بیماری‌های گیاهی / جلد ۵۷ / شماره ۴ / سال ۱۴۰۰: ۲۸۱-۲۸۹ DOI: 10.22034/IJPP.2022.545383.376

مقاله پژوهشی

تعیین ترادف ژنوم چند جدایه جدید ویروس پیچیدگی برگ کنجد از استان کرمان و شناسایی چند میزبان جدید برای این ویروس*

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(تاریخ دریافت: ۱٤۰۰/۱۰/٦؛ تاریخ پذیرش: ۱٤۰۰/۱۱/۱۷)

چکیده

ویروس پیچیدگ برگ کنجد (*Sesame curly top virus*, SeCTV) یک گونهٔ جدید برای جنس *Turncurtovirus* (خانوادهٔ *Geminiviridae*) است و تاکنون تنها کنجد بعنوان میزبان طبیعی این ویروس شناخته شده است. در یک مطالعه، به منظور شناسائی جمینی ویروس های آلوده کنندهٔ گیاهان زراعی، سبزیجات و گیاهان زینتی در شرق ایران، آلودگی طبیعی یونجه، لوبیا، بادمجان، باقلا، گل ساعتی، تربچه و هندوانه به SeCTV با استفاده از آزمون واکنش زنجیره ای پلی مراز و سپس تعیین ترادف ژنوم کامل جدایه های بدست آمده به اثبات رسید. میزان شباهت طول کامل ژنوم این هفت جدایه با سایر جدایه این ویروس که قبلا از ایران و کشور پاکستان گزارش شده اند بترتیب بیش از ۹۷ و ۸۷-۸۸ درصد تعیین گردید. همسانهٔ عفونت زای این ویروس طراحی و ساخته شد و بیماری زائی آن روی چندین گیاه با روش بمباران سازهٔ عفونت زا یا مایه کوبی سلول های آگروباکتریوم حاوی سازهٔ ساخته شده اثبات گردید. بر اساس نتایج بدست آمده از این تحقیق، SeCTV مانند دو ترنکرتوویروس دیگر، دارای دامنهٔ میزبانی وسیعی در طبیعت بوده و در میان میزبان های فوق، هندوانه به لحاظ میزان آلودگی و اثرات آن به شدت تحت تأثیر این ویروس قرار می گیرد.

کلیدواژه: میزبان های جایگزین، جنس Turncurtovirus، ویروس پیچیدگ برگ کنجد، ایران

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DOI: 10.22034/IJPP.2022.545383.376

Research Article

Genome sequence of new isolates of sesame curly top virus in Kerman province and identification of new hosts of the virus*

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(Received: 27.12.2021; Accepted: 6.2.2022)

Abstract

Sesame curly top virus (SeCTV) is a new species in the genus *Turncurtovirus* (family *Geminiviridae*). To date, sesame was the only natural host of the virus. During a survey for identification of geminiviruses infecting crop, vegetable or ornamental plants in south-eastern Iran, the sesame curly top virus infection of alfalfa, bean, eggplant, faba bean, blanket flower, radish and watermelon was confirmed by PCR and sequencing. The full-length genome of seven new SeCTV isolates share >97 and ~87-88% nucleotide identity with other sequences from Iran and Pakistan, respectively. An infectious clone of SeCTV was constructed, biolistically- or *Rhizobium*-inoculated to several host plant species and the pathogenesis of the virus was demonstrated. Results of this study indicated that, similar to known turncurtoviruses, SeCTV has a wide natural host range in Iran and among the alternative hosts, the rate of infection and severity of disease in watermelon are remarkable.

Keywords: Alternate hosts, *Turncurtovirus*, sesame curly top virus, Iran

* A part of the PhD thesis of the first author submitted to College of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran.

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Introduction

Geminiviruses (family *Geminiviridae*) are a unique and large group of plant viruses infecting vegetables, crops, ornamental plants and fruit trees worldwide (Brown et al. 2012). These viruses are responsible for substantial losses of many economically plant species (Varma and Malathi 2003). Geminiviruses are classified into 14 genera (*Becurtovirus*, *Mastrevirus*, *Topocuvirus*, *Curtovirus*, *Begomovirus*, *Eragrovirus*, *Turncurtovirus*, *Capulavirus*, *Grablovirus*, *Citlodavirus*, *Maldovirus*, *Mulcrilevirus*, *Opunvirus* and *Topilevirus*) based on their host range, insect vector and the genome organisation (Roumagnac *et al*. 2021). Furthermore, over the last decade, a number of novel geminiviruses have been identified that are yet to be classified into new genera. Genomes of geminiviruses consists of one or two circular single stranded (ss) DNA molecules 2.5-5.2 kb in size encapsidated in twinned (geminate) particles with the dimension of ~22 × 38 nm (Hesketh et al. 2018; Zhang et al. 2001).

