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Genome characterization and genetic diversity of beet curly top Iran virus: a geminivirus with a novel nonanucleotide

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Abstract Beet curly top Iran virus (BCTIV) was previously reported as a distinct curtovirus in Iran. Complete nucleotide sequences of three BCTIV isolates, one each from central, southern, and south eastern Iran were determined to be 2844, 2844, and 2845 nt long, respectively. BCTIV shared highest nucleotide sequence identity (52.3%) with *Spinach curly top virus* (SpCTV) and lowest identity (46.6%) with *Horseradish curly top virus* (HrCTV). The BCTIV genome comprises three virion-sense (V1, V2, and V3) and two complementary-sense (C1 and C2) ORFs. ORFs C3 and C4 were not found in BCTIV genome. Based on a comparison of nucleotide sequence identity of individual genes, the three virion-sense ORFs were 72.7–79.9% related to the corresponding ORFs of curtoviruses, whereas no significant relationship was found between the C1 and C2 ORFs of BCTIV and curtoviruses. These two ORFs, however, were only distantly related with those of mastreviruses. Similar to the latter viruses, the BCTIV genome comprises two intergenic regions. The BCTIV large intergenic region included a sequence capable of forming a stem loop structure and a novel nonanucleotide (TAAGATT/CC) with a unique nick site. Phylogenetic analysis using deduced amino acid sequence of individual ORFs revealed that the V2 and V3 ORFs are monophyletic and the V1 ORF is classified with the related ORF of curtoviruses. Whereas the two complementary-sense ORFs are grouped with those of mastreviruses. Computer-based prediction suggested that BCTIV has a chimeric genome which may have arisen by a recombination event involving curto- and mastrevirus ancestors. Percent nucleotide sequence identities of the coat protein gene of ten isolates of BCTIV, collected from a wide range of geographical regions in Iran, varied from 87.1 to 99.9, with the isolates being distributed between two subgroups. Based on biological and molecular properties, BCTIV is proposed as a new member of the genus *Curtovirus*.

Keywords *Curtovirus* · *Geminiviridae* · Beet curly top Iran virus · Nonanucleotide · Recombination

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Introduction

The family *Geminiviridae* comprises a large number of plant viruses that cause significant yield reduction in economically important crops [reviewed by 5]. Geminiviruses have twinned virions each containing a circular ssDNA, ranging in size from 2.5 to 3 kb. Viruses in the genera *Mastrevirus*, *Curtovirus*, and *Topocuvirus* have a single genomic component while those in the genus *Begomovirus* have either one or two components [14]. In addition to 4–7 ORFs, the genome of the geminiviruses, with the exception of that of the mastreviruses, contains an intergenic region (IR) between the 5′ ends of virion- and complementary-sense genes. The mastrevirus genome, on the other hand, contains two intergenic regions, one large (LIR) and another small (SIR), located at opposite sites on the viral genome [reviewed by 5]. ssDNA synthesis is initiated by cleavage of the virion-sense strand using the virus-encoded replication-associated protein (Rep) immediately downstream of the 3′ thymidine residue in an absolutely conserved TAATATT/AC sequence located in the loop of a potential stem-loop structure within the IR (LIR). The Rep nick site is also conserved in the DNA β satellites associated with the Old World monopartite begomoviruses [14].

Based on full-length nucleotide sequence identity and biological properties, the genus *Curtovirus* consists of four species *Beet curly top virus* (BCTV, formerly California/Logan), *Beet severe curly top virus* (BSCTV, formerly CFH), *Beet mild curly top virus* (BMCTV, formerly Worland), and *Horseradish curly top virus* (HrCTV) [9, 14, 15]. Recently a fifth member of the genus, *Spinach curly top virus* (SpCTV), has been reported [1]. The genome of curtoviruses is 2.9–3.0 kb in size and encodes six to seven proteins. The three proteins on the virion-sense strand are the coat protein (CP) (ORF V1) that encapsidates the virion-sense ssDNA genome and is involved in virus movement and insect vector transmission; V2 protein that is involved in the regulation of the relative levels of the ssDNA and dsDNA; and a movement protein (MP) (ORF V3). The complementary-sense strand encodes the Rep protein (ORF C1); C2 protein that acts as a pathogenicity factor in some hosts; a replication enhancer protein (REn, ORF C3); and C4 protein, which is a symptom determinant and is implicated in cell-cycle control [14]. HrCTV is the only curtovirus to lack C3 ORF [9].

