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Original Article

Molecular Detection of *Beet Curly Top Iran Becurtovirus* in Insect Vector (*Circulifer Haematocpes*) and Analysis of Synonymous Codon Usage Bias

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Abstract

Background and Aims: *Beet curly top Iran virus* is a member of the genus *Becurtovirus* in the family *Geminiviradae*, and is a major pathogen of sugar beet in Iran. BCTIV is transmitted by beet leafhopper *Circulifer haematocpes* in a persistent manner. The primary objective of the work was to monitor the occurrence and incidence of BCTIV in vector leafhoppers, which provides useful information on the potential risk to other economically important hosts. A secondary objective was to analysis of CUB using CP gene sequences available in Genbank. This information will further our understanding of the importance of the beet leafhopper in diseases affecting sugar beet and other host crops. In addition, CUB analyses reveal novel information about the evolutionary fitness of BCTD.

Materials and Methods: Total nucleic acid was extracted from 10 leafhoppers individually, which were collected from West Iran. The partial coat protein (CP) genes were amplified using specific primers. For molecular analysis, CP sequences of 164 isolates worldwide, including BCTIV (n=53), and BCTV (n=111) isolates obtained from the GenBank database, were used for codon usage bias -CUB analysis. To clarify the genetic diversity of BCTIV and BCTV, CP sequences were aligned using CLUSTALX2. The CodonW 1.4.2 package was used for assessing of the nucleotide mixtures at the 3rd codon position (A3, C3, T3, and G3%). The Emboss explorer (http://www.bioinformatics.nl/emboss-explorer/) was used for calculating GC content at the first, second, and third codon positions (GC1s, GC2s, GC3s), where the average of GC1 and GC2s is indicated by GC1,2s.

Results: After polymerase chain reaction (PCR) an expected DNA band of about 600bp was amplified, which confirmed the infection of six leafhoppers with BCTIV. A constant and conserved genomic composition CP coding sequences were inferred by low codon usage bias. Nucleotide composition analysis indicates the frequency of amino acid coded by A/U ended optimal codon. This unequal use of nucleotides composition, effective number of codons (ENC), and principal component analysis (PCA) plots indicates that the combination of mutation pressure and natural selection are deriving the codon usage patterns in the CP gene but the role of selection pressure is more important.

Conclusion: Our PCR method would be useful in monitoring and detection of BCTIV in this important insect vector, and the data regarding viruliferous vectors can be applied in disease forecasting and management. In addition, our findings showed that overall codon usage bias within BCT CP genes is slightly biased. The evolution of BCT perhaps reflects a dynamic process of mutation and natural selection to adapt their codon usage to different environments and hosts. This research makes an essential contribution to the understanding of plant virus evolution andeal the novel information about their evolutionary fitness.

Keywords: BCT viruses, insect vector, beet leafhopper, PCR, codon usage patterns

Introduction

The Family *Geminiviridae* is the second largest of plant virus families distributed worldwide (1). This family includes seven genera of plant-infecting viruses with circular single-stranded DNA (ssDNA) genomes that are encapsidated within geminate virions. Beet curly top disease (BCTD) is a destructive, yield-limiting viral infection widespread in Central America and the Middle East (2-4). The curly top has an extraordinarily