Iran is a likely hotspot for geminiviruses in the genus *Turncurtovirus*. To date, members of three turncurtovirus species; *Sesame curly top virus*, *Turnip curly top virus* and *Turnip leaf roll virus* have been identified in insects and/or crop plants (Roumagnac *et al*. 2021). Turncurtoviruses have the monopartite genome with a relatively wide genetic variation which comprises of six open reading frames (ORFs), two on the viral and four on the complementary coding strands. SeCTV, TCTV and TLRV are transmitted by the leafhopper, *Circulifer haematoceps* (Mulsant and Rey 1855) (family: *Cicadellidae*) (Briddon et al. 2010; Kamali et al. 2016; Razavinejad et al. 2013).

Until now, sesame was the only known natural host of SeCTV (Hasanvand et al. 2018). During the monitoring and sampling of different crop growing farms, for study of the geminivirus infection, we identified previously unknown SeCTV hosts. In this research, SeCTV has been detected in a number of crops in Kerman province (south-eastern Iran) and the infectivity of the virus fulfills Koch's postulates.

Materials and methods

Plant sampling

Between 2017 and 2019 symptomatic plant samples including alfalfa, bean, eggplant, faba bean, blanket flower, radish and watermelon showing various viral-like symptoms were collected in 35 crop- and vegetable-growing farms in Kerman (Bagher-Abad), Sirjan, Jiroft, Roodbar-e-Jonob (Kerman province, south-eastern Iran). In case of blanket flower, four symptomatic samples were collected at Shahid Bahonar University of Kerman. The symptoms of the collected samples comprised of mild yellowing, vein clearing and leaf rolling in alfalfa [n=13] (Fig. 1a), chlorotic spots and cup-shaped leaves in bean [n=7] (Fig. 1b), interveinal chlorosis, leaf curling, vein swelling on the underside of the leaves and dwarfing in eggplant [n=6] (Fig. 1c), green vein banding, general yellowing and cup-shaped leaves (in case of faba bean) in blanket flower [n=4] and faba bean [n=12] (Figs. 1d and 1e, respectively), cup-shaped leaves and general yellowing in radish [n=14] (Fig. 1f) and severe leaf rolling across longitudinal vein, brittle leaves and dwarfing in watermelon [n=10] (Figs. 1g and 1h).

Genome amplification

Total DNA from leaf samples was extracted using CTAB method (Zhang et al. 1998) and the circular DNA molecules in the DNA extracts were amplified by rolling circle amplification (RCA) technique using Phi29 DNA polymerase (TempliPhi, GE Healthcare, USA) as previously described by Shepherd et al. (2008). Viral infection in the samples was tested by PCR using the RCA product as template with conserved back-to-back primer pair LHEcoRV-F: 5'-GATATC ACT TTG GTA ATT GAA GAG AGG GCC-3'/LHEcoRV-R: 5'-GATATC GAA GCC GAA GAG GAT-3'and Platinum Pfx DNA polymerase (Thermo Fisher Scientific, USA). This primer pair was designated based on the alignment of all SeCTV sequences available in GenBank (Hasanvand et al. 2018). The PCR amplicons of seven isolates (one isolate per host) with the size of ~3 kb (Fig. 2A) were recovered from the 0.7% agarose gel, EcoRV restricted and ligated into the EcoRV site of pGreen0029 plasmid (Hellens et al. 2000). The resulting recombinant plasmids bearing the full-length SeCTV genome were sequences from both directions at Bioneer Company (South Korea). To check the nucleotide sequence of the genome at the position of back-to-back primer pair, a part of the genome of all seven isolates bearing the primer

