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Partial Characterization of Watermelon Chlorotic Stunt Virus from *Trichosanthes cucumerina* in Al-Ahsa, Saudi Arabia

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Abstract: Leaf samples were collected from the symptomatic snake gourd (*Trichosanthes cucumerina*) plants showing plant stunting, leaf yellowing and mottling symptoms during 2016-17 in Al-Ahsa region of the Kingdom of Saudi Arabia. Total genomic DNA was extracted from three samples and was analyzed further for the presence of begomovirus infection through rolling circle amplification (RCA), PCR and Sanger sequencing. Universal primer pairs AC1048/AV494 and PCRc1/PBL1v2040 were used to amplify the partial coat protein region of DNA-A and partial DNA-B, respectively. All three samples were tested positive for the presence of both DNA-A and DNA-B components of begomoviruses. Three partial PCR amplicons from two plants were further confirmed through Sanger sequencing. The sequence analysis revealed that the partial DNA-A shared the highest nucleotide (nt) sequence identities of 97.1-97.7% with that of watermelon chlorotic stunt virus (WmCSV) DNA-A (JN618984) identified from squash in Oman. Whereas, the three partial DNA-B amplicons shared the highest nt sequence identities of 94.2-95.8% with three WmCSV isolates reported from Iran (KT272770) and Oman (JN618980 and HG969288). The partial nt sequences of DNA-A and DNA-B were well-grouped with other WmCSV isolates in the phylogenetic dendrograms. This identification of WmCSV from the *T. cucumerina* plants reveals that WmCSV may become a major threat to the cucurbit crops in Saudi Arabia.

Keywords: Begomovirus, watermelon chlorotic stunt virus, *Trichosanthes cucumerina*, Saudi Arabia.

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1 Introduction

The genus *Begomovirus* is the largest genus of plant viruses in the family *Geminiviridae* with >350 species (Brown et al., 2015; Zerbini et al., 2017), which widely infect important dicotyledonous crop plants worldwide (Sattar et al., 2013; Sattar et al., 2017; Rey et al., 2012). There are nine genera reported so far in the family *Geminiviridae* including *Becurto*-, *Begomo*-, *Capula*-, *Curto*-, *Eragro*-, *Grablo*-, *Mastre*-, *Topocu*- and *Turncurto-virus* (Zerbini et al., 2017). These viruses are responsible to cause diseases to many economically important crop plants grown in tropical and sub-tropical to temperate regions (Briddon et al., 2018; Malathi, 2017). Begomoviruses have circular, single-stranded DNA (cssDNA) genomes encapsidated in twinned-icosahedron protein coats (Hanley-Bowdoin et al., 2013; Mubin et al., 2019). Based upon the genome structure, the genus *Begomovirus* is further categorized into monopartite and bipartite begomoviruses (Zubair et al., 2017). The genome of bipartite begomoviruses is comprised of two almost equally sized genomic components DNA-A and DNA-B (~2.6-2.7kb each), which are jointly encapsidated into the twinned-icosahedrons (Roshan et al., 2017; Zerbini et al., 2017). Whereas, monopartite begomovirus genome is an equivalent of DNA-A of bipartite begomovirus. The begomovirus replication, encapsidation and pathogenesis are encoded by 5-6 open reading frames (ORFs) (Iqbal et al., 2012; Saeed et al., 2018; Silva et al., 2017). These ORFs are encoded by DNA-A in the opposite orientation i.e. two ORFs (AV1 and AV2) in the

virion sense while four ORFs (AC1, AC2, AC3 and AC4) in the complementary sense orientation (Hanley-Bowdoin et al., 2013; Sattar, 2012). All ORFs are transcribed from a common promoter in the common region (CR) of the genome (Ashraf et al., 2014). The DNA-B of bipartite begomoviruses only encodes two ORFs (BV1 and BC1) in the opposite orientation (Zhou, 2013). The essential role of DNA-B in the viral pathogenesis is to regulate *in planta* viral trafficking across the cellular passages (Briddon et al., 2010). The DNA-A and DNA-B of bipartite begomoviruses share a ~200-400 bp CR and a stem-loop with nona-nucleotides (TAATATTAC).

