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ORIGINAL ARTICLE

Molecular diversity of turncurtoviruses in Iran

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Abstract Turnip curly top virus (TCTV) is the only member of the newly established genus *Turncurtovirus* (family *Geminiviridae*). As part of an ongoing study to identify additional plant hosts and the diversity of turncurtoviruses, between 2012 and 2014, we sampled symptomatic turnip plants and other crops in the provinces Fars and Khorasan Razavi (southern and northeastern Iran, respectively). Infection by turncurtoviruses was tested by PCR and/or rolling-circle amplification (RCA) coupled with restriction enzyme digests. Turncurtoviruses were identified in turnip as well as seven other field crops, including eggplant, basil, radish, lettuce, sugar beet, red beet and spinach. Full turncurtovirus genomes were recovered from 25 of these samples, leading to the identification of TCTV and a new putative turncurtovirus, turnip leaf roll virus (TLRV; 13 isolates), which shares <80 % genome-wide pairwise identity with TCTV. Agroinoculation of plants with an infectious clone of TLRV demonstrated that this virus could infect several plant hosts under greenhouse conditions and could be transmitted by the leafhopper *Circulifer haematoceps* (Mulsant and Rey, 1855) from agroinoculated to healthy plants.

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Introduction

Geminiviridae is a large family of plant-infecting viruses, and its members are associated with substantial crop losses of economically important food and fiber crops worldwide [40]. This family is divided into seven genera: *Mastrevirus*, *Curtovirus*, *Topocuvirus*, *Begomovirus*, *Becurtovirus*, *Eragrovirus* and *Turncurtovirus* [41]. Furthermore, over the last few years, several novel geminiviruses have been discovered infecting alfalfa, *Euphorbia caput-medusae*, faba bean, and grapevine as well as apple, citrus and mulberry trees [3, 24–26, 37]. Geminiviruses have twinned quasi-isometric particles with a diameter of approximately 20 nm and a length of 30 nm [6, 44]. Their genomes consist of one or two circular single-stranded DNA molecules with a length of ~2.5-3.0 kb [8].

Over the last decade, the use of rolling-circle amplification (RCA) coupled with restriction enzyme digests has enabled the identification of a large number of diverse circular DNA viruses, especially geminiviruses [4, 7, 18, 20, 24, 25, 27, 38, 42]. Using these methods, our research group has identified turnip curly top virus (TCTV) infecting turnip (*Brassica rapa* L. var. *rapa*), radish (*Raphanus sativus* L.) and five weeds species [7, 12, 35]. TCTV is the type member of the genus *Turncurtovirus* of family *Geminiviridae*, and it is vectored by the leafhopper *Circulifer haematoceps* [7, 35, 36, 41].

The 20 TCTV genome sequences that have been characterized previously [7, 36] share >87 % pairwise identity,

Table 1 Nucleotide sequences of turncurtovirus isolates determined in this study (in bold) and those from previous studies, with accession numbers, isolation source and sampling locations