Fig. 1. Symptoms observed as a result of SeCTV infected plants in Kerman province in a) alfalfa showing mild yellowing leaf rolling and vein clearing; b) bean showing cup-shaped leaves and chlorotic spots; c) eggplant showing cup-shaped leaves, green vein banding and swelling of veins on the lower surface of leaves; d) Blanket flower showing green vein banding and general yellowing; e) faba bean showing cup-shaped leaves, mild necrosis, general yellowing and vein banding; f) radish showing cup-shaped leaves and general yellowing; g and h) watermelon showing severe leaf curling, dwarfing and brittle leaves.

position was amplified in PCR assay using primer pair LH 1459-F (5'-TGG GTT CCC GTA ATT GTG-3')/SeCTV-OR (5'-GCA GAC AAA CCT CAA ATA CGG-3') and directly sequenced. Seven full-length genomes of new SeCTV isolates were deposited in GenBank (accession numbers MT041691-MT041697) (Table 1).

Construction of the infectious clone

A partial dimer strategy was used for the construction of a SeCTV infectious clone. A recombinant plasmid bearing full-length SeCTV genome of the sesame isolate (IR:Jir:JIR36:Ses:17; accession number MH595443) (Hasanvand et al. 2018) was

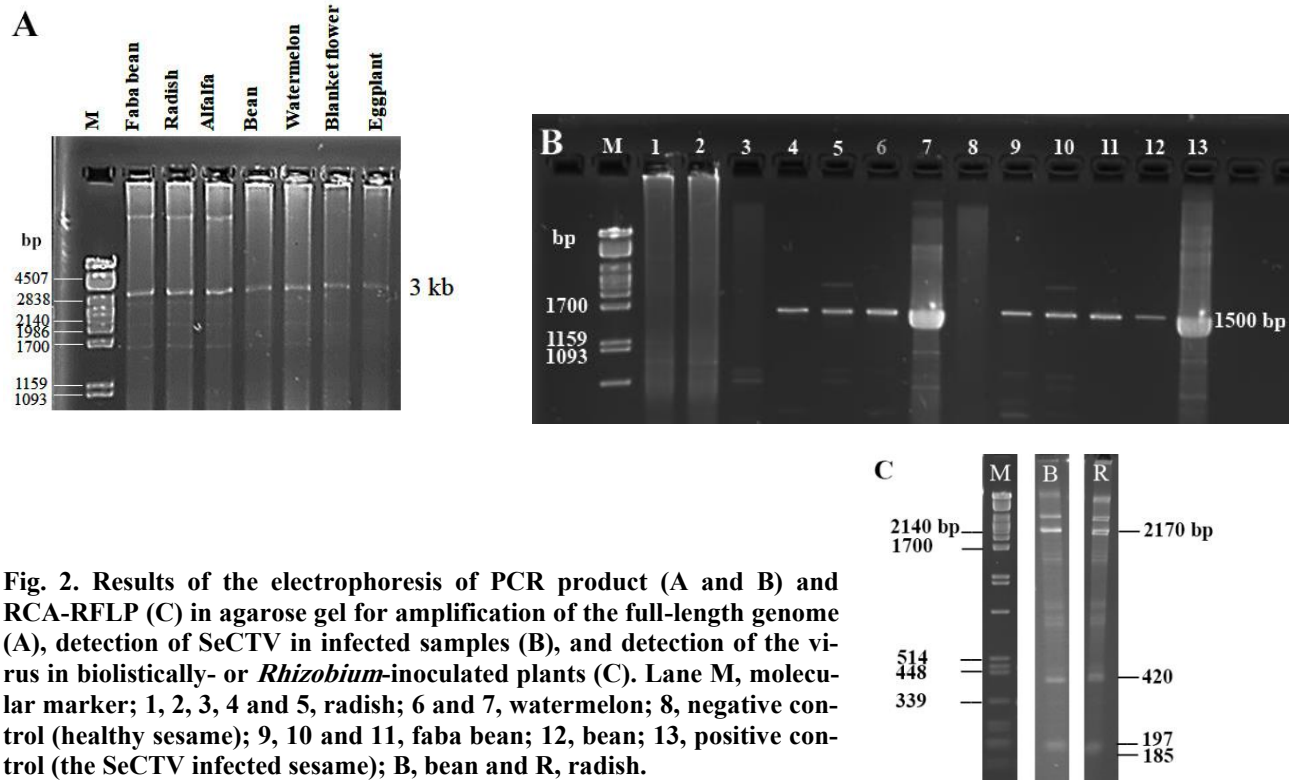

Fig. 2. Results of the electrophoresis of PCR product (A and B) and RCA-RFLP (C) in agarose gel for amplification of the full-length genome (A), detection of SeCTV in infected samples (B), and detection of the virus in biolistically- or *Rhizobium*-inoculated plants (C). Lane M, molecular marker; 1, 2, 3, 4 and 5, radish; 6 and 7, watermelon; 8, negative control (healthy sesame); 9, 10 and 11, faba bean; 12, bean; 13, positive control (the SeCTV infected sesame); B, bean and R, radish.

