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MICROBIOLOGY

Molecular characterization of viruses associated to leaf curl disease complex on zucchini squash in Iraq reveals Deng primer set could distinguish between New and Old World Begomoviruses

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Abstract: The emergence of begomoviruses has led to significant losses in vegetable production in the recent years. Squash leaf curl disease (SqLCD) is caused by begomoviruses, and the infected plants show leaf curl symptoms on zucchini squash. In this study, we characterized the begomoviruses responsible for SqLCD symptoms and economic losses in zucchini major growing area near Baghdad and Babylon provinces. PCR amplification was performed to screen for begomovirus infection using Deng (a begomovirus specific) primer set. Sequence comparison confirmed the detection of two begomoviruses; *Tomato leaf curl Palampur virus* (ToLCPMV) and *Squash leaf curl virus* (SLCuV), in symptomatic zucchini samples when shared 99.14 and 99.50% maximum nucleotide (nt) identities with coat protein CP gene, respectively. All samples collected from Baghdad/Al-Jadriya were ToLCPMV infected while those collected from Babylon/Jibela were SLCuV infected. Moreover, mixed infection of the two viruses was detected in all zucchini squash samples collected from Baghdad/Yusufiyah. Evidence is provided here of the relevance of the wild species *Malva neglecta* and *Datura stramonium* as reservoir of begomoviruses that cause epedemics of leaf curl disease in zucchini squash in Iraq. Neighbor joining (NJ) Phylogenetic analysis confirmed the relatedness when diverged virus sequences in separated groups based on CP gene. The high nt identity suggests the two begomoviruses may recently be introduced to Iraq and could be a serious threatening to squash cultivation.

Key words: Geminiviridae, *Begomovirus*, plant viruses, Tomato leaf curl Palampur virus, Squash leaf curl virus.

INTRODUCTION

Zucchini or courgetti squash *Cucurbita pepo* L. is cultivated worldwide due to its high nutritional value. It originated from and was domesticated in Mexico and North America about 7000 years ago (Paris 1989, Hirst 2020). In Iraq, zucchini is grown both in protected (greenhouse) and open fields, throughout the year (Al-Kuwaiti 2017). Based on local statistical data released by the Iraqi Central Statistical Organization (CSO) in 2020, zucchini production decreased up to 79.6 %, in the past few years, from 126,000 tons in 2014 to 25,700 tons; this decline in zucchini production may be attributed to factors including viral diseases (CSO 2020). At least, 61 plant viruses, belonging to 39 different viral groups, were found to infect cucurbits, including zucchini squash (ICTV 2020, Al-Ani et al. 2009). The genus *Begomovirus*, member of the family *Geminiviridae,* is the largest amongst the other plant virus genera, as it includes 424 definite species (Brown et al. 2015, ICTV 2020). Begomoviruses comprise either mono- or bi-partite DNA genomes encapsidated