Bipartite begomoviruses, mostly prevailing in the New World (NW), usually have five ORFs in their DNA-A component (Al-Aqeel et al., 2018). Whereas, most of the monopartite begomoviruses have been reported from the Old World (OW) (Rey et al., 2012). Monopartite begomoviruses are also found associated with DNA-satellites alphasatellite, betasatellite and recently deltasatellites (Briddon et al., 2018; Fiallo-Olive et al., 2016; Malathi et al., 2017). Although, in OW mostly monopartite begomoviruses have been reported however, some bipartite begomoviruses have also been found (Brown et al., 2015). Watermelon chlorotic stunt virus (WmCSV) is a bipartite begomovirus, which was firstly reported from Yemen during late 1980s (Rojas et al., 2018; Walkey et al., 1990). Soon after its first emergence in Yemen, WmCSV spread out rapidly into the neighboring Middle-Eastern and North African countries (Ali-Shtayeh et al., 2014). In the subsequent years, it was reported from Israel (Abudy et al., 2010), Jordan (Al-Musa et al., 2011), Lebanon and Oman (Khan et al., 2012) and Palestine (Ali-Shtayeh et al., 2014). In Saudi Arabia, WmCSV was reported from watermelon crop recently (Al-Saleh et al., 2014).

Vegetable crops are under a continuous threat from begomoviruses in the tropical and sub-tropical regions (Leke et al., 2015). *Trichosanthes cucumerina* (family Cucurbitaceae) or snake gourd is well-known annual vegetable crop, which has been grown in many Asian countries (Liyanage et al., 2016). The fruit is pharmacologically and therapeutically very active and rich in flavonoids, carotenoids, various phenolics and dietary fibers. The present study was designed to isolate and characterize partial genome of WmCSV from *T. cucumerina* in the Eastern region of Kingdom of Saudi Arabia.

Fig. 1. ***Trichosanthes cucumerina*** **plants showing disease symptoms of plant stunting, leaf yellowing and mottling in Al-Ahsa, Saudi Arabia.**

2. Materials and Methods

2.1. Sample Collection

During the growing season of 2016-17, three *T. cucumerina* plants showing plant stunted growth, leaf yellowing and mottling symptoms were found in a field in the Al-Ahsa region of Kingdom of Saudi Arabia (Fig. 1). The youngest leaves were harvested from symptomatic (three) and asymptomatic (one) plants and were immediately transferred into the plastic bags and later used for downstream analysis on the same day.

2.2. Genomic DNA isolation and agarose gel electrophoresis

All the collected leaf samples were used for total genomic DNA isolation using CTAB method of DNA-extraction as previously described (Doyle et al., 1990). The isolated DNA-pellet was dissolved finally in 50 µl of autoclaved double distilled water. The integrity and quality of the isolated DNA was analyzed using 1% agarose gel electrophoresis in 0.5x TBE buffer.

2.3. RCA and PCR amplifications

The isolated DNA from all the plants was subjected to rolling circle amplification (RCA) using Phi-29 DNA-polymerase using Illustra Templiphi Kit (GE healthcare, UK) as described earlier (Qurashi et al., 2017). The RCA quantification was carried out by electrophoresing 0.5 µl RCA product. The confirmed

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RCA product was further used to carry out PCR amplification using universal degenerate primers, which usually amplify ~550 bp partial fragment of *coat protein* (CP) gene of DNA-A component of begomovirus genome(AC1048/AV494) (Brown et al., 2001). Another set of degenerate primers PCRc1/PBL1v2040 (Rojas et al., 1993) were also employed to amplify ~600 bp (the CR and partial replication associated protein) of DNA-B component. The successful PCR-amplicons were separated in 1% agarose gel and stained with ethidium bromide solution to illuminate under UV light in UV trans-illuminator (ThermoScientific).

2.4. Cloning and sequencing

The obtained PCR-amplicons were excised from the agarose gel and the excessive salts and/or gel impurities were removed by purification using QIAquick PCR purification kit (Qiagen, Germany). The purified DNA fragments were subsequently cloned into the pGEM-T Easy vector (Promega, USA) following the manufacturer's protocol. The recombinant plasmids containing the inserted DNA-fragments were transformed into the DH5α sub-cloning efficiency *Escherichia coli* cells (Invitrogen, USA). The transformed bacteria with recombinant plasmids were selected with blue-white selection on LB-agar media. The selected clones for DNA-A and DNA-B partial components were sent for complete sequencing through Sanger sequencing platform (Macrogen, Korea).

2.5. Comparative Sequence Analysis and Phylogenetic Dendrograms

The obtained sequences of partial DNA-A and DNA-B genomes were initially compared by BLASTn tool available in NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) with the most closely related begomovirus sequences. The top 20 most closely related DNA-A and DNA-B nt sequences were retrieved from NCBI and were used for sequence comparison in Sequence Demarcation Tool (SDT v1.2) (Muhire et al., 2013) to obtain pairwise nt sequence identities. The muscle algorithm was used for sequence comparison and the comparative plots were created in three-color mode with cut-off value set at 91%. The evolutionary inferences were obtained by constructing phylogenetic dendrograms in Mega7 software using neighbor-joining (NJ) algorithm and maximum likelihood model (Kumar et al., 2016). The integrity of the phylogenetic trees was tested by bootstrap method with 1000-bootstrap iterations.