GenBank accession no.	Isolate	Host	Location
KT388064	**TCTV-C [IR:Lap:L14:Tur:12]**	***Brassica rapa***	**Lapouei, Fars province (southern Iran)**
KT388065	**TCTV-B [IR:Hom:Th8:Tur:12]**	***Brassica rapa***	**Homayejan, Fars province (southern Iran)**
KT388066	**TCTV-D [IR:Hom:Th9:Tur:12]**	***Brassica rapa***	**Homayejan, Fars province (southern Iran)**
KT388067	**TCTV-D [IR:Hom:Th10:Tur:12]**	***Brassica rapa***	**Homayejan, Fars province (southern Iran)**
KT388068	**TCTV-B [IR:Zaf:Z2-4:Sug:12]**	***Beta vulgaris***	**Zafar Abad, Fars province (southern Iran)**
KT388069	**TCTV-B [IR:Zaf:Z2-26:Let:12]**	***Lactuca sativa***	**Zafar Abad, Fars province (southern Iran)**
KT388070	**TCTV-C [IR:Zaf:Z3:Tur:12]**	***Brassica rapa***	**Zafar Abad, Fars province (southern Iran)**
KT388071	**TCTV-E [IR:Zaf:Z4-7:Rad:12]**	***Raphanus sativus***	**Zafar Abad, Fars province (southern Iran)**
KT388072	**TCTV-E [IR:Zaf:Z4-9:Sug12]**	***Beta vulgaris***	**Zafar Abad, Fars province (southern Iran)**
KT388073	**TCTV-E [IR:Zaf:Z4-15:Bas:12]**	***Ocimum basilicum***	**Zafar Abad, Fars province (southern Iran)**
KT388074	**TCTV-E [IR:Zaf:Z4-22:Rad:12]**	***Raphanus sativus***	**Zafar Abad, Fars province (southern Iran)**
KT388075	**TCTV-C [IR:Zaf:Z6:Tur:12]**	***Brassica rapa***	**Zafar Abad, Fars province (southern Iran)**
KT388076	**TLRV-A [IR:Hom:Th2:Tur:12]**	***Brassica rapa***	**Homayejan, Fars province (southern Iran)**
KT388077	**TLRV-A [IR:Hom:Th5:Tur:12]:**	***Brassica rapa***	**Homayejan, Fars province (southern Iran)**
KT388078	**TLRV-D [IR:Zaf:Z2-15:Tur:12]**	***Brassica rapa***	**Zafar Abad, Fars province (southern Iran)**
KT388079	**TLRV-C [IR:Zaf:Z2-22:Let:12]**	***Lactuca sativa***	**Zafar Abad, Fars province (southern Iran)**
KT388080	**TLRV-D [IR:Zaf:Z2-23:Let:12]**	***Lactuca sativa***	**Zafar Abad, Fars province (southern Iran)**
KT388081	**TLRV-D [IR:Zaf:Z2-24:Let:12]**	***Lactuca sativa***	**Zafar Abad, Fars province (southern Iran)**
KT388082	**TLRV-D [IR:Zaf:Z2-49:Red beet:12]**	***Beta vulgaris* subsp. *maritima***	**Zafar Abad, Fars province (southern Iran)**
KT388083	**TLRV-D [IR:Zaf:Z4-10:Sug:12]**	***Beta vulgaris***	**Zafar Abad, Fars province (southern Iran)**
KT388084	**TLRV-D [IR:Zaf:Z4-12:Sug:12]**	***Beta vulgaris***	**Zafar Abad, Fars province (southern Iran)**
KT388085	**TLRV-B [IR:Zaf:Z7:Tur:12]**	***Brassica rapa***	**Zafar Abad, Fars province (southern Iran)**
KT388086	**TLRV-A [IR:Zaf:Z11-Bgl:Tur:12]**	***Brassica rapa***	**Zafar Abad, Fars province (southern Iran)**
KT388087	**TLRV-A [IR:Zaf:Z11-4:Tur:12]**	***Brassica rapa***	**Zafar Abad, Fars province (southern Iran)**
KT388088	**TLRV-D [IR:Ney:N8-5:Tur:14]**	***Brassica rapa***	**Neyshabour, Khorasan Razavi (north-eastern Iran)**
GU456685	TCTV-A [IR:Zaf:B11:06]	*Brassica rapa*	Zafar Abad, Fars province (southern Iran)
GU456686	TCTV-B [IR:Hom1:2K:09]	*Brassica rapa*	Homayejan, Fars province (southern Iran)
GU456687	TCTV-A [IR: Hom2:8K:09]:	*Brassica rapa*	Homayejan, Fars province (southern Iran)
GU456688	TCTV-A [IR: Hom3:4K:09]	*Brassica rapa*	Homayejan, Fars province (southern Iran)
GU456689	TCTV-A [IR: Hom3:7K:09]	*Brassica rapa*	Homayejan, Fars province (southern Iran)
JQ742019	TCTV-B [IR:Hom:T57K:Tur:10]	*Raphanus sativus*	Homayejan, Fars province (southern Iran)
KC108893	TCTV-A [IR:Lap:L13:Tur:12]	*Brassica rapa*	Lapoui, Fars province (southern Iran)
KC108894	TCTV-A [IR:Zaf:Z8:Tur:12]	*Brassica rapa*	Zafar Abad, Fars province (southern Iran)
KC108895	TCTV-A [IR:Lap:L2-P:Tur:12]	*Brassica rapa*	Lapoui, Fars province (southern Iran)
KC108896	TCTV-B [IR:Lap:L16:Tur:12]	*Brassica rapa*	Lapoui, Fars province (southern Iran)
KC108897	TCTV-B [IR:Zaf:Z9:Tur:12]	*Brassica rapa*	Zafar Abad, Fars province (southern Iran)
KC108898	TCTV-B [IR:Zaf:Z2-14:Tur:12]	*Brassica rapa*	Zafar Abad, Fars province (southern Iran)
KC108899	TCTV-B [IR:Hom:H6:Tur:12]	*Brassica rapa*	Homayejan, Fars province (southern Iran)
KC108900	TCTV-B [IR:Zaf:Z2-18:Tur:12]	*Brassica rapa*	Zafar Abad, Fars province (southern Iran)
KC108901	TCTV-C [IR:Hom:H1:Tur:12]	*Brassica rapa*	Homayejan, Fars province (southern Iran)
KC108902	TCTV-D [IR:Hom:H9:Tur:12]	*Brassica rapa*	Homayejan, Fars province (southern Iran)
KC108903	TCTV-C [IR:Zaf:Z10:Tur:12]	*Brassica rapa*	Zafar Abad, Fars province (southern Iran)
KC108904	TCTV-B [IR:Hom:H8:Tur:12]	*Brassica rapa*	Homayejan, Fars province (southern Iran)
KC108905	TCTV-C [IR:Zaf:Z2-1:Tur:12]	*Brassica rapa*	Zafar Abad, Fars province (southern Iran)
KC108906	TCTV-C [IR:Zaf:Z5-2:Tur:12]	*Brassica rapa*	Zafar Abad, Fars province (southern Iran)

and they can be assigned to four strains: TCTV-A (n = 7), -B (n = 8), -C (n = 4) and -D (n = 1). Except for an isolate of TCTV-B that was found in radish, all isolates have been identified only in turnip.

To complement the dataset of 20 full-length genomes of TCTV from previous studies [7, 35, 36] for more in-depth evolutionary analysis with a greater diversity of turncurtoviruses, we have continued to sample plants of turnip and other crops displaying symptoms similar to those induced by TCTV. In this study, we report the identification of a new turncurtovirus (13 isolates) and also demonstrate that the leafhopper *C. haematoceps* is able to transmit this virus from agroinoculated plants.