in geminate particles. They are transmitted in a persistent manner by the whitefly *Bemesia tabaci*, the only known vector. At least 21 begomoviruses were found to infect cucurbits worldwide causing serious diseases including leaf curl diseases (Kurowski et al. 2015, Fortes et al. 2016, Pandey & Verma 2017, ICTV 2020, Sanchez-Chavez et al. 2020). In Iraq, begomoviruses have been found to cause a significant damage to vegetable crops (Al-Ani et al. 2011a, b, Al-Kuwaiti 2017). Leaf curl diseases have threatened zucchini squash in many growing areas including the Middle Eastern countries (Lapidot et al. 2014, Medina-Hernández et al. 2019). These diseases are caused by a number of begomoviruses including *Squash leaf curl virus* (SLCuV) (Al-Kuwaiti 2017) and *Tomato leaf curl Palampur virus* (ToLCPMV) (Heydarnejad et al. 2013). SLCuV is a New World (NW) begomovirus, possibly originated in Mexico (Medina-Hernández et al. 2019, Vargas-Salinas et al. 2019). It comprises a bipartite genome DNAA and DNAB. The DNAA encodes five proteins, a coat protein (AV1), replication-associated Rep (AC1), transcriptional activator protein Trap (AC2), replication enhancer protein REn (AC3) and AC4. Whereas DNAB includes 2 ORFs encoding nuclear shuttle protein NSP (BV1) and movement protein MP (BC1). Besides, SLCuV genome includes non-coding intergenic regions (IRs) common regions (CR) A (or IR) and B (long intergenic region LIR) and short intergenic region SIR (ICTV 2020). Zucchini squash plants infected with SLCuV exhibit typical symptoms including a severe chlorotic mosaic or mottle on foliar, curling, malformation and thickened vein-banding of the leaf, stunting, flower drops and fruit set failure (CABI 2020, Medina-Hernández et al. 2019). Beside cucurbits, SLCuV has been reported to infect host plants within the families Solanaceae, Malvaceae, Fabaceae, Euphorbiaceae and Chenopodiaceae (Duffus & Stenger 1998, Al-Musa et al. 2008, Awad et al. 2019, CABI 2020). ToLCPMV belongs to Old World (OW) bipartite begomoviruses. It infects tomato, beans and many cucurbitaceous hosts (Heydarnejad et al. 2009, 2013). DNAA contains 6 ORFs encoding AV2 (precoat protein), CP, REn, TrAp, Rep and AC4. DNAB includes 2 ORFs encoding NSP and MP (Malik et al. 2011). These two DNAs share a common sequence of approximately 200 bp within the intergenic region (termed the "common region", CR), encompassing the conserved stem-loop with the 5′-TAATATTAC-3′ sequence at the virion strand origin of replication v-*ori* (ICTV 2020).

Squash leaf curl disease (SqLCD), caused by SLCuV, was confirmed in Iraq based on partial DNA sequencing (Al-Kuwaiti 2017). Since then, this disease has threatened zucchini squash in recent growing seasons. SqLCD symptoms were observed in zucchini plants in growing areas in Baghdad and Babylon provinces with a high disease incidence (ca. 100%). Thus, this study was initiated to investigate whether these symptoms are caused by a single infection of SLCuV or another begomovirus.

MATERIALS AND METHODS

Sample collection

Leaf samples were collected from zucchini squash exhibiting leaf curl disease symptoms in major growing areas in Baghdad (33.333° N 44.383° E; elevation 43m) and Babylon (32° 32'11'N 44° 25'15'E). There were seven symptomatic samples from Babylon/Jibela area (Fig. 1a), two from Baghdad/Al-Jadriya area (Fig 2a-c) and fourteen from Baghdad/Yusufiyah area (Fig. 3a-c). Samples from symptomatic weed plants present in Zucchini fields were also collected (Figs. 2d and 3d).