Curly top disease of sugar beet (*Beta vulgaris* L.) was first reported in Iran by Gibson [4]. During the past four decades, it has been responsible for reduced performance in sugar beet and other crops in this country [4, 8]. Briddon et al. [3] compared the nucleotide sequence of an infectious clone of a beet curly top virus isolate from Iran with the genome of other curtoviruses and concluded that the virus is a strain of BSCTV (BSCTV-I). Recently a new curly top virus isolate, the beet curly top Iran virus (BCTIV, formerly Iranian beet curly top virus), was found infecting sugar beet, tomato, spinach, turnip, and some weed species in central and southern provinces of Iran [7]. The virus was transmitted by the leafhopper, *Circulifer haematcepts* [6]. Analysis of CP nucleotide sequence identity of BCTIV showed that it was distinct from known curtoviruses [7]. We report here the complete nucleotide sequence of the BCTIV genome and the analysis of gene organization within the virus. Furthermore, we discuss the phylogenetic relationship of BCTIV ORFs with the related ORFs of other geminiviruses and the diversity of virus isolates collected from different regions of Iran.

Materials and methods

Collection of field samples

BCTIV infected leaves of sugar beet, tomato, spinach, and turnip were collected in widely separated areas from Iran during 2004 to 2006 (Table 1). All BCTIV infected crop plants showed typical symptoms of curly top disease as described by, for example, Bennett [2].

PCR amplification and cloning

DNA extraction of infected samples was carried out according to the method of Zhang et al. [16]. The CP gene of all isolates was amplified by PCR using primer pair IBCTV-F/CTV-R and sequenced as described by Heydarnejad et al. [7]. The resulting sequence data plus corresponding sequences of BCTIV-[K], BCTIV-[Y], and BCTIV-[Sh1] isolates [7] were used to analyze genetic diversity of BCTIV in Iran. Isolates BCTIV-[K], BCTIV-[Y], and BCTIV-[Sh2] were used to obtain full-length sequences of their genomes using primer pair F20/R10 (5′-ACATTGTGGAAGGACACTGG3′/5′TCCTCGTCCA ACTGGCCGGAG-3′) designed by alignment of nucleotide sequence of their CP genes. About 2300 bp segment of each isolate genome was amplified. The amplification program for F20/R10 primers was the same as that for amplification of CP gene, but extension time was increased to 95 s. The amplification was carried out using a TC-312 Thermal Cycler (Techne, UK). Amplified IBCTV-F/CTV-

Table 1 Location, original host plant, isolate designation, and GenBank accession numbers of field-collected BCTIV isolates

Location	Host plant	Isolate designation	GenBank accession number
Kerman, Kerman Province, south-eastern Iran	Sugar beet	BCTIV-[K][a]	EU273818
Karaj, Tehran Province, northern Iran	Sugar beet	BCTIV-[Kj]	EU263013
Yazd, Yazd Province, central Iran	Sugar beet	BCTIV-[Y][a]	EU273816
Chenaran, Razavi Khorasan Province, north-eastern Iran	Sugar beet	BCTIV-[Kh]	EU263012
Isfahan, Isfahan Province, central Iran	Sugar beet	BCTIV-[Is]	EU263008
Shiraz, Fars Province, southern Iran	Sugar beet	BCTIV-[Sh1][a]	EF059897
Shiraz, Fars Province, southern Iran	Sugar beet	BCTIV-[Sh2]	EU273817
Boushehr, Boushehr Province, southern Iran	Tomato	BCTIV-[To]	EU263010
Shiraz, Fars Province, southern Iran	Spinach	BCTIV-[Sp]	EU263009
Shiraz, Fars Province, southern Iran	Turnip	BCTIV-[Tu]	EU263011