Materials and methods

Sampling and recovery of turncurtovirus genomes

Symptomatic leaves ($n = 220$) were sampled as part of ongoing field surveys between 2012 and 2014 in turnip-growing areas and vegetable farms around turnip plantations in the provinces Fars and Khorasan Razavi, which are located in southern and northeastern Iran, respectively. Total DNA of all 220 samples was extracted from leaves using the CTAB method as described by Zhang *et al.* [45]. DNA from the samples was enriched for circular molecules by RCA using Phi29 DNA polymerase (TempliPhi, GE Healthcare, USA) as described in Shepherd *et al.* [38]. The high-molecular-weight RCA amplicons from each sample were digested with different restriction endonucleases, including *Pst*I, *Kpn*I, *Hind*III, *Bgl*II or *Eco*RI, to yield unit-size molecules. In addition to this, a specific back-to-back primer pair (TCTV-F: 5'-AGG TTT GTC TGC CAC TCC TTT-3'/TCTV-R 5'-GCA GAC AAA CCT CAA ATA CGG-3') was used to recover complete genomes using the RCA DNA as template with KAPA HiFi DNA polymerase (Kapa Biosystems, USA). The following thermal cycling protocol was used: initial denaturation at 95 °C for 3 min, followed by 25 cycles of 98 °C for 20 s, 60 °C for 15 s, and 72 °C for 3 min, and then a final extension at 72 °C for 3 min. According to restriction enzyme digests, *Bgl*II or *Eco*RI restriction enzymes were used to clone the full-length genome of IR:Zaf:Z11-Bgl:Tur:12, IR:Zaf:Z11-4:Tur:12 and IR:Lap:L14:Tur:12 isolates. The ~3-kb fragments amplified by RCA reaction or PCR for 25 symptomatic leaves from various hosts in Homayejan ($n = 5$; lat.: 30° 12′ 35.9″N, long.: 52° 03′ 59.1″O), Zafar-Abad ($n = 18$; lat.: 29° 24′ 34.8″N, long.: 52° 35′ 39″O), Lapouei ($n = 1$; lat.: 29° 48′ 44.5″N, long.: 52° 37′ 40.1″O) and Neyshabour ($n = 1$; lat.: 36° 14′ 41.4″N, long.: 58° 46′ 50″O) were ligated into pTZ57R or pJET1.2 vectors (Thermo Fisher Scientific, USA) followed by transformation of *Escherichia coli* strain XL1blue or DH5α. One clone per isolate was sequenced from both directions by primer walking at either Bioneer or Macrogen Inc. (South Korea).

Construction of an infectious clone of a divergent turncurtovirus

Since a subset of divergent turncurtoviruses was identified in this study (n = 13), an infectious clone of isolate IR:Zaf:Z11:12 (GenBank accession no. KT388087) was constructed as described previously [20] in order to study biological properties of this isolate and compare it to TCTV-[IR:Zaf:B11:06] (GenBank accession no. GU456685) from our previous study [36]. In brief, the genome of Z11 was cloned as a dimer into *Eco*RI-digested pGreen0029 plasmid [19]. The resulting recombinant pGreen0029 plasmid together with a pSoup helper plasmid was used to transform *Agrobacterium tumefaciens* strain C58.

Three to four leaf seedlings of turnip (*B. rapa* cv. Purple top white globe), cabbage (*Brassica oleracea* L. cvs Capitata and Kohlrabi white vienna), sugar beet (*Beta vulgaris* L. cv. Universe), red beet (*Beta vulgaris* subsp. esculenta (L.) Arcang. cv. Conditiva), spinach (*Spinacia oleracea* L. cv. Winter giant), pepper (*Capsicum annuum* L. cv. California Wonder), radish (*R. sativus* cv. Sparkler), okra (*Abelmoschus esculentus* (L.) Moench cv. Clemson Spineless), cowpea (*Vigna unguiculata* (L.) Walp. cv. Mashhad), eggplant (*Solanum melongena* L. cv. Black Beauty), lettuce (*Lactuca sativa* L. cv. Attraction), tomato (*Solanum lycopersicum* L. cv. Moneymaker), canola (*Brassica napus* L. cv. Mendel), thale cress (*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia) and *Nicotiana benthamiana* L. were agroinoculated with the infectious clone of the turncurtovirus IR:Zaf:Z11:12 isolate according to the method described by Grimsley *et al.* [16]. Similarly, turnip and radish seedlings were agroinoculated with *A. tumefaciens* cultures bearing pGreen plasmid without the turncurtovirus genome and used as negative controls. Agroinoculated plants were kept in a greenhouse at ~25 °C and monitored for the appearance of curly top symptoms. Total DNA from symptomatic and non-symptomatic plants (top non-inoculated leaves) was extracted 4 weeks post-agroinoculation, and PCR assays were used to confirm turncurtovirus infection.