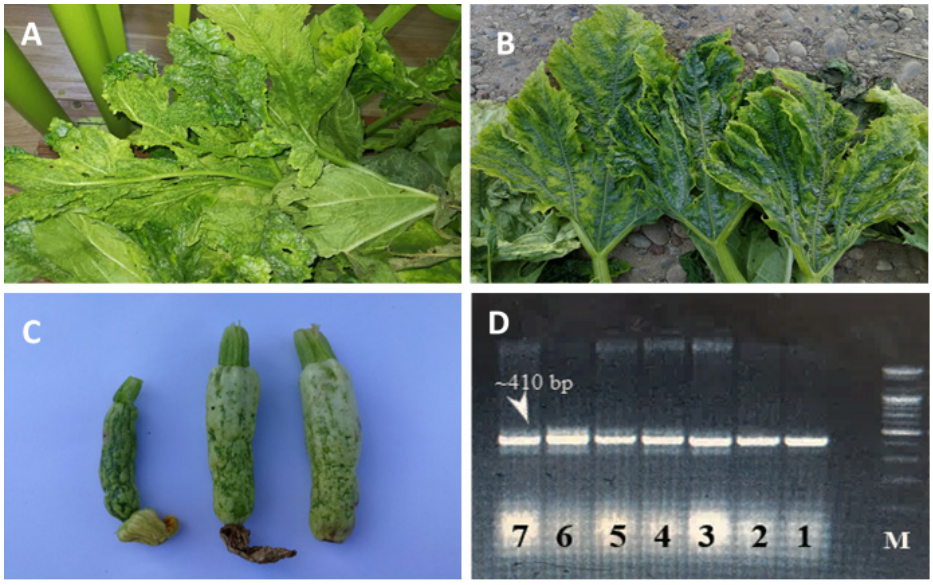

Figure 1. Naturally infected zucchini squash plants exhibiting leaf curl disease caused by SLCuV collected from Babylon/Jibela (a & b), SLCuV symptoms on fruit (c) and Gel electrophoresis pattern showing ~410 DNA fragments amplified from zucchini (1-7). M: 100 bp DNA marker (d).

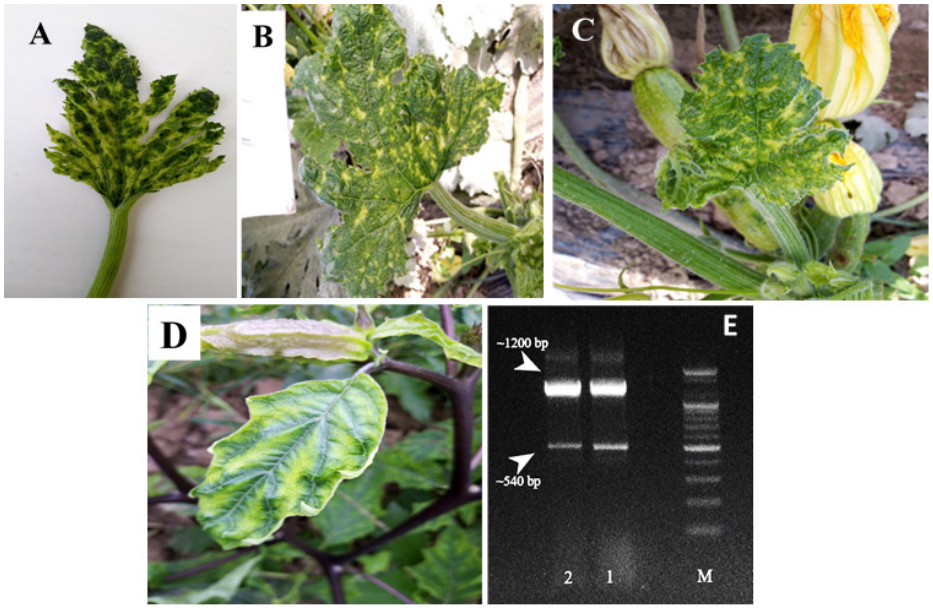

Figure 2. Naturally infected zucchini squash (a-c) from plants exhibiting leaf curl like symptoms collected from Baghdad/Al-Jadriya caused by ToLCPMV. ToLCPMV on Datura plant (d). Gel electrophoresis pattern showing ~540 bp DNA fragments amplified from zucchini lane (1) and datura lane (2). M: 100 bp DNA marker (e).

DNA extraction, PCR amplification and sequencing

Total DNA was extracted using commercial DNA extraction kit (Bioneer, South Korea) following the manufacturer`s instructions. PCR amplification was performed using AccuPower PCR PreMix commercial kit (Bioneer, South Korea) using a bigomovirus specific primer set (Deng et al. 1994) and following the protocol described by Al-Kuwaiti (2017). PCR products were visualized by ethidium bromide agarose gel electrophoresis following Sambrook & Russell (2006) standard protocol. PCR products were sent to Macrogen, South Korea for sequencing. Sequence analysis was performed using MEGAX (Kumar et al. 2018) and Sequence Demarcation Tool Version 1.2 (SDTv1.2) (Muhire et al. 2014) software packages.