[a] Nucleotide sequences of coat protein gene for these samples are derived from Heydarnejad et al. [7]

R and F20/R10 segments of each isolate were ligated into pTZ57R/T plasmid using InsT/Aclone PCR Product Cloning Kit (Fermentas, Lithuania). After transformation of *Escherichia coli* strain XL blue, bacterial clones bearing recombinant plasmids were identified by color in the presence of X-gal and then by PCR using primer pairs IBCTV-F/CTV-R or F20/R10. Recombinant plasmids were extracted and sequenced on both strands by automatic sequencer ABI3730XL using primer walking strategy. A total of 10 CP segments belonging to 10 BCTIV isolates as well as three full-length genomes were used for various analyses and construction of phylogenetic trees.

Sequence analyses

Sequence comparisons were made using multiple alignments of individual genes or complete sequences. To analyze genetic diversity of BCTIV, CP nucleotide sequences were aligned using DNAMAN software package (Lynnon Biosoft, Quebec, Canada) and percent nucleotide sequence identity was calculated. Furthermore, the nucleotide sequence of the CP gene of all BCTIV isolates was translated into amino acid sequences using the standard genetic code. Amino acid sequences were aligned using the T-Coffee computer program [12] and percent amino acid similarities were calculated. Aligned amino acid sequences were used as guides to manually align nucleotide sequences as described by Baliji et al. [1]. Sequence comparisons included the CP sequences of the ten different isolates of BCTIV and corresponding curtovirus sequences available in GenBank: BCTV, BSCTV, BSCTV-I, BMCTV, SpCTV, and HrCTV. The CP sequence of tomato yellow leaf curl virus (TYLCV), tomato pseudo-curly top virus (TPCTV), and squash leaf curl virus (SLCV) was also included in the sequence analysis (Tables 1 and 2). To compare BCTIV genome with other geminiviruses, the full-length BCTIV-[Y] genome was compared with that for geminiviruses. In addition, percent nucleotide and amino acid sequence identity for individual genes were calculated and phylogenetic trees were constructed based on the amino acid sequences of the individual BCTIV genes. Phylogenetic analysis was done by the neighbor-joining method using DNAMAN software package. The RDP3 Beta 18 (recombination detection program) [10] was used to search for recombination events among geminiviruses by detecting potential recombined sequences, identifying the likely parent sequence, and localizing possible recombination break points. The RDP settings used were: window size 30, highest acceptable probability 0.05 together with internal and external reference sequences.

Results

Genetic diversity of BCTIV in Iran

All samples of plants showing typical curly top symptoms, collected for the present study, tested positive for the presence of BCTIV by IBCTV-F/CTV-R primed PCR yielding fragments of expected size, i.e., 680 bp. The sequence of the CP gene from seven isolates and three previously reported sequences of BCTIV isolates [7] were aligned using DNAMAN software package. Percent nucleotide sequence identity for the 672 nt fragments from 10 isolates ranged from 87.1 to 99.9%. Similar values (88.0 to 99.0%) were obtained when the CP amino acid sequences were compared. Percent nucleotide and amino acid sequence identities between the 672 nt fragment of all ten BCTIV isolates and a previously reported BSCTV isolate from Iran (BSCTV-I) were calculated to be 74.1–75.6% and

Table 2 The names and acronyms of viruses and accession numbers of sequences used in this study