digested with EcoRV and SpeI restriction enzymes and resulted in a 1927 bp fragment bearing replication origin was recovered from 0.7% agarose gel followed by ligation into the pGreen0029 plasmid. The resulted plasmid was named pGreen0.65 and used for the transformation of *Escherichia coli* strain XL1 blue. The pGreen0.65 plasmid was extracted and digested with the *Eco*RV restriction enzyme. The recovered full-length SeCTV genome from the agarose gel was ligated into *Eco*RV digested pGreen0.65 plasmid and the resulted plasmid was named pGreen1.65. This and pSoup (helper) plasmids were used simultaneously for transformation of the *Rhizobium radiobacter* (updated name for *Agrobacterium tumefaciens*) cells strain C58 using freeze/thaw method (Boulton 2008).

Rhizobium-inoculation assay

R. radiobacter cells bearing pGreen1.65 and pSoup plasmids were cultured in liquid LB medium at 28°C for 48-72 hours and harvested bacterial cells were suspended into sterile deionized water. Approximately 10 µl of bacterial suspension (OD_{600} of 0.4) was injected by needle into the

Table 1. Characteristics of SeCTV isolates, accession numbers and natural host plants collected in Kerman province (south-eastern Iran).

Isolate	Host	Location in Kerman province	GPS-coordinates of samples	Accession number
IR:Jir:23Jir:Alf:17	*Medicago sativa L.*	Jiroft	28.630447 N, 57.786371 E	MT041691
IR:Jir:26Jir:Bea:17	*Phaseolus vulgaris L.*	Jiroft	28.547297 N, 57.859380 E	MT041692
IR:Bag:13Ba:Egg:17	*Solanum melongena L.*	Bagher-Abad	30.221206 N, 57.028321 E	MT041693
IR:Ker:D6:Bla:17	*Gaillardia aristata Pursh*	Kerman	30.256456 N, 57.103276 E	MT041694
IR:Sir:49Sir:Fab:17	*Vicia faba L.*	Sirjan	29.614947 N, 55.820922 E	MT041695
IR:Sir:54Jir:Rad:18	*Raphanus raphanistrum subsp. sativus (L.) Domin*	Sirjan	28.645099 N, 57.763240 E	MT041696
IR:Roo:Hen1:Wat:19	*Citrullus lanatus (Thunb.) Matsum. & Nakai*	Roodbar-e-Jonob	28.004598 N, 58.015973	MT041697

shoot and main vein of the leaves of the three to four-leave stage seedling of sesame, *Nicotiana benthamiana*, spinach, watermelon, eggplant, bean and radish as described by Grimsley et al. (1986). greenhouse at ~20–25°C for two months and checked for appearance of symptoms. The SeCTV infection of the *Rhizobium*-inoculated plants was tested by the PCR assay using extracted DNA of top and new grown leaves and primer pair LHEcoRV-F/LHEcoRV-R followed by checking the expected amplicon size of ~3 kb.

Biolistic inoculation

To bind SeCTV infectious DNA clones to gold particles for biolistic inoculation, 60 mg of gold particles (1.0 µm in diameter) were incubated 10 min with 70% ethanol, followed by three washing steps with water and resuspended in one ml of 50% (v/v) glycerol. Fifty µg of resuspended gold particles were mixed with 10 µl the pGreen1.65 plasmid (1 µg), 8 mM spermidine, and 1.0 M $CaCl_2$ incubated for three min at room temperature, washed twice with 70% ethanol and resuspended in 98% ethanol. This solution was used for particle bombardment. Sesame (*Sesamum indicum* L.) and tobacco (*N. benthamiana* Domin) seedlings in the two-leaf stage were biolistically inoculated with the particle gun PDS1000/HE (Bio-Rad, München, Germany) using 900 psi rupture discs under a vacuum pressure of 27-inch Hg as previously described by Unseld et al. (2001). The same procedure was used for bombardment of plant seedlings with gold particles without any DNA as negative control. Inoculated seedlings were maintained in greenhouse at ~20–25 °C for 30 days and the SeCTV infection of the inoculated plants was checked by RCA-RFLP using HpaII restriction enzyme and the extracted DNA from top new grown leaves.