Leafhopper transmission of the turncurtovirus IR:Zaf:Z11:12 isolate

Symptomatic turnip plants agroinoculated with the infectious clone of the IR:Zaf:Z11:12 isolate were used for leafhopper transmission using *C. haematoceps* under

Fig. 1 Symptoms of crop plants with natural infections of turncurtoviruses. (A) Mild yellowing in TCTV-infected eggplant, (B) dwarfing and severe yellowing in TCTV-infected basil, (C) outward leaf curling in TCTV-infected radish, (D) dark green vein banding in TLRV-infected lettuce, (E, F and G) inward curling of leaf margin in sugar beet, red beet and spinach, infected with TLRV, TLRV and TCTV, respectively

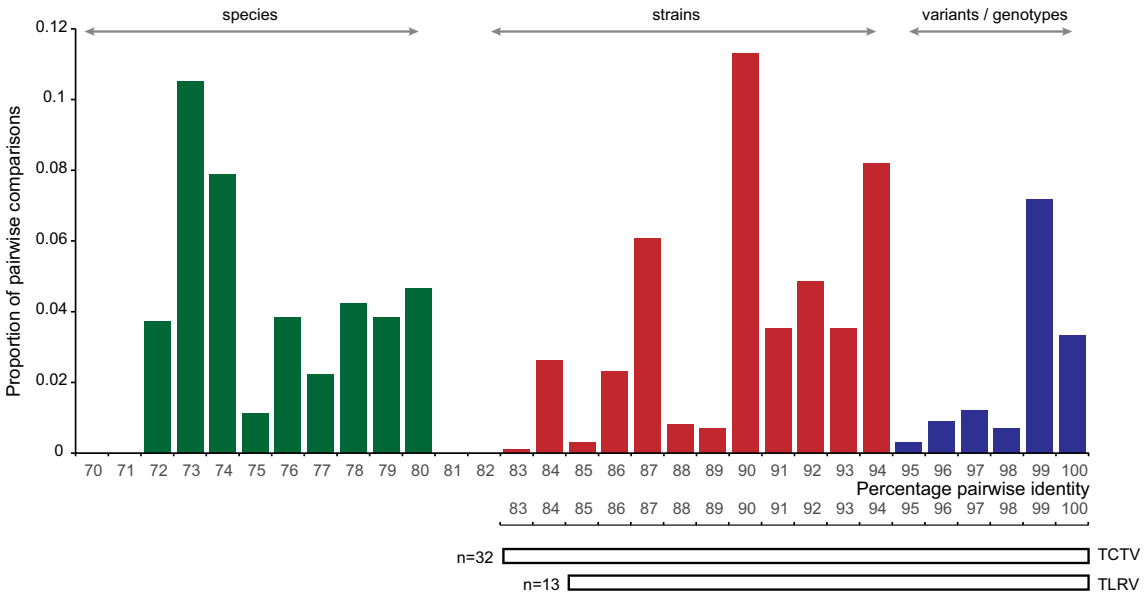


Fig. 2 Distribution of 991 pairwise identities of turncurtoviruses calculated using SDT v1.2 [30] with the MUSCLE option, and the diversity of TCTV (n = 32) and TLRV (n = 13) is illustrated