Virus	Genus	Acronym	Accession number
Beet curly top virus-California [Logan]	*Curtovirus*	BCTV	M24597
Beet severe curly top virus–[Cfh]	*Curtovirus*	BSCTV	U02311
Beet severe curly top virus–[Cfh Beta]	*Curtovirus*	BSCTV-I	X97203
Beet mild curly top virus–[Worland]	*Curtovirus*	BMCTV	U56975
Horseradish curly top virus	*Curtovirus*	HrCTV	U49907
Spinach curly top virus	*Curtovirus*	SpCTV	AY548948
Tobacco yellow dwarf virus	*Mastrevirus*	TYDV	M81103
Tomato yellow leaf curl virus-Iran	*Begomovirus*	TYLCV	AJ132711
Tomato pseudo-curly top virus	*Topocuvirus*	TPCTV	X84735
Squash leaf curl virus	*Begomovirus*	SLCV	M38183
Wheat dwarf virus	*Mastrevirus*	WDV	AJ783960
Bean yellow dwarf virus	*Mastrevirus*	BeYDV	Y11023
Sugarcane streak Egypt virus-[Giza]	*Mastrevirus*	SSEV	AF037752
Maize streak virus	*Mastrevirus*	MSV	EF547092

70.0–74.0%, respectively. Phylogenetic analysis based on the nucleotide sequence of the CP gene showed that all BCTIV isolates were contained within two subgroups. BCTIV-[Sh2], BCTIV-[Tu], BCTIV-[Kj], and BCTIV-[Kh] isolates were placed in the first subgroup with percent nucleotide identity of 86.9 to 99.0 and the rest of the isolates in the second subgroup with percent nucleotide identity of 98.2 to 99.9 (data not shown).

Characterization of the BCTIV genome

The IBCTV-F/CTV-R and F20/R10 primed fragments of BCTIV-[Sh2], BCTIV-[Y], and BCTIV-[K] were assembled to obtain full-length sequences of 2844, 2844, and 2845 nt, respectively. The extra nucleotide of the BCTIV-[K] genome is located within the LIR (Table 3). Comparison of the complete nucleotide sequence of BCTIV with those of curtoviruses indicated that BCTIV has the smallest genome among known curtoviruses and shares the highest and lowest nucleotide sequence identity with SpCTV (52.3%) and HrCTV (46.6%), respectively. By analogy with other geminiviruses, the BCTIV genome comprises three overlapping genes (V1, V2, and V3) present in the virion-sense strand and two complementary-sense genes (C1 and C2). Comparison of nucleotide and amino acid sequences of BCTIV virion-sense ORFs and their putative products showed that these ORFs are most similar to counterpart ORFs in curtoviruses and may, by implication, have similar functions (Table 4). The C1 and C2 ORFs, however, were more distantly related to those of mastreviruses although occupying approximately the same position in the genome. In contrast to curtoviruses, the BCTIV genome lacks C3 and C4 ORFs which encode replication enhancer and symptom determinant proteins, respectively [14]. Despite a 1–2 nucleotide variation in starting point, each virion-sense ORF had the same length regardless of isolate (Table 3). Like other geminiviruses, the BCTIV genome contains C1, the largest complementary-sense ORF. The C2 ORF of BCTIV is located between C1 and the SIR. Phylogenetic analysis using deduced amino acid sequences for individual ORFs showed that the three BCTIV virion-sense genes are monophyletic (V2 and V3) or classified with corresponding proteins of curtoviruses (V1) (Figs. 1, 2), whereas the C1 and C2 genes are

Table 3 Comparison of the gene organization between three isolates of beet curly top Iran virus (BCTIV-[Sh2], BCTIV-[K], and BCTIV-[Y]) and *Beet curly top virus* (BCTV-[Cal])

Virus	Full-length genome (nt)	ORF							Intergenic region	
		V1	V2	V3	C1	C2	C3	C4	LIR	SIR
BCTIV-[Sh2]	2844	487–1239	243–608	167–436	2713–1805	1790–1485	NF	NF	2712–166	1240–1484
		753	366	270	909	306			299	245
BCTIV-[K]	2845	488–1240	244–609	168–437	2714–1806	1791–1486	NF	NF	2713–167	1241–1485
		753	366	270	909	306			300	245
BCTIV-[Y]	2844	486–1238	242–607	166–435	2713–1805	1790–1485	NF	NF	2712–165	1239–1484
		753	366	270	909	306			298	246
BCTV-[Cal]	2994	697–1461	477–785	401–667	1917–2993	1604–2125	1486–1896	2579-2836	2837–400	–
		765	309	267	1077	522	411	258	558	