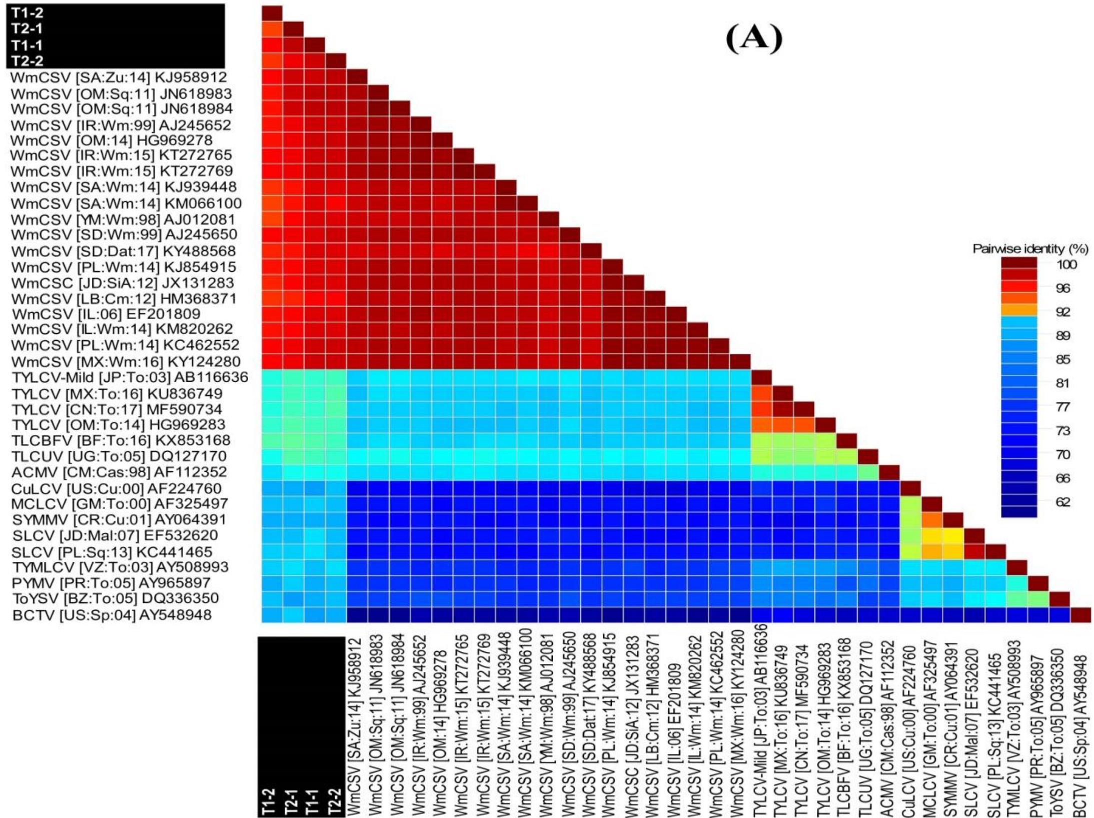
3. Results and Discussion

3.1. Plant Screening and Sample Collection

During the growing season of 2016-17 some *T. cucumerina* plants were found showing typical begomovirus symptoms of stunted growth, leaf yellowing and mottling in an open field in the vicinity of Al-Ahsa region, Kingdom of Saudi Arabia. The presence of heavy whitefly infestation and plant symptoms pointed towards begomovirus infection in the plants. For confirmation, four (including three symptomatic and one asymptomatic) plants were randomly selected from the field and were subjected to DNA extraction in the College of Agriculture and Food Science, King Faisal University, Al-Ahsa, Saudi Arabia. Total genomic DNA was extracted from one asymptomatic plant T0 and three symptomatic samples T1, T2 and T3. Using universal primers the partial DNA-A and DNA-B components were successfully amplified for suspected begomovirus/es. The consecutive attempts were failed to amplify any associated DNA-satellites using universal primers UN101/UN102 (Bull et al., 2003) and Beta01/Beta02 (Briddon et al., 2002) for alphasatellite and betasatellite, respectively. However, the PCR using universal primers AC1048/AV494 and PCRc1/PBL1v2040 yielded ~500 and ~600 bp fragments, respectively. The asymptomatic plant sample could not produce any amplification.