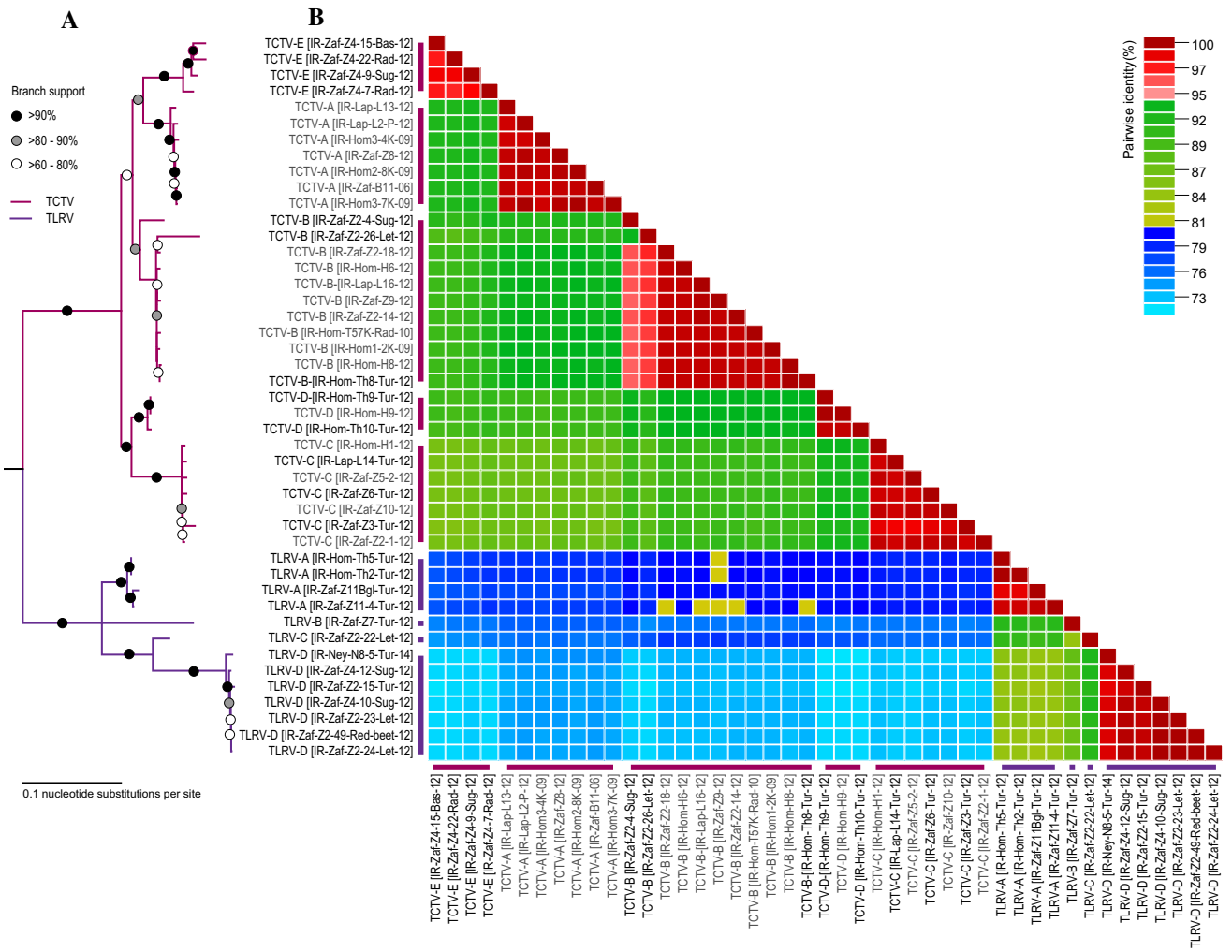


Fig. 3 (A) Neighbor-joining phylogenetic tree of complete genome sequences of turncurtoviruses rooted with genome sequences of eragroviruses. Branches with <60 % bootstrap support have been collapsed. (B) A pairwise identity matrix of turncurtoviruses calculated using SDT v1.2. All viral genomes determined in this study are in bold

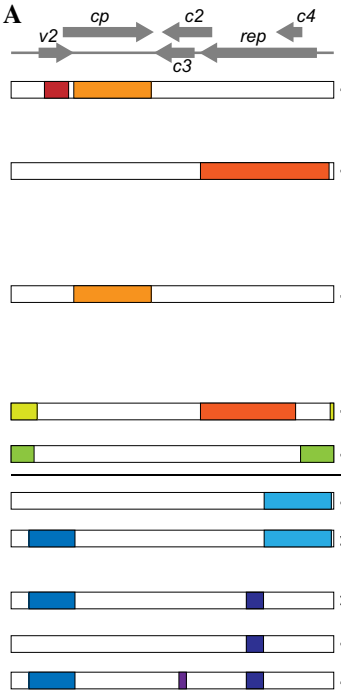
insect-proof conditions in a greenhouse at ~25 °C as described previously [20]. Non-viruliferous leafhoppers (nymph stage and/or adults) were caged with agroinoculated infected turnip plants showing typical curly top symptoms for one week to allow for acquisition of the virus. The constructed cage contained a plastic cylinder sealed at one end with cotton mesh. Following acquisition, leafhoppers were transferred to healthy turnip or radish seedlings (one leafhopper per seedling), and the inoculated plants were checked four weeks later for appearance of curly top symptoms. In addition, the agroinoculated plants were tested by PCR using the specific primer pair TCTV11-1676-F-GCGTAAATCCCTCACCACAGC/TCTV11-2699-R-CAGCTGCAGAACCTCGCCTGT, which direct the amplification of a ~1060-bp fragment of the genome of the IR:Zaf:Z11:12 isolate to confirm turncurtovirus infection. PCR conditions consisted of initial denaturation at 95 °C for 3 min and 35 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1.5 min, followed by one cycle of 72 °C for 10 min.

Turncurtovirus sequence analysis

The genomes of turncurtoviruses available in GenBank (n = 20) together with the ones determined in this study (n = 25) were aligned using MUSCLE [11]. The aligned genome sequences were used to construct a neighbor-joining tree using the Jukes-Cantor model with 1000 bootstrap replicates. The tree was rooted with the genome sequences of eragroviruses [42]. Branches with less than 60 % support were collapsed.

The aligned turncurtovirus dataset was also used for analysis of evidence of recombination in the genomes using RDP4 [28, 29], which implements the methods RDP