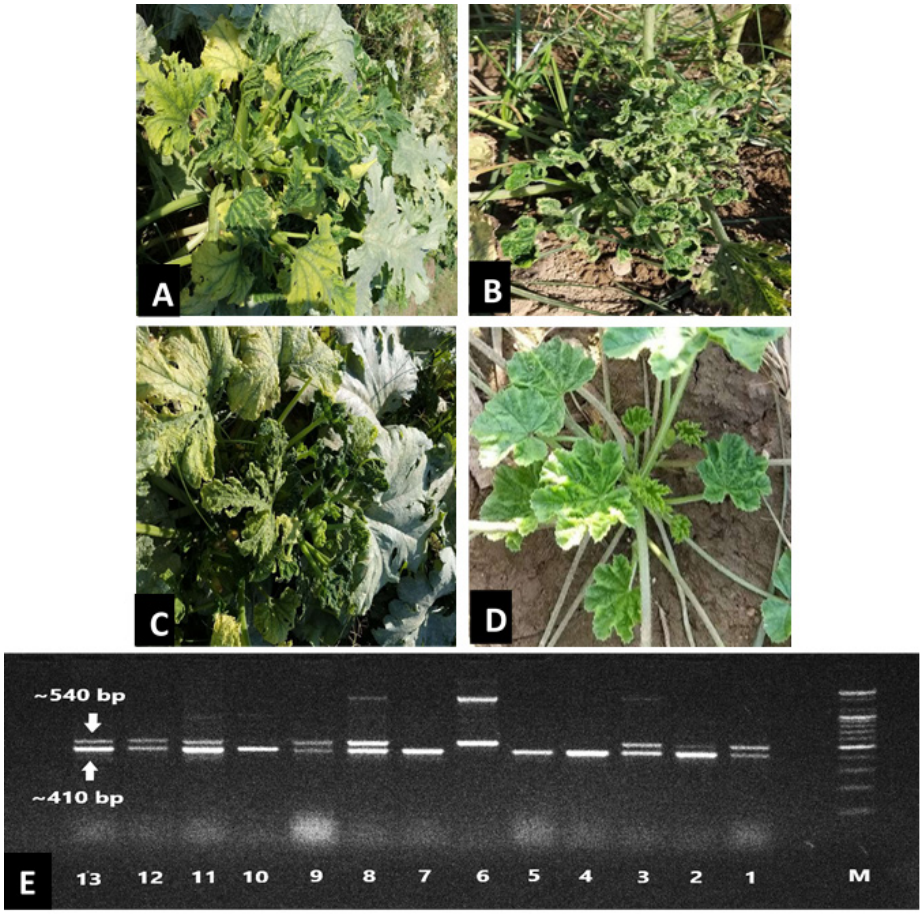

Figure 3. Naturally infected zucchini squash plants exhibiting leaf curl disease caused by SLCuV and ToLCPMV mixed infection; samples were collected from Baghdad/Yusufiyah growing area (a-c), d: Mallow plant exhibiting vein thickness symptoms collected from the same field that is infested by two begomoviruses. e: Gel electrophoresis pattern showing ~410 and 450 bp DNA fragments amplified from zucchini indicating mixed infections lanes (1-13). Lane 5: mallow sample. M: 100 bp DNA marker.

RESULTS

Begomoviruses were detected in SqLCD symptomatic plants

PCR results indicated that the leaf samples collected from symptomatic plants (Figs. 1a& b, 2a-d and 3a-d) were infected with begomovirus. Surprisingly, Deng primer set amplified two different DNA fragments sizes (Figs. 1d, 2e and 3e). These DNA fragments ranged ~410-540 bp indicating presence of two different begomoviruses in infected samples (Deng et al. 1994, Al-Kuwaiti 2017).

Sequence comparison confirmed the detection of two begomoviruses when the obtained sequences shared 99.51 and 98.59 % maximum nucleotide (nt) identity percent with the equivalent SLCuV and ToLCPMV GenBank sequences, respectively, in partial CP gene (Table I). SDT analysis showed that sequences isolated are belonging to two different begomoviruses, based on pair wise alignment of partial CP amino acid sequences (Fig. 4). Phylogenetic analysis confirmed the begomoviral species relatedness due to the information of two groups, SLCuV and ToLCPMV lineages (Fig. 5).