Sequence analyses

The seven genomes of SeCTV identified in this study were aligned with 13 genomes available in GenBank. The alignment was used to infer a maximum likelihood phylogenetic tree with PHYML (Guindon et al. 2010) with TN93+G as the best substitution model determined using jModelTest (Darriba et al. 2010). Branches with less than 60% bootstrap support were collapsed with TreeGraph2 (Stover and Muller 2010). The tree was rooted with TCTV and TRLV sequences. Genome-wide pairwise identities were determined using SDT v1.2 (Muhire et al. 2014).

Results and discussion

The PCR assay of symptomatic collected samples using primer pair LHEcoRV-F/LHEcoRV-R showed the SeCTV infection of 5/13 alfalfa, 5/7 bean, 2/6 eggplant, 3/4 blanket flower, 2/12 faba bean, 6/14 radish and 10/10 watermelon samples and the PCR amplicon with the size of ~1500 bp was detected (Fig. 2B). Generally, dwarfing, yellowing and leaf curling are attributed to virus, virus-like and phytoplasma diseases or abiotic disorders. Thus, probably the symptomatic SeCTV non-infected plants were either infected with these groups of pathogens or affected by abiotic disorders. Prior to this study, sesame had been also reported as the natural host of the virus (Hasanvand et al. 2018). Of considerable interest is the fact that among the SeCTV infected hosts, the severity and the infection rate of watermelon is remarkable at 100% (Figs. 1g and 1h). In some cases, the watermelon growing farms had been completely affected. To assure the lack of co-infection of severe watermelon affected plants with other geminiviruses commonly found in Iran; turnip curly top virus (TCTV), turnip leaf roll virus (TRLV), beet curly top Iran virus (BCTIV) and a number of begomoviruses, we tested the samples by PCR using specific primers (in the case of BCTIV) and degenerate primers (in the case of begomoviruses) and none of these viruses were detected. Roodbar-e-Jonob (Kerman province, south-eastern Iran) with hot climatic conditions is a center for production of crops and vegetables such as cucurbits. Hence, the economic impact of SeCTV on watermelon production in these regions is noticeable. Based on the results of this study, SeCTV has a significant incidence and wide host range in south-eastern Iran and infects crops, vegetables and an ornamental plant.

Biolistically- or *Rhizobium*-inoculation of various plant seedlings with the infectious clone of SeCTV led to appearance of symptoms including downward leaf curling, vein swelling, brittle leaves and dwarfing in 25% (6/24) of sesame plants (variety Paloma) (Fig. 3a) as the main host; severe dwarfing and little leaves in 19.4% (6/31) of *N. benthamiana* (variety Jasmin tobacco) (Fig. 3b);