A

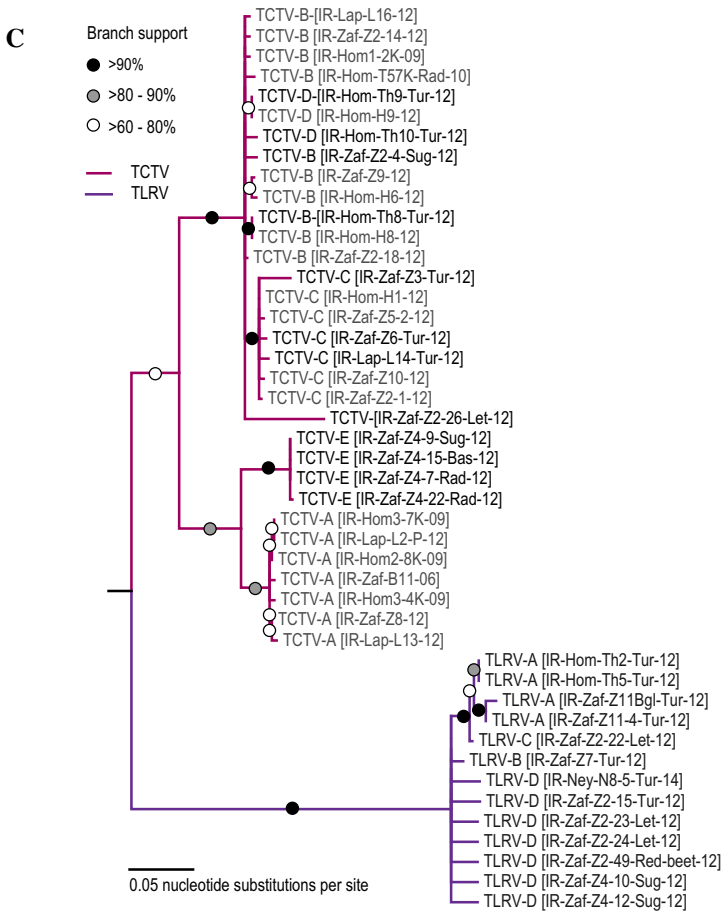


B

Recombinant sequence(s)	Recombination breakpoint Begin	End	Minor parental sequence(s)	Major parental sequence(s)	Detection methods	p-value
TCTV-E [IR-Zaf-Z4-7-Rad-12]	268	496	TCTV-B TCTV-C TCTV-D	TCTV-E [IR-Zaf-Z4-15-Bas-12]	RGMCST	2.54 x10^{-34}
TCTV-B	1746	2951	TCTV-A	TCTV-D TCTV-C	RGBMCST	3.23 x10^{-48}
TCTV-E TCTV-A [IR-Hom3-4K-09] TCTV-A [IR-Lap-L13-12] TCTV-A [IR-Hom2-8K-09] TCTV-A [IR-Zaf-B11-06] TCTV-A [IR-Zaf-Z8-12] TCTV-A [IR-Lap-L2-P-12] TCTV-A [IR-Hom3-7K-09]	530	1331	TCTV-B TCTV-D	Unk	RGMCST	4.22 x10^{-23}
TCTV-B [IR-Zaf-Z2-26-Let-12]	2955	211	Unk	TCTV-B TCTV-D	RGBMCST	2.62 x10^{-16}
TCTV-C [IR-Zaf-Z3-Tur-12]	2711	196	Unk	TCTV-C	RGMCT	4.58 x10^{-09}
TLRV-D TLRV-C	2335	2958	Unk	TLRV-A TLRV-B	RGMCST	4.07 x10^{-52}
TLRV-C TLRV-A	148	546	TCTV	TLRV-B TLRV-D	RGMCST	1.80 x10^{-78}
TLRV-A TLRV-B	2188	2336	TLRV-D TLRV-C	Unk	RGMST	5.76 x10^{-08}
TLRV-A [IR-Zaf-Z11Bgl-Tur-12]	1531	1594	Unk	TLRV-A [IR-Hom-Th2-Tur-12] TLRV-A [IR-Hom-Th5-Tur-12] TLRV-A [IR-Zaf-Z11-4-Tur-12]	GMCST	6.96 x10^{-08}

R= RDP; G=GENECONV; B=Bootscan; M=Maxchi; C=Chimaera; S=SiSscan; T=3Seq

C



◀**Fig. 4** (A) Cartoon illustration and (B) details of recombination events detected within turncurtovirus genomes, using RDP 4. Recombinant regions and details are colour coded. (C) Recombination-free maximum-likelihood phylogenetic tree of turncurtoviruses. The tree was rooted with genome sequences of eragroviruses. Branches with <60 % bootstrap support have been collapsed. All viral sequences determined in this study are in bold

[31], GENECONV [33], Bootscan [30], Maxchi [39], Chimaera [34], Siscan [14] and 3Seq [5]. Recombination events detected by RDP4 were deemed credible if there was strong phylogenetic support for the recombination event and the event was detected by a minimum of three methods with p-values of $<10^{-3}$.

A maximum-likelihood phylogenetic tree with recombinant regions removed from the genomes was constructed using PHYML 3.0 [17], using the T92+G nucleotide substitution model inferred using jModelTest [9] and 1000 bootstrap replicates. Branches with less than 60 % support were collapsed.

Maximum-likelihood phylogenetic trees of the amino acid sequences of replication-associated protein (Rep) and coat protein (CP) were constructed using PHYML 3.0 [17] with the WAG+G substitution model, determined as the best model using Prottest [1], and with an approximate-likelihood ratio test (aLRT) for branch support. Branches with aLRT branch support <80 % were collapsed.

The pairwise identities of the genome nucleotide sequences as well as amino acid sequences of CP and Rep were determined using SDT v1.2 [32].

Results

Turncurtovirus host range and symptoms

The surveys and molecular detection of turncurtoviruses revealed that in addition to turnip and radish [7, 36], a number of crops in vicinity of turnip-growing farms are hosts to turncurtoviruses (25 out of 220 samples). With the exception of turnip, turncurtoviruses were identified in eggplant showing mild yellowing (Fig. 1A), basil (*Ocimum basilicum* L.) with symptoms of dwarfing and severe yellowing (Fig. 1B), radish showing inward rolling of the leaf margins, brittle leaves and swelling of veins on the lower leaf surfaces (Fig. 1C), lettuce showing dark green vein banding (Fig. 1D), and sugar beet, red beet and spinach