3.2. Sequencing and Sequence Analysis of Partial DNA-A Component

The PCR amplicons corresponding to the CP region of begomoviruses from two symptomatic plants T1 and T2 were selected for cloning into the pGemT-easy PCR cloning vector. After bacterial transformation and cloning, two clones T1-1 and T1-2 from the plant T1 whereas, two clones T2-1 and T2-2 from the plant T2 were selected for sequencing. An initial sequence analysis in NCBI GenBank database showed that all four clones represented typical begomovirus partial CP region. The top most similar sequences were retrieved and were further employed for SDT and phylogenetic analysis. The SDT analysis revealed that all four clones were 92.5-97.7% identical to each other whereas, three clones T1-1, T2-1 and T2-2 shared the highest nt sequence identities at 97.7, 97.1 and 97.4% respectively to WmCSVDNA-A (JN618984) identified from squash in Oman (Fig. 2A). Nevertheless, the clone T1-2 shared the highest nt sequence identity with WmCSV isolate (KC462552) identified from watermelon in Palestine (Fig. 2A).



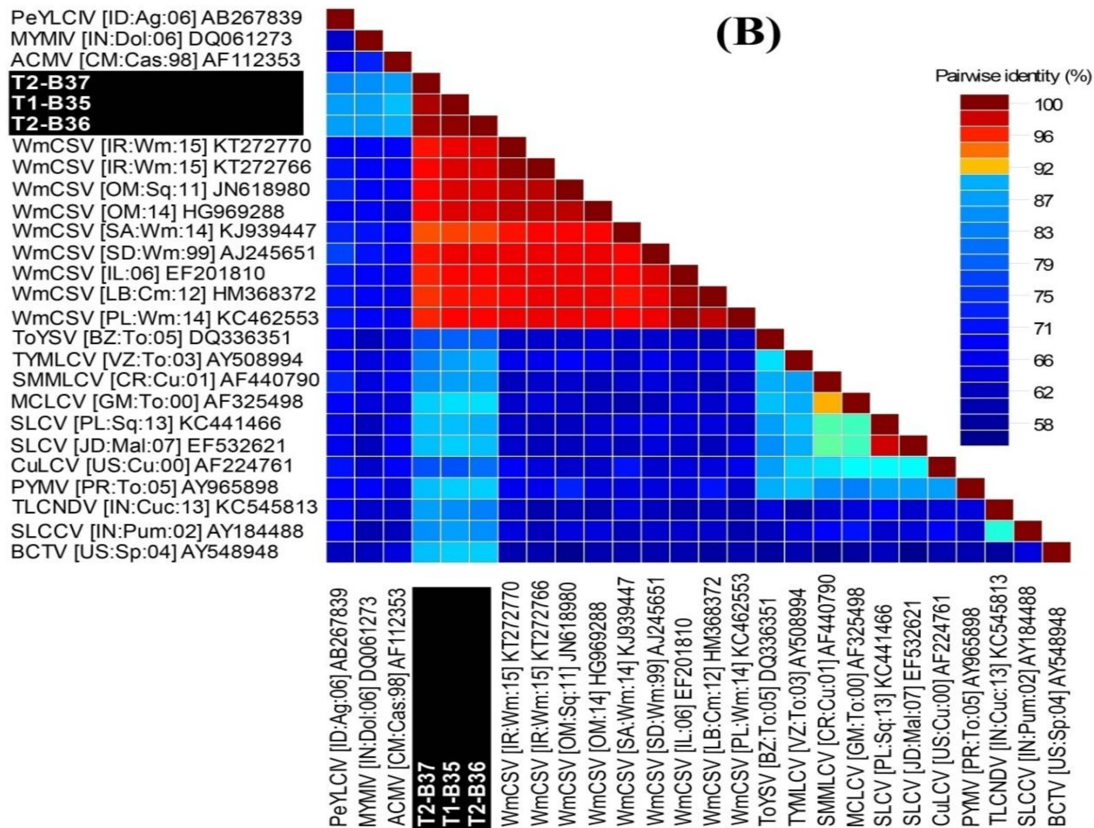
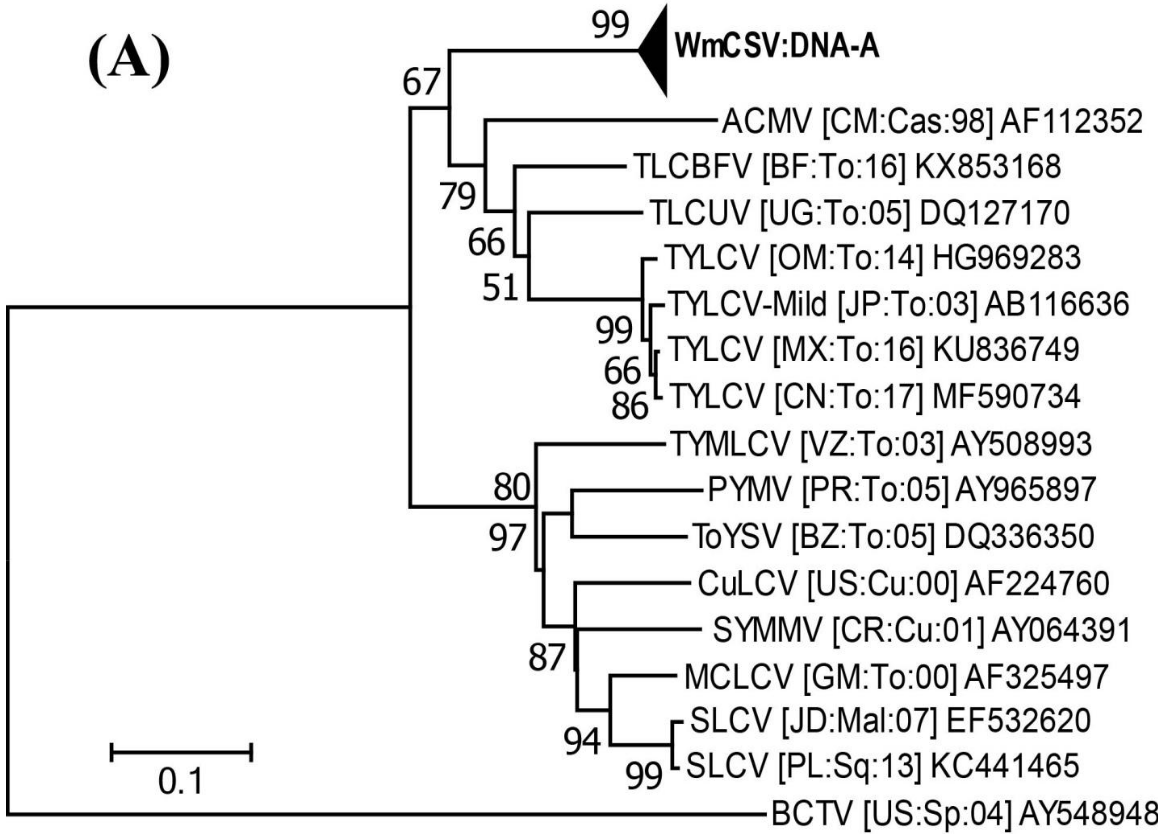
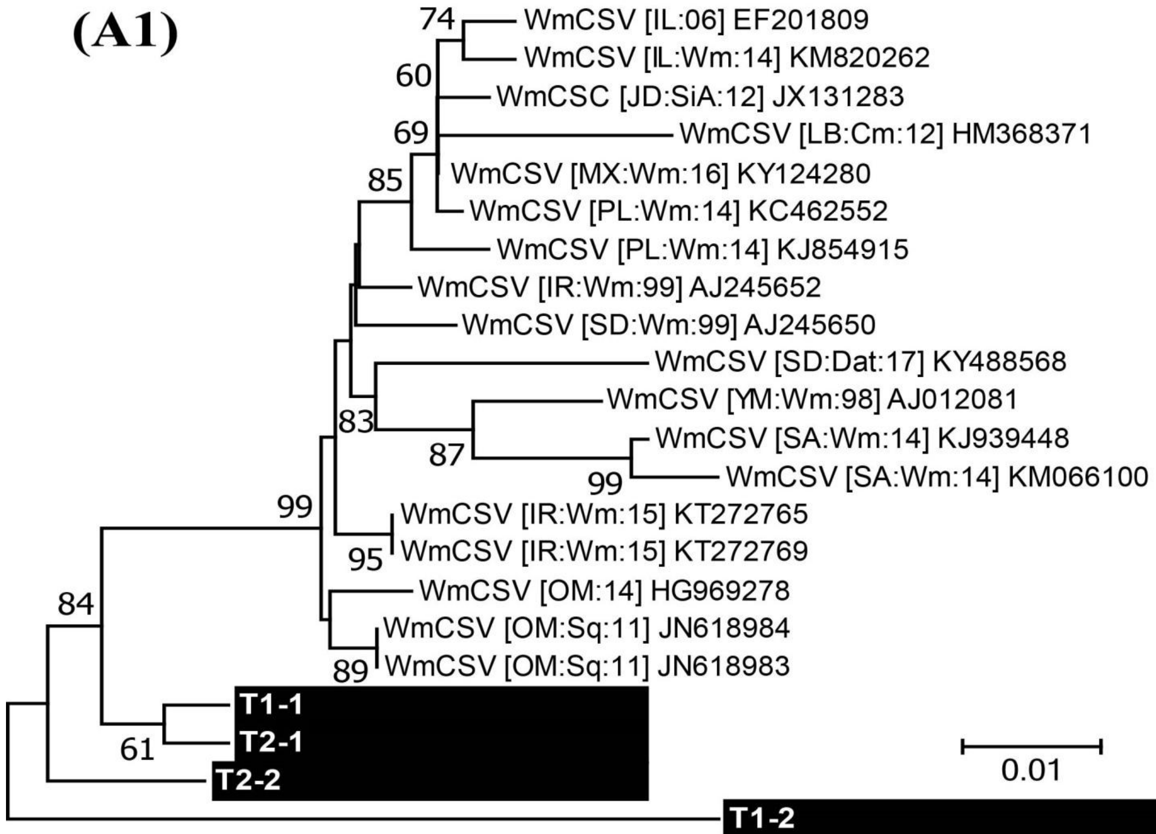