An extra ~1200 bp DNA fragments were amplified from samples infected with ToLCPMV both in single and mixed infection samples (Figs. 2e and 3e). Sequence comparison confirmed that the ~1200 bp was amplified from partial CP/AC4 genomic regions. The AC4 sequences obtained scored 97.4-98.78% maximum nt identities when compared to the equivalent GenBank sequences (Table I).

Table I. Begomovirus sequences isolated from symptomatic plants.

GenBank Acc. Code	isolate	host	location	virus	DNA fragment size/Gene	Identity%
MH277018-MH277031	SqLCV1-SqLCV14	Zucchini	Babil/Jibela	SLCuV	410/CP	90.82-99.50
MK606691-MK606696	TLCPV sequash1-TLCPV sequash6	Zucchini	Baghdad/AL-Jhadryaa	ToLCPMV	530/CP	98.39-99.14
MK606697-MK606698	TLCPV Datura1-TLCPV Datura2	Datura				97.59-98.59
MT248389-MT248405	SLCVY2-SLCVY14a	Zucchini*	Baghdad/ Yusufiyah	SLCuV	410/CP	89.68-99.51
MT248390-MT248391	SLCV Y6- SLCV Y6a	Mallow				93.89-99.02
MT248372-MT248377	TLCPV Y1- TLCPV Y10	Zucchini*		ToLCPMV	530/CP	91.87-96.98
MW147674-MW147675	TLCPV sequash1-TLCPV sequash2	Zucchini	Baghdad/AL-Jhadryaa	ToLCPMV	1200/AC4	96.65-97.4
MW314140-MW314141	TLCPV7- TLCPV7a	Zucchini*	Baghdad/ Yusufiyah	ToLCPMV	1200/AC4	98.33-98.78

***Samples detected with mixed begomovirus infection.**

Mixed infection by begomoviruses caused the most severe symptoms in zucchini

Mixed infection of SLCuV and ToLCPMV was detected in most zucchini samples collected from Baghdad/Yusufiyah. Gel electrophoresis revealed two DNA fragments were amplified from these samples (Fig. 3e) compared to single begomoviral infections in zucchini samples collected from Babylon/Jibela (Fig. 1) and Baghdad/Al-Jadriya (Fig. 2e). Sequence comparison confirmed the mixed infection when traces of ToLCPMV CP sequences were present with SLCuV positive zucchini samples. The sequence bits obtained, ranged 75-100 bp (data not shown) and were identical to ToLCPMV CP region. Similarly, SLCuV CP sequence bits were detected in ToLCPMV positive zucchini samples. A similar gel pattern was obtained in a previous study (Al-Kuwaiti 2017) but mixed infection was not confirmed.

Mallow (*Malva neglecta*) is an alternative host to SLCuV, but Datura (D*atura stramonium*) is an alternative host for ToLCPMV

Single SLCuV infection was detected in mallow (*Malva neglecta*) sample collected from the same zucchini field in Baghdad/Yusufiyah. Mallow, therefore, could be an alternative host for SLCuV (Al-Musa et al. 2008) but not for ToLCPMV. Sequence analysis confirmed that *D. stramonium* that exhibited leaf yellowing and green vein thickness (Fig. 2d) was ToLCPMV positive (Table I), which suggest a new alternative solanaceous host that has not been reported before.