Abbreviation: C1, C2, C3, and C4, complementary-sense ORFs; LIR, large intergenic region; NF, not found; nt, nucleotide; SIR, small intergenic region; ORF, open reading frame; V1, V2, and V3, viral-sense ORFs; and – indicates BCTV lacks the SIR. See Table 2 for accession numbers

Table 4 Percent nucleotide sequence identities of three virion-sense (V1, V2, and V3) and two complementary-sense (C1 and C2) ORFs of beet curly top Iran virus (BCTIV) relative to curto- and mastreviruses, respectively

Virus	Curtoviruses			Mastreviruses		Virus
	V1	V2	V3	C1	C2	
BCTV	85.2 (73.0)	77.2 (65.0)	77.1 (62.0)	49.8 (64.0)	58.0 (64.0)	TYDV
BMCTV	86.5 (74.0)	80.2 (67.0)	77.1 (62.0)	50.5 (65.0)	53.3 (68.0)	MSV
BSCTV	86.4 (74.0)	80.6 (66.0)	77.7 (63.0)	50.3 (62.0)	56.5 (67.0)	WDV
BSCTV-I	86.0 (73.0)	80.2 (68.0)	77.3 (63.0)	52.9 (55.0)	58.9 (60.0)	BeYDV
SpCTV	86.1 (74.0)	77.6 (64.0)	81.6 (65.0)	52.7 (57.0)	54.9 (65.0)	SSEV
HrCTV	83.4 (70.0)	77.1 (62.0)	77.1 (60.2)			

Percent amino acid identities are in parentheses. See Table 2 for abbreviations and accession numbers

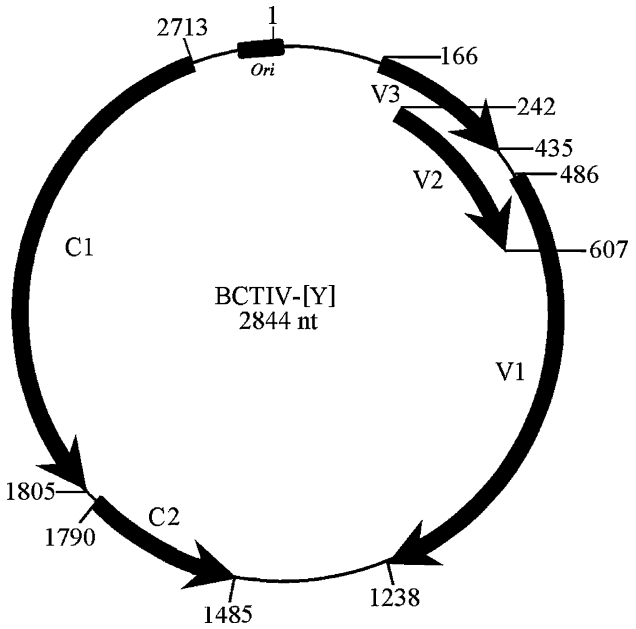

Fig. 1 Genome organization of beet curly top Iran virus, Yazd isolate (BCTIV-[Y]), showing position of potential virion-sense (V1, V2, and V3) and complementary sense (C1 and C2) ORFs. The arrows indicate the size and polarity of each potential ORF. The start and stop sites of specific ORFs are indicated by numbers. The arc shows the location of the DNA origin of replication (*ori*) within the large intergenic region