Fig. 2. Pairwise nucleotide (nt) sequence identities of the partial DNA-A (A) and DNA-B (B)with the retrieved sequences from the NCBI GenBank database, respectively.


(A)
WmCSV:DNA-A
ACMV [CM:Cas:98] AF112352
TLCBFV [BF:To:16] KX853168
TLCUV [UG:To:05] DQ127170
TYLCV [OM:To:14] HG969283
TYLCV-Mild [JP:To:03] AB116636
TYLCV [MX:To:16] KU836749
TYLCV [CN:To:17] MF590734
TYMLCV [VZ:To:03] AY508993
PYMV [PR:To:05] AY965897
ToYSV [BZ:To:05] DQ336350
CuLCV [US:Cu:00] AF224760
SYMMV [CR:Cu:01] AY064391
MCLCV [GM:To:00] AF325497
SLCV [JD:Mal:07] EF532620
SLCV [PL:Sq:13] KC441465
BCTV [US:Sp:04] AY548948
0.1


(A1)
WmCSV [IL:06] EF201809
WmCSV [IL:Wm:14] KM820262
WmCSC [JD:SiA:12] JX131283
WmCSV [LB:Cm:12] HM368371
WmCSV [MX:Wm:16] KY124280
WmCSV [PL:Wm:14] KC462552
WmCSV [PL:Wm:14] KJ854915
WmCSV [IR:Wm:99] AJ245652
WmCSV [SD:Wm:99] AJ245650
WmCSV [SD:Dat:17] KY488568
WmCSV [YM:Wm:98] AJ012081
WmCSV [SA:Wm:14] KJ939448
WmCSV [SA:Wm:14] KM066100
WmCSV [IR:Wm:15] KT272765
WmCSV [IR:Wm:15] KT272769
WmCSV [OM:14] HG969278
WmCSV [OM:Sq:11] JN618984
WmCSV [OM:Sq:11] JN618983
T1-1
T2-1
T2-2
T1-2
0.01

