Spain	ES:Mlg:TY11:Pep2003	KC953602
Barboza *et al.* (2014)	*Solanum lycopersicum*	Costa Rica	CR:5241-14:2012	KF533857
NCBI	*Tomato*	Kuwait	–	KJ830842
This work	Tomato	Egypt	TY10	KT921303
NCBI	*Solanum lycopersicum*	Morocco	33	LN846615
NCBI	not stated	Israel	Sardinia	TYLCSV DQ845787
Noris *et al.* (1994)	*Lycopersicon esculentum*	Spain	TYLCV-M	TYLCSV Z25751
Briddon *et al.* (1996)	*Solanum nigrum*	UK	K77B-11	TPCTV X84735