grouped with the related proteins of the mastreviruses. Similar to mastreviruses, the BCTIV genome comprises both an LIR and an SIR. The number of nucleotides in the LIR differs in the three BCTIV isolates. The length of the SIR, however, is the same for the BCTIV-[Sh2] and BCTIV-[K] isolates (Table 3). Surprisingly, the BCTIV rep protein nick site within the loop contains a novel nonanucleotide that differs from those of all known geminiviruses and their satellites. The fourth and eighth nucleotides of the BCTIV Rep protein nick site are G and C, respectively, instead of T and A in other geminiviruses (Fig. 3). Searching for possible recombination events using the RDP3 revealed that BCTIV comprises a hybrid genome in which a segment (nucleotides 1275 to 2837) overlapping to the complementary-sense ORFs was probably donated by a BeYDV-like and remaining sequence by a BSCTV-I-like ancestors as the minor and major parents, respectively (RDP P-value $= 3.7 \times 10^{-15}$).

Discussion

In spite of having unusual genome and low full-length sequence identity relative to curtoviruses, BCTIV possesses some biological and molecular characteristics that make it more similar to members of the genus *Curtovirus* than to those of other genera comprising the family *Geminiviridae*. It causes typical symptoms of curly top disease in sugar beet and other crops such as tomato, spinach, and turnip as previously reported by Bennett [2]. Similar to most curtoviruses, it has a wide host range among the cultivated and wild species [7]. This property cannot be generalized to all curtoviruses. HrCTV, for example, has a narrow host range [9]. BCTIV is leafhopper transmissible [6] similar to curtoviruses [14]. Moreover, gene organization of BCTIV virion-sense ORFs is similar to that of the curtovirus genome. Possession of three virion-sense ORFs on the single genome component differentiates BCTIV from members of the genera *Mastrevirus*, *Begomovirus,* and *Topocuvirus*. Some bipartite begomoviruses comprise the third virion-sense ORF (BV1) situated on the B component [14]. While all three virion-sense ORFs of BCTIV are moderately related to those of other curtoviruses, the two complementary-sense ORFs are an unusual feature of the BCTIV genome relative to curtoviruses which contain three to four complementary-sense ORFs [14]. The two complementary-sense BCTIV ORFs showed a distant relationship with those of the mastreviruses. Recombination analysis predicted that BCTIV has been generated by recombination between curto- and mastrevirus ancestors. Phylogenetic analysis and comparison of sequence identity of individual BCTIV ORFs with the corresponding ORFs of members of the two genera, *Curtovirus* or *Mastrevirus*, supported this hypothesis. Based on nucleotide and amino acid sequence identities, the three BCTIV virion-sense ORFs were most related to counterpart ORFs of the curtoviruses while the BCTIV C1 and C2 ORFs were most similar to the related ORFs of the mastreviruses (Table 4). Likewise, constructed phylogenetic trees using amino acid sequences of the C1 and C2 ORFs showed that BCTIV could be grouped with the corresponding ORFs of the mastreviruses (Fig. 2). The complementary-sense strand of the mastreviruses encodes the Rep protein, expressed from ORFs C1 and C2 by transcript splicing [14]. RepA (the C1 ORF) and the C2 ORF (also called RepB) encode the amino- and carboxy-terminal parts of Rep protein, respectively [11]. The Rep protein of other geminiviruses is encoded in a single ORF which resembles the splice product of RepA and RepB ORFs of mastreviruses. The Rep protein of the latter viruses contains a specific LxCxE motif which lies very close to the splicing site [reviewed by 5]. The LxCxE motif was also detected in the putative product of BCTIV ORF C1. The *cis*-acting elements of BCTIV LIR are somewhat similar to those of other curtoviruses. Although the 5′-proximal motif (GGAG) of the BCTIV Rep binding site is identical to those of BCTV, the 3′-proximal motif (GGGA) of BCTIV is different from all curtoviruses GGT(A)G [1, 15]. More nucleotide substitutions are found in the central portion and the 3′-proximal motif of the *cis*-acting elements of curtoviruses [15]. All three BCTIV isolates had the same