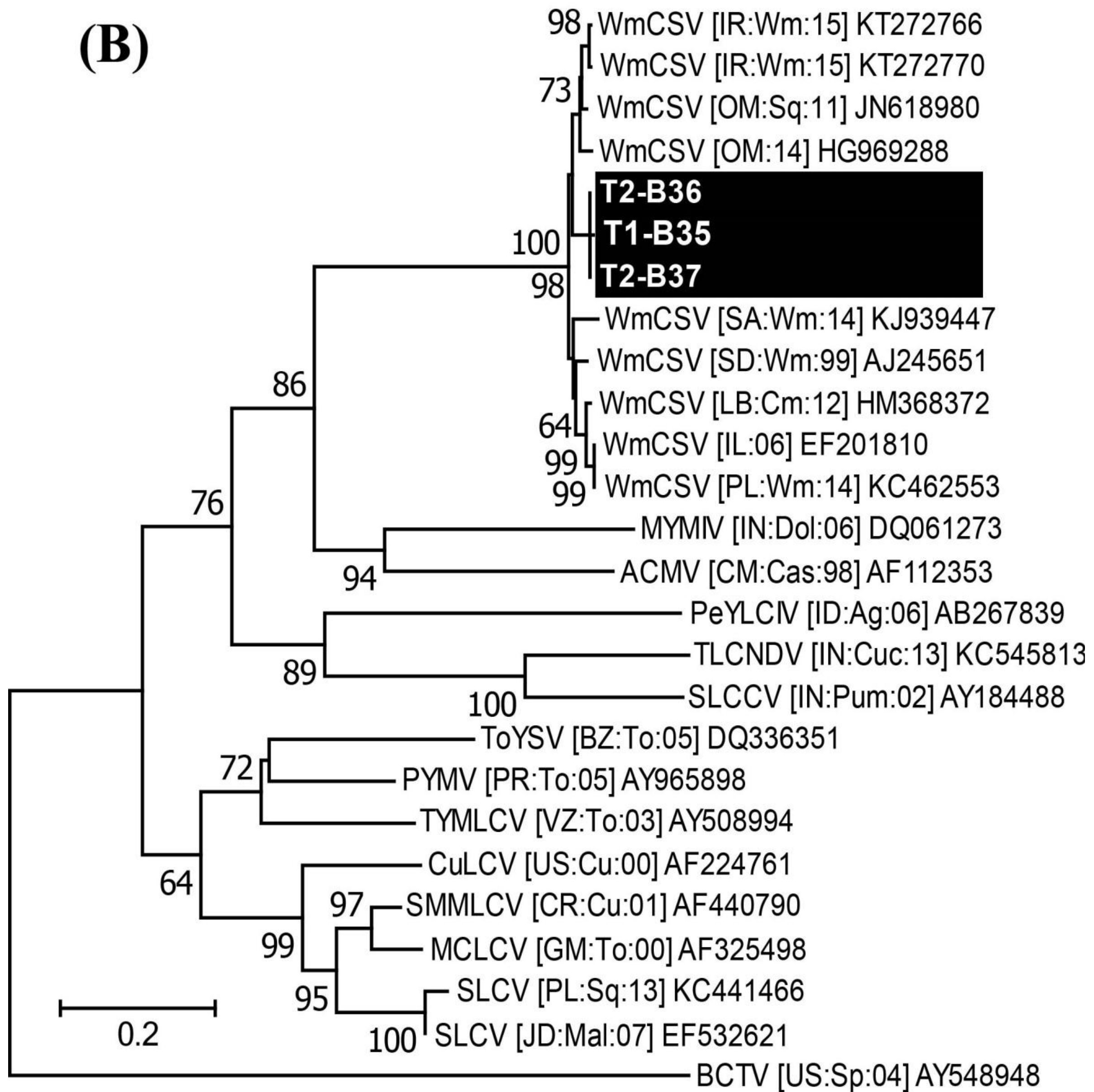


Fig. 3.The phylogenetic dendrograms showing evolutionary relationships of watermelon chlorotic stunt virus (WmCSV) compoennts. The phylogenetic trees were constructed for the coat protein (CP) of DNA-A **(A)** of WmCSV isolates with the selected begomovirus sequences retrieved from NCBI GenBank database. Furthermore, the WmCSV clade was resolved to reveal the diversification of begomovirus isolates in this study (**A1**). The software Mega7 was used to inferr the molecular evolutionary genetic analysis with neighbor joining algorithm. The isolates identified in this study are shown in white text on a black background. The numberic branch nodes are showing percent (%) bootstrap values more than 60% with 1000 replicates and the nt substitutions have been shown as horizontal lines. The viral isolates were named and abbreviated as per suggestions from the International Committee for Taxonomy of Viruses (ICTV). Beet curly top virus (BCTV) DNA-A and DNA-B seqeucnes were used as an out-group to root the tree, respectively. Begomovirus acronyms used are African cassava mosaic virus (ACMV), cucurbit leaf curl virus (CuLCV), melon chlorotic leaf curl virus (MCLCV), mungbean yellow mosaic India virus (MYMIV), pepper yellow leaf curl Indonesia virus (PeYLCIV), potato yellow mosaic virus (PYMV), squash leaf curl virus (SLCV), squash leaf curl China virus (SLCCV), tomato leaf curl Burkina Faso virus (TLCBFV), tomato leaf curl New Delhi virus (TLCNDV),tomato leaf curl Uganda virus (TLCUV), tomato yellow leaf curl virus (TYLCV), tomato yellow margin leaf curl virus (TYMLCV), tomato yellow spot virus (ToYSV) andwatermelon chlorotic stunt virus (WmCSV).