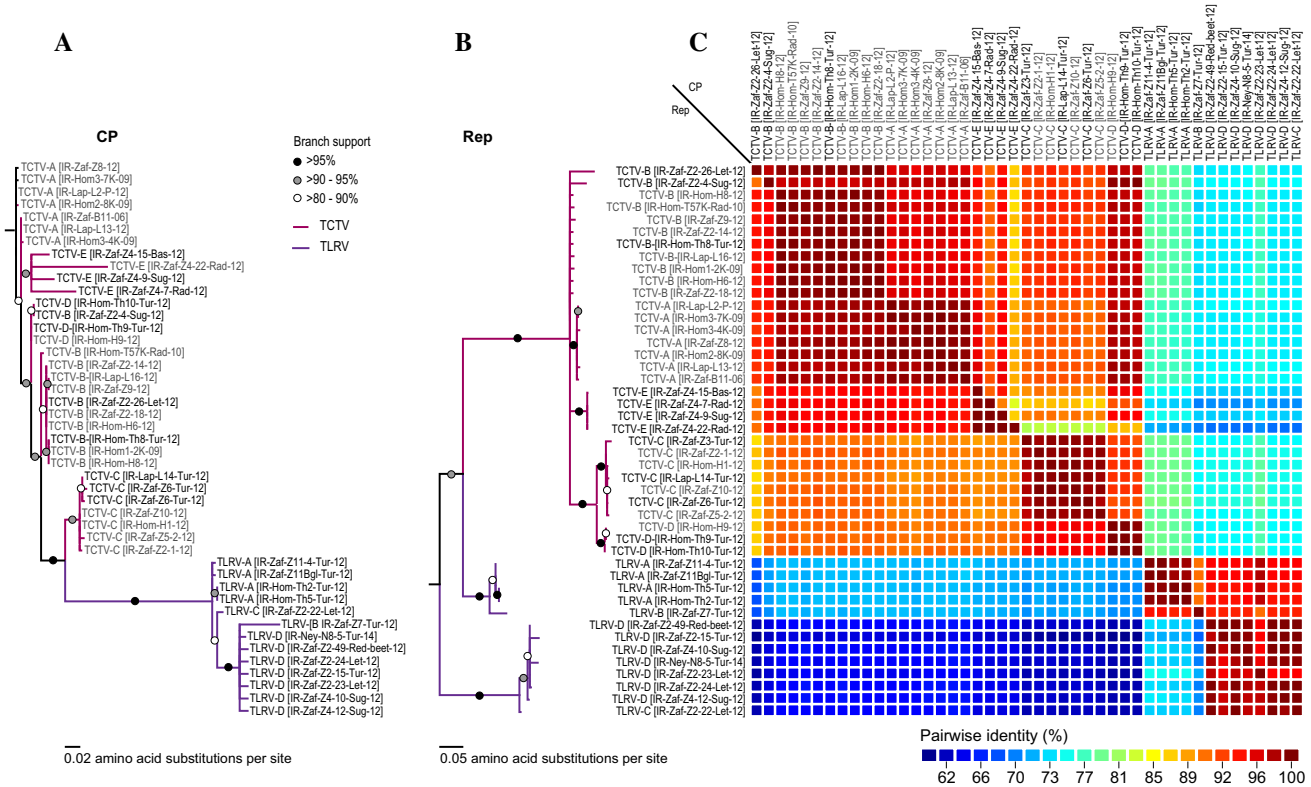


Fig. 5 Maximum-likelihood phylogenetic tree of amino acid sequences of (A) CP and (B) Rep rooted with Reps and CPs of eragroviruses, respectively. Branches with <80 % aLRT support have been collapsed. All viral sequences determined in this study are in bold. (C) A pairwise matrix of amino acid identities for Rep and CP of turncurtoviruses determined using SDT v1.2

with typical curly top symptoms (Fig. 1E, F and G, respectively).

Turncurtoviruses were identified in *Beta vulgaris* (n = 4), *B. vulgaris* subsp. esculenta (n = 1), *Brassica rapa* var. rapa (n = 13), *Lactuca sativa* (n = 4), *Ocimum basilicum* (n = 1) and *Raphanus sativus* (n = 2). Taking into account previously identified turncurtoviruses, the amongst collected samples, the dominant host is *B. rapa* (n = 32; n = 13 from this study and n = 19 from previous studies) (Table 1).

Diversity of turncurtoviruses

Twenty-five turncurtovirus genomes were recovered, and their sequences were analyzed together with 20 turncurtovirus genomes from previous studies. A pairwise identity analysis revealed that 12 of the turncurtovirus genomes from this study grouped with TCTV isolates, sharing >85 % pairwise identity. In addition, a group of 13 turncurtoviruses recovered from *B. rapa* var. rapa, *L. sativa*, *B. vulgaris* subsp. esculenta and *B. vulgaris* shared <80 % pairwise identity with TCTV isolates and had among them a diversity of ~17 % (TCTV isolates showed 15 % diversity; Fig. 2). We analyzed 991 pairwise comparisons of these turncurtovirus isolates, and the distribution of these identities shows a trough between 81-82 % and 95 % (Fig. 2). We recommend that turncurtoviruses sharing less than 80 % pairwise identity to known turncurtoviruses be considered members of tentative new species. The diverse group was well supported in the neighbor-joining phylogenetic tree (Fig. 3). Based on the phylogenetic support coupled with the distribution of pairwise identities and rough recommendations set out by Varsani et al. [41], these diverse isolates would be classified as members of a different species, and we propose the name turnip leaf roll virus (TLRV) for this virus.

Further, the 12 TCTV sequences from this study can be grouped, as outlined by Razavinejad et al. [36] and Varsani et al. [41], into the existing strains TCTV-B (n = 3), TCTV-C (n = 3), TCTV-D (n = 2) and the new strain TCTV-E (n = 4). Similarly, the 13 TLRV sequences can be grouped into the four strains TLRV-A (n = 4), TLRV-B (n = 1), TLRV-C (n = 1) and TLRV-D (n = 7).

Overall, based on the 45 genomes of turncurtoviruses, TCTV-A (n = 7), TCTV-C (n = 7), TCTV-D (n = 3), TLRV-A (n = 4) and TLRV-B (n = 1) have only been recovered from *B. rapa* (Table 1).