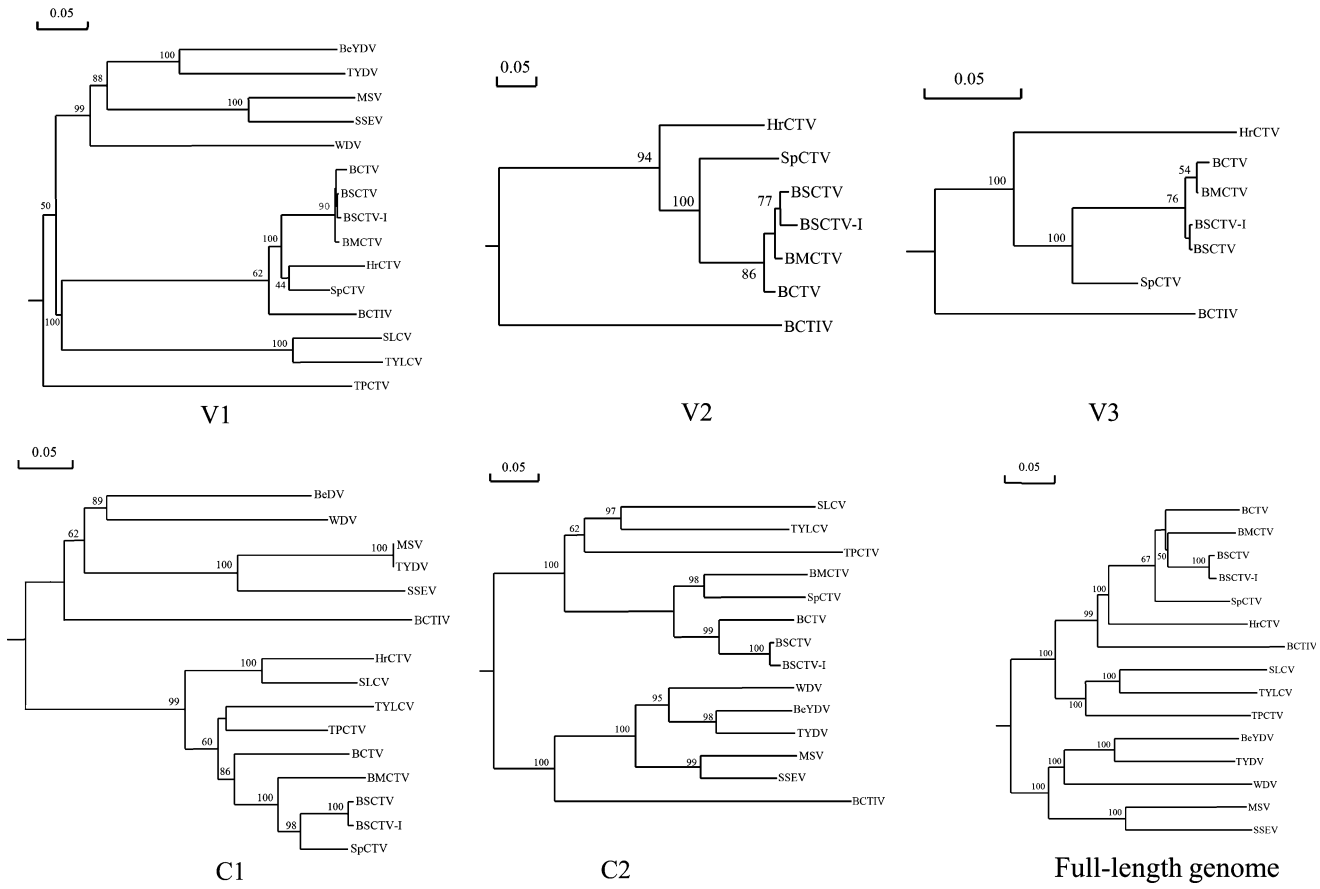

Fig. 2 Phylogenetic trees constructed on the basis of the amino acid sequences of individual virion-sense (V1, V2 and, V3) and complementary-sense (C1 and C2) ORFs of beet curly top Iran virus (BCTIV) and selected geminiviruses. The phylogenetic tree constructed using the full-length genome shows the position of BCTIV relative to selected geminiviruses. Trees were generated by DNAMAN software using neighbor-joining method. Tree branches were bootstrapped with 1000 replications. Horizontal distances are proportional to sequence distances. See Table 2 for abbreviations and accession numbers