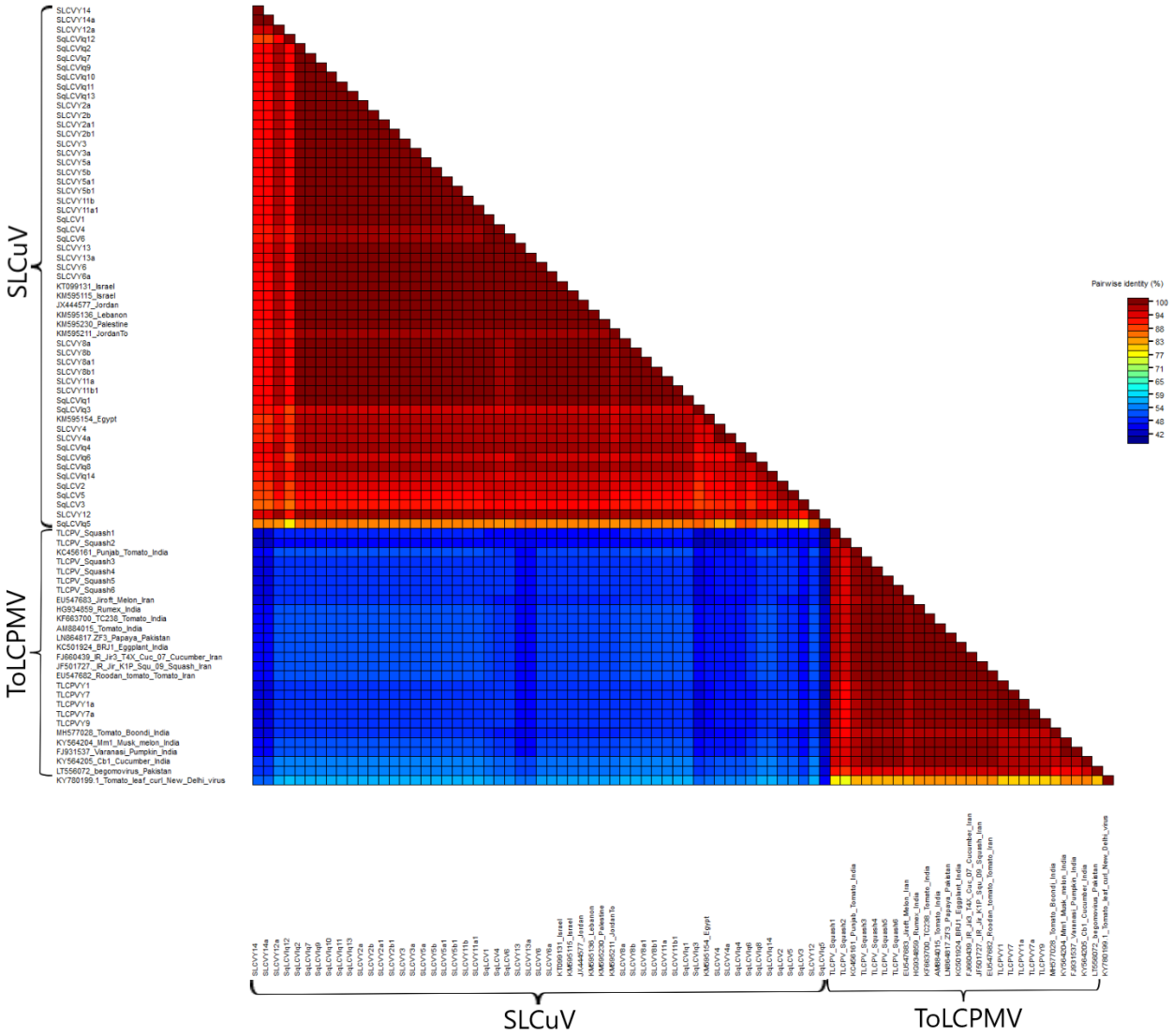

Figure 4. Three color mode matrix showing SLCuV and ToLCPMV identities, constructed from partial CP deduced amino acid sequences of isolated and the equivalent sequences from the GenBank. *Tomato leaf curl New Delhi virus* TLCNDV was used as an out-group comparison.