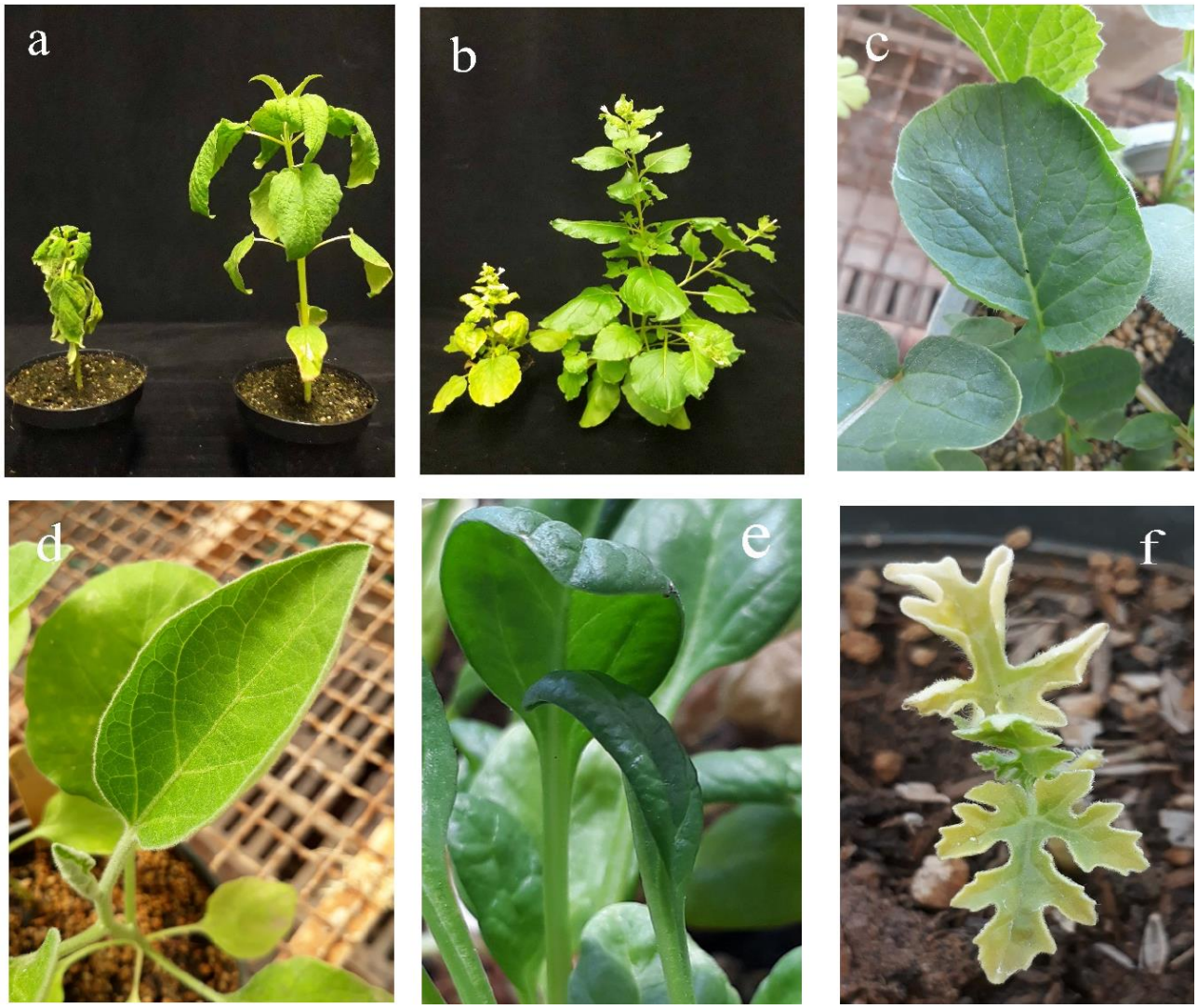

Fig. 3. Symptoms of the biolistically- or *Rhizobium*-inoculated plants six weeks post-inoculation with partial dimer construct. a) severe dwarfing and epinasty (left) of sesame in compare to healthy plant (right); b) tobacco (*N. benthamiana*) showing general yellowing and severe dwarfing (left) in compare to healthy plant (right); c and d) upward cup-shaped leaves of radish and eggplant, respectively; e) downward cup-shaped leaves of spinach and f) severe dwarfing and yellowing and upward leaf curling of watermelon.

cap-shape leaves of radish (variety Champion) and eggplant (variety Shadow) in 73.33% (11/15) and 66.66% (4/6), respectively (Figs. 3c and 3d); downward leaf curling and vein swelling underside of the leaves in 60% (9/15) of spinach plants (variety Viroflay) (Fig. 3e); and upward leaf curling, sever dwarfing and yellowing in 66.7% (8/12) of watermelon plants (variety Crimson sweet) (Fig.3f) six weeks post-inoculation. The SeCTV infection of the inoculated plants was confirmed in the PCR assay and the RCA-RFLP using HpaII restriction enzyme (Fig. 2C). Accordingly, all symptomatic inoculated plants using two inoculation methods were tested positive. However, no symptoms and no amplicon in the PCR or band in the RCA-RFLP were detected when the *Rhizobium* bearing the pSoup and pGreen0029 plasmids (with no insert) or in the case of biolistic inoculation gold particles without any DNA were used for inoculation. Although *Rhizobium*-inoculation of radish, eggplant, spinach and watermelon plants was more efficient than biolistically-inoculation of sesame and *N. benthamiana* plants to cause infection, no definitive conclusion can be drawn between two methods due to inoculation of different plant species for two methods.