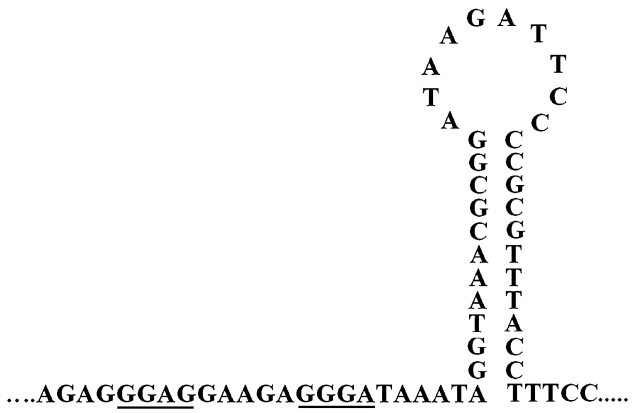

Fig. 3 Nucleotide sequence of Rep binding site (*underlined letters*) and potential stem loop structure within the large intergenic region of beet curly top Iran virus genome. The loop includes the novel nonanucleotide sequence (TAAGATT/CC) compared to absolutely conserved TAATATT/AC sequence in all known geminiviruses

nucleotide substitutions within the *cis*-acting elements and identical sequences in the potential stem loop structure. Our study showed that in curtoviruses the number of nucleotides of AT-rich region between the potential stem loop and the Rep binding site for a specific curtovirus may be related to the length of genome. BCTIV and HrCTV which possess the smallest and largest genome, respectively, contain 5 and 35 nt between the stem loop and the Rep binding site, respectively. A similar relation can be found for other curtoviruses. Geminivirus Rep proteins have a site-specific nicking activity within the invariant 9-nt sequence of the loop. This activity is directly responsible for the initiation of replication on virion-sense strand [14, reviewed by 5]. The most important difference in the BCTIV genome relative to all known geminiviruses is possession of a unique nick site (TAAGATTCC) within the LIR. Among plant viruses two families the *Geminiviridae* and the *Nanoviridae* which have circular ssDNA genomes, replicate by a rolling circle mechanism and contain highly conserved sequences, either TAATATTAC (geminiviruses) or predominantly TAGTATTAC (nanoviruses) in the loop of the putative stem-loop structure [14]. Therefore, although the Rep nick site of BCTIV genome is unique even among all known geminiviruses, based on biological and molecular properties we

believe that it should be classified within the genus *Curtovirus*.

Curly top disease of sugar beet has been reported since 1967 in Iran [4] and BCTIV was identified to be the dominant curly top agent in this country [7]. In spite of the report of Briddon et al. [3] concerning the close relationship of Iranian curly top agent and BSCTV, we were unable to detect BSCTV-I or any related curtoviruses besides BCTIV in Iran. However, the presence of other beet curly top virus variants on sugar beet and other crops cannot be ruled out. The ten BCTIV isolates collected from four crops in widely separated areas in Iran varied with respect to nucleotide and deduced amino acid sequence of the CP gene. This gene sequence is an informative marker for curtovirus identity when a full-length genomic sequence is not available [13]. Differences between the CP sequences of BCTIV isolates reflect genetic diversity of the virus in Iran.

Percent nucleotide sequence identity of full-length BCTIV genome relative to other curtoviruses was calculated to be between 46.6 (HrCTV) and 52.3% (SpCTV). This value is well below the curtovirus species demarcation threshold of 89% established by the *Geminiviridae* Working Group of the International Committee on the Taxonomy of Viruses [14]. We proposed, therefore, that the virus we investigated in this study and which, based on our findings, we believed to be a curtivirus, should be designated as a new species of the genus *Curtovirus*, namely beet curly top Iran virus.

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