DISCUSSION

The association of SLCuV with other begomoviruses on squash has previously been reported (Sufrin-Ringwald & Lapidot 2011, Medina-Hernández et al. 2019), but not with ToLCPMV. Moreover, mixed infection of ToLCPMV and other begomoviruses was detected in pumpkin (Namrata Jaiswal et al. 2012). Based on gel electrophoresis and sequence analysis, shorter DNA fragments could be amplified due to the absence of AV2 gene in SLCuV compared to ToLCPMV. The extra ~1200 bp DNA fragments amplified from samples infected with ToLCPMV was from partial CP/AC4 genomic regions. The AC4 sequences obtained scored 97.4-98.78% maximum nt identities when compared to the equivalent GenBank sequences (Table I). Neighbor joining phylogentic tree grouped the AC4 sequences isolated to those from the GenBank confirming its relationship (Fig. 6). These results suggest Deng primer set can be a

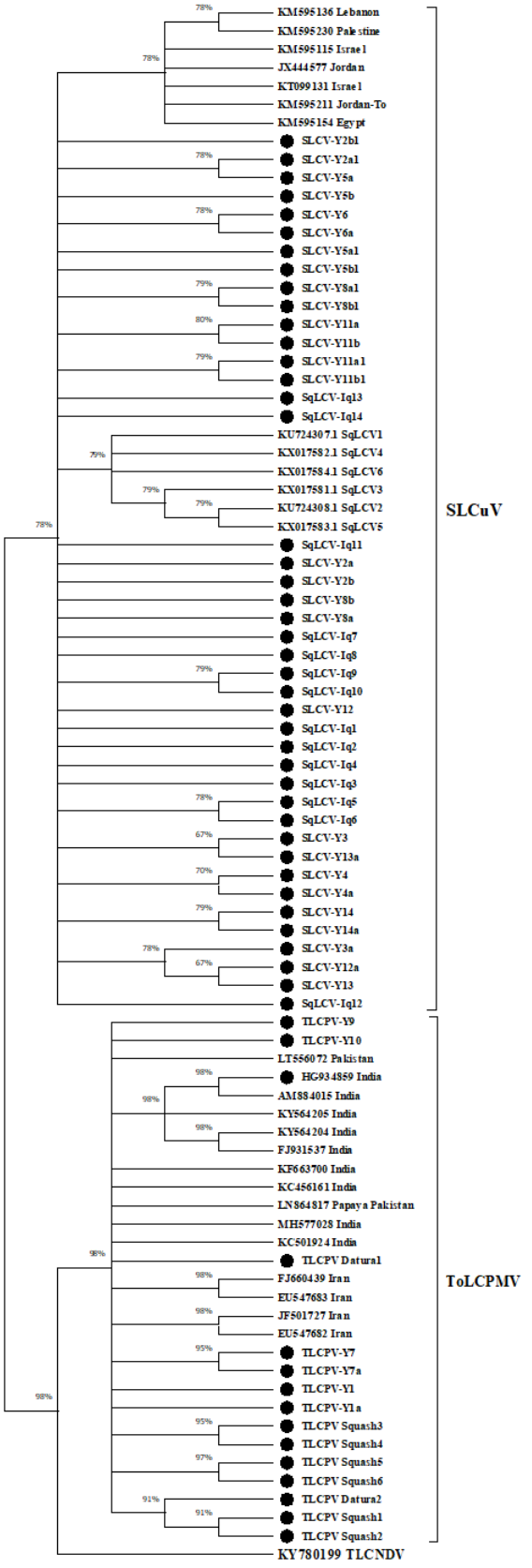

Figure 5. Neighbor joining phylogenetic tree constructed from partial begomoviral CP nucleotide sequences of SLCuV and ToLCPMV isolates from Iraq (marked with ●) and equivalent GenBank sequences. Tomato leaf curl New Delhi virus TLCNDV was used as an out-group comparison.

useful tool to distinguish between NW and OW begomoviruses infecting zucchini, both in mixed and single infection based on gel profile of the DNA fragments amplified.

Mixed infection caused by SLCuV and ToLCPMV can be a serious threat to zucchini squash production in Iraq and Middle eastern countries as these two begomoviruses are highly epidemic (Heydarnejad et al. 2013, Lapidot et al. 2014). This disease complex suggests SLCuV and ToLCPMV have been introduced to Iraq and settled in the recent past. These two begomoviruses may have been moved from bordering countries to Iraq through infected plant materials carrying whiteflies (Lapidot et al. 2014). It is possible that SLCuV has been introduced through Middle Eastern countries west Iraq, as this begomovirus has not been reported, neither in Iran nor Tukey (Lapidot et al. 2014). ToLCPMV could have been introduced from Iran east Iraq based on data available regarding the high incidence of this begomovirus in Iran only. This OW recombinant begomovirus could have been originated from India then moved to Iran (Heydarnejad et al. 2009).