The phylogenetic dendrogram also grouped all four isolates together with other WmCSV isolates reported previously into a well-supported clade with 99% bootstrap value (Fig.3A). Moreover, when WmCSV clade was further resolved by using only WmCSV isolates the four clones in this study grouped monophyletically making a separate branch from WmCSV isolates reported so far (Fig.3A1). It indicates that the diversity of WmCSV is probably very high in the Middle-Eastern countries (Lapidot et al., 2014).Thus, it can be speculated that the WmCSV isolates reported from Saudi Arabia are quite distinct and have different evolutionary origin. The spread of WmCSV in the cucurbit fields might be due to the exchange of infected vegetative plant materials or through the viruliferous whitefly vectors from the neighboring countries. The dominating whitefly species in the Al-Ahsa region in the Eastern Saudi Arabia is the Middle-East Asia Minor 1 (previously called B-biotype) (Alhudaib et al., 2014). However, in the Western border regions with Yemen the Mediterranean species (previously called Q-biotype) is the most dominating whitefly species (Ragab, 2013). Thus, it is speculated that the spread of these distinct isolates of WmCSV is due to the diverse whitefly population in this region, particularly in the Eastern province.

3.2. Sequencing and Sequence Analysis of Partial DNA-B Component

Besides, three clones T1-B35, T2-B36 and T2-B37 were also selected for sequencing from plants T1 and T2, respectively. The sequencing analysis revealed that all three clones represent DNA-B component of WmCSV isolate. These three clones were 98.1-99.5% identical to each other whereas, they shared the maximum nt sequence identities of 94.2-95.8% with three WmCSV isolates reported from Iran (KT272770) and Oman (JN618980 and HG969288), respectively (Fig. 2B). The phylogenetic dendrogram also grouped these clones together into a well-supported (100% bootstrap value) clade with other previously reported isolates of WmCSV DNA-B (Fig.3B).

Begomoviruses have been increasing their host range from weeds (Kyallo et al., 2017; Sohrab, 2017), crops (Barboza et al., 2018; Munir et al. 2018; Sahu et al., 2018), ornamental (Khatri et al., 2014; Kumari and Mishra, 2017; Marwal et al., 2014; Marwal et al., 2018) aromatic, medicinal (Saeed and Samad, 2017) and the woody plants (Sattar et al., 2018). Diversification is the main force behind the begomovirus evolution and hence it is necessary to regularly carry out population diversity surveys (Islam et al., 2018; Kumar et al., 2017; Lima et al., 2017; Mubin et al., 2019). The Middle-Eastern countries have faced two begomovirus invasions in cucurbit production during last couple of decades (Lapidot et al., 2014). These include squash leaf curl virus (SLCV) originated from the NW and WmCSV, which was originated in the OW. Both these invading begomoviruses are being established as epidemic in the cucurbit corps simultaneously in the Middle-East. The identification of WmCSV in various Middle-Eastern countries and Saudi Arabia reflects that the virus inoculum is getting higher due to spread of more efficient whitefly vector in these regions (Alhudaib et al., 2014). The situation is becoming more alarming with the recent identification of WmCSV from watermelon (Al-Saleh et al., 2014) and *T. cucumerina* in the Saudi Arabia (present study). A most recent report of WmCSV from Mexico is also pointing towards the serious spread of WmCSV across the continents and around the world (Dominguez-Duran et al., 2018). Such begomovirus epidemic spreads are quite common between two different countries or even across the continents (Sattar et al., 2013; Sattar et al., 2017). Thus, a detailed survey of WmCSV is highly necessary to understand the population structure, amount of variability and the factors supporting the high diversity of WmCSV in this region.

4. Conclusion

Although *T. cucumerina* have been reported as a host to begomoviruses, this report presents the first identification of DNA-A and DNA-B of bipartite begomovirus WmCSV from the *T. cucumerina* plants in the Al-Ahsa region of Saudi Arabia. The results of present study further extend our knowledge about the existing etiology of WmCSV in the agro-climatic regions of Middle East. However, this study is a partial characterization of WmCSV from *T. cucumerina* plants thus, a detailed survey for the identification of full-length genomes of WmCSV is highly needed. Besides, the current situation, pattern of distribution and the host range of WmCSV in Saudi Arabia are also very important to be investigated for futuristic resistance strategies against this virus.

List of Abbreviations: bp, base pair; CP, coat protein; CR, common region; cssDNA, circular, single-stranded DNA; CTAB, cetyletrimethyle ammonium bromide; NJ, neighbor-joining; nt, nucleotide; NW, new world; ORFs, open reading frames; OW, old world; RCA, rolling circle

amplification; SDT, species demarcation tool; SLCV, squash leaf curl virus; WmCSV, watermelon chlorotic stunt virus.

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