Inter- and intraspecies recombination in turncurtoviruses

Amongst the turncurtoviruses, evidence of recombination was found for almost all isolates, with recombinant regions accounting for 5 to 40 % of the genome. In total, nine well-supported detectable recombination events were detected (summarized in Fig. 4), with up to three recombinant regions in a genome. The genomes of TLRV-A and TLRV-C isolates were found to have a ~400-nt recombinant region derived from TCTV.

Nonetheless, taking recombination into account, it is clear from the recombination-free maximum-likelihood phylogenetic tree that TCTV and TLRV are members of two distinct species and that the bulk of the diversity between these two viruses arose primarily by recombination (Fig. 4).

Analysis of CP and Rep sequences revealed that the CPs of both TCTV and TLRV are more conserved than Rep (Fig. 5). The Rep sequences of TCTV and TLRV isolates

Fig. 6 Symptoms after inoculation of seedlings with the infectious clone of TLRV-A[IR:Zaf:Z11:12]. A) Inward rolling of leaf margin and swelling of veins on the lower leaf surfaces in turnip, B) cup-shaped leaves in radish, and C) local lesions on cowpea. All images were obtained at 21 dpi

share >87 % and >74 % pairwise amino acid sequence identity, respectively, whereas the CP sequences of TCTV and TLRV isolates share >88 % and >95 % pairwise amino acid sequence identity, respectively. Overall, the CP sequences of turncurtoviruses show 30 % diversity, whereas the Rep sequences have a diversity of 38 %.

Agroinfection studies with the infectious clone of TLRV-A[IR:Zaf:Z11:12]

Agroinoculation of all of the hosts with the infectious clone of TLRV-A[IR:Zaf:Z11:12] resulted in systemic infection only in turnip (7/10), radish (3/16), rapeseed (5/10), thale cress (3/13) and *N. benthamiana* (10/17). Infected plants of turnip and radish showed inward rolling of the leaf margin and swelling of veins on the lower leaf surfaces (Fig. 6A and B). For *N. benthamiana*, thale cress and rapeseed, virus could be detected by PCR in systemic top leaves, but no symptoms were observed on the plants. Agroinoculated cowpea seedlings (5/15) showed formation of local lesions on the leaves (Fig. 6C), and virus was not detected in top non-inoculated leaves. Viral infection was not detected in cabbage, sugar beet, red beet, spinach, pepper, okra, eggplant, lettuce, tomato and canola.

Leafhopper transmission of TLRV-A[IR:Zaf:Z11:12]

Nymphs and/or adults of *C. haematoceps* successfully transmitted TLRV-A[IR:Zaf:Z11:12] from infected turnip plants to 83.3 % (30/36) and 72.2 % (13/18) of the turnip and radish seedlings, respectively. Leafhopper-inoculated plants showed typical turncurtovirus symptoms similar to those of agroinoculated plants. The turncurtovirus infection was confirmed by PCR using back-to-back primers.

Discussion

Amongst geminiviruses, host range varies from wide to restricted [8]. While beet curly top virus (BCTV) has a broad host range, horseradish curly top virus infects only cruciferous species [10]. Prior to this study, it was believed that in contrast to other reported curly top viruses from Iran, i.e., BCTV and beet curly top Iran virus (BCTIV), TCTV would have a limited natural host range [2, 13, 35]. Based on the results of this study, in addition to the main host (turnip), TCTV and/or TLRV were detected in radish, sugar beet, red beet, lettuce, eggplant, spinach and basil. However, most of these plants, i.e., sugar beet, red beet, lettuce, eggplant and spinach, did not become infected after agroinoculation with TLRV-A [IR:Zaf:Z11:12] in the greenhouse. Furthermore, our earlier studies have shown that, besides turnip, radish and sugar beet, the infectious clone of TCTV-A[IR:Zaf:B11:06] could not establish an infection in other experimental test plants, and only 6.7 % of the agroinoculated sugar beet seedlings became infected [36]. The reason may be due to different cultivars used in the field as compared to the greenhouse studies. In addition, the reaction of these plants was tested against only one TLRV genotype (TLRV-A[IR:Zaf:Z11:12] isolate) using agroinoculation. Therefore, due to the genetic variability of turncurtovirus isolates, the infectivity of agroinoculated clones may differ between TCTV and TLRV genotypes.

Fig. 7 *Circulifer haematoceps*, the vector of four curly top viruses in Iran

Iran is a part of the Old World with a long history of agriculture coupled with hot and dry climatic conditions. The climate is influenced by Iran's location between the subtropical aridity of the Arabian Desert areas and the subtropical humidity of the eastern Mediterranean area. This is favorable for crop cultivation, but also for the leafhopper *C. haematoceps* [23, 43], the vector of several curtoviruses, becurtoviruses and turncurtoviruses [21, 22, 35]. Four geminiviruses causing curly top symptoms, i.e., BCTV, BCTIV, TCTV and TRLV, have been reported from sugar beet and turnip of farms in Iran [4, 7, 15], and all can be vectored by *C. haematoceps* (Fig 7). Adaptation of vector and virus to a system with long-term agricultural activities provides suitable conditions for variability and emergence of distinct viruses, and it appears that the Middle East region is a diversity hotspot for the emergence of new curly top viruses where recombination is playing a significant role in generating diversity.

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