This study confirmed that leaf curl disease on squash in Iraq is caused by at least two different begomoviruses, SLCuV and ToLCPMV. The high identity percentage, low sequence variability, weed and mixed infections suggest that these two begomoviruses have been introduced into and settled in Iraq in the recent past. SLCuV and ToLCPMV can be highly epidemic in Iraq as

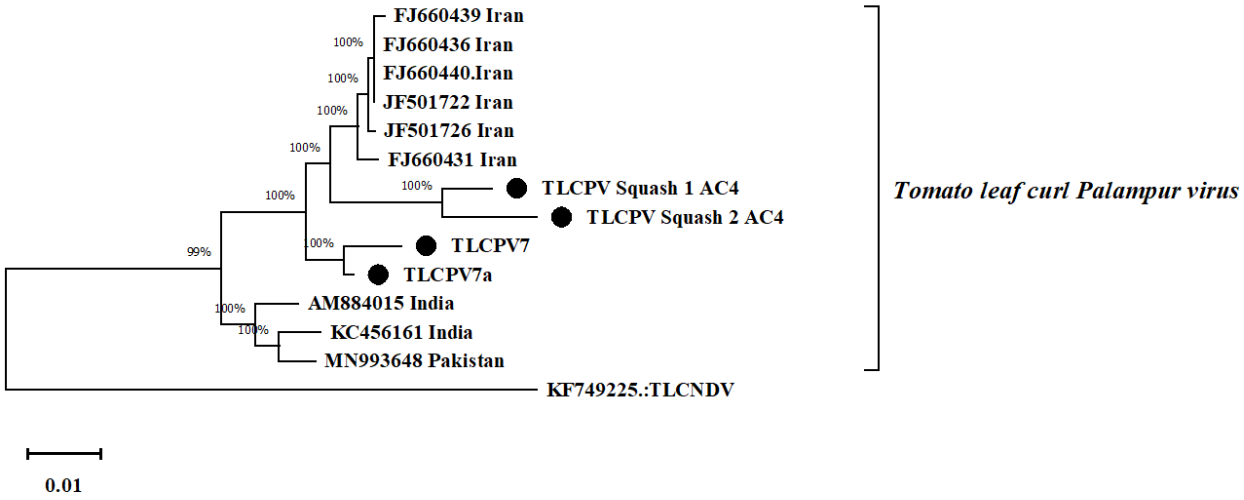

Figure 6. Neighbor joining phylogenetic tree constructed from partial begomoviral AC4 nucleotide sequences of ToLCPMV isolates from Iraq (marked with ●) and equivalent GenBank sequences. Tomato leaf curl New Delhi virus TLCNDV was used as an out-group comparison.

they can infect plants from different families (Heydarnejad et al. 2013, Lapidot et al. 2014). Host biodiversity, the agricultural intensification due to the water shortage and the high activity of the vector (*B. tabaci*) can provide a constant source of infection and recombination events of these two begomoviruses in Iraq (Seal et al. 2006). Thus, the use of resistance varieties may be insufficient to control begomoviruses in Iraq. Rapid action and precaution procedures must be taken to protect zucchini and other crops against begomoviruses in Iraq and the Middle East. Further studies, including full length genome amplification and agroinoculation are required to investigate the leaf curl disease infecting zucchini squash in Iraq and maybe the neighboring countries.

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Author contributions

Doaa Mohammed: contributed in laboratory work and writing of the original draft. Mustafa Adhab: participated in the molecular laboratory methodology, sampling, conceptualization and review and editing the draft. Nawres Alkuwaiti: contributed in laboratory work, conceptualization, supervision, writing, review and edition of the draft.