The full-length genome of all seven SeCTV isolates from seven new natural hosts were 2972 nts

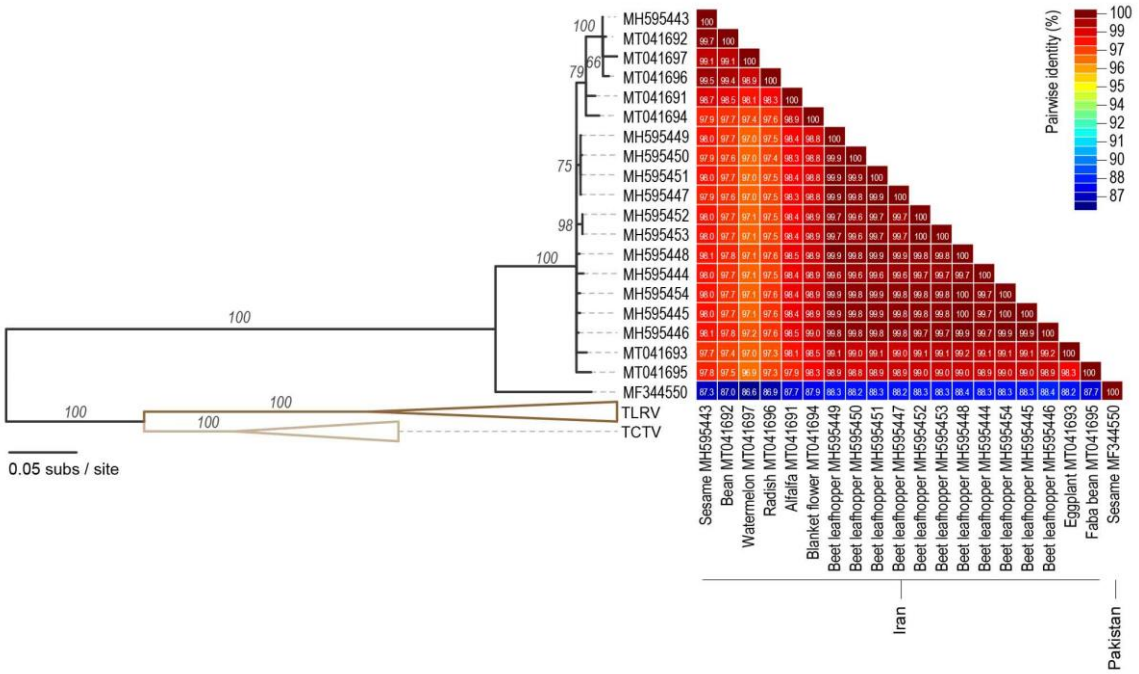

Fig. 4. Maximum Likelihood phylogenetic tree of aligned SeCTV genome sequences rooted with TCTV and TLRV sequences. The genome-wide pairwise identity matrix is provided to the right of the phylogenetic tree. Host the virus and sample collection country is provided with accession numbers on the bottom section of pairwise identity matrix.

in size and share >97% genome-wide nucleotide identity with other sequences from Iran and ~87-88% identity with an isolate from Pakistan (Fig. 4). This result indicates that SeCTV in contrast to two Viruses in all three species of the genus *Turncurtovirus*; SeCTV, TCTV and TLRV (Hasanvand et al. 2018; Kamali et al. 2016; Razavinejad et al. 2013; Razavinejad and Heydarnejad 2013) have a wide host range. However, turnip (in the case of TCTV and TLRV) and sesame as well as watermelon (in the case of SeCTV) crop plants are more severely affected by these viruses. The wide host range of turncurtoviruses is reflected by the polyphagous nature of *Circulifer haematoceps* as a other turncurtovirus members; TCTVs and TLRVs, have a low genetic diversity in Iran. However, all three species have a wide natural host range.

Iran appears to be a hotspot of turncurtoviruses. vector for SeCTV, which is active in many agricultural ecosystems specially those of with hot and dry climate (Health EPoP 2015).

Acknowledgments

This study was supported by a grant from Shahid Bahonar University of Kerman, Kerman, Iran.

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