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large number of economic and wild hosts (more than 300 species from at least 44 families). BCTD had been reported to be caused by mainly two geminivirus species, *Beet curly top virus* and *Beet curly top Iran virus* (BCTIV) are agents of CTD in Iran (5).
BCTV, belonging to the genus *Curtovirus* and BCTIV (genus *Becurtovirus*). Although these two viruses have different genome sequences and organizations, however, they are the same in biological properties, such as leafhopper transmission and host range (3). BCTV occurs in many regions of both the New and Old Worlds however BCTIV is so far reported only from Iran and Turkey (6,7,8). Both viruses transmitted by leafhoppers *Circulifer tenellus* Baker and *C. haematoceps* (Homoptera: Cicadellidae) (8). BCTD transmitted by leafhoppers were the first group of plant diseases considered to be associated with an insect vector.
Leafhopper-transmitted diseases show a high degree of specificity for their host plants and a distinctly close biological relationship between the vector and pathogen. BCTIV is a dominant and widespread virus in Iran and is responsible for the significant annual loss and yield reduction for agricultural production (5).
The leafhopper *C. haematoceps* is known as the main vector of BCTIV in Iran (3). The infection of BCTIV has been reported from sugar beet, spinach, tomato, turnip, and several weed species (3). BCTV and BCTIV have the same BCTD symptoms on the sugar beet such as vein swelling (enation), leaf curling, and stunted growth (5). The genetic variability and population structure of BCTIV have already been studied (5).
However, the synonymous codon usage patterns and selection pressure analysis, which provides important information about the virus evolution as well as gene expression and function, has not been reported. Understanding the evolution of viruses is so important, due to rapid evolution through genetic recombination, mutation, the potential of adaption to new or resistant hosts (9,10), fast adaptation to the different environmental conditions, and mostly lack effective chemical compounds (11). As the virus translation is dependent on the host cellular machinery, the interaction of a virus with a particular host must be studied on the basis of its codon usage bias (CUB). A significant role of CUB in the evolution of viruses was reported (12) The studies on CUB and its role in the evolution of plant viruses are limited (13). The recent advancement in sequencing technologies, allow studying the codon usage behavior of viral diseases (14-18). It is presumed that viral coat proteins (CP) evolved more rapidly than proteins involved in replication and expression of virus genomes (19), thus providing a strong incentive to study the diversity of viruses based on CP genes.
The primary objective of the work was to detect BCTIV in the beet leafhopper in west Iran using specific primers. A secondary objective was to analysis of CUB using CP gene sequences available in Genbank.
This information will further our understanding of the importance of the beet leafhopper in diseases affecting sugar beet and other host crops. In addition, CUB analyses reveal novel information about the evolutionary fitness of BCTD.

Methods

Sample collection, nucleic acids extraction, polymerase chain reaction: Beet leafhoppers were collected on the yellow sticky traps weekly at three sites in West Iran. Nucleic acids were extracted from leafhoppers as previously described. Individual *C. haematoceps* leafhoppers (Fig. 1) were ground in 200 ul of STE buffer (100 mM NaCl, 10 mM Tris-HCl(pH 8.0), and 1 mM EDTA) in a 1.5 ml microtube with a glass pestle.



Fig.1. Leafhopper *Circulifer haematoceps*, the main vector of *Beet curly top Iran virus*

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The resulting suspension was clarified by centrifugation at 14,000 g for 5 min, and the supernatant was extracted with an equal volume of phenol/chloroform.

After centrifugation, nucleic acids were recovered by ethanol precipitation, suspended in 20 ul of PCR grade water and 5 ul was used in the PCR. Nucleic acid preparations (5 ul) were initially tested for the presence of BCTIV by polymerase chain reaction (PCR) using oligonucleotide primers designed for the coat protein (CP) region of the virus.

Forward primer F/10 (5'-CTCCGGCCAGTTGGACGAGGA-3') and reverse primer R/20 (5'-CCAGTGTCCTTCCACAATGT-3') were used in 50 ul PCR reactions containing 5 ul of 10X PCR buffer (CinnaGen, Iran), 1 ul of 50 mM MgCl2, 2 ul of 10 mM dNTP mix, 1 ul (20 pmol) of each forward and reverse primers, 1 ul (5 U) of *Pfu* DNA polymerase (CinnaGen, Iran), and 34 ul of nuclease-free water.

PCR was performed under the following conditions: 94˚C for 5 min; 35 cycles of 95˚C for 30 s, 58˚C for the 30s, and 72˚C for 30 s; and 72˚C for 10 min. PCR amplification was assessed by electrophoresis in a 1% agarose gel in TBE buffer containing ethidium bromide (final concentration 1 ug/ml).

Viral isolates: The CP gene sequences of 164 BCT and BCTI isolates worldwide obtained from the GenBank database, were used for CUB analysis (Table S1, Supplementary Materials). To clarify the genetic diversity of BCTIV, 54 CP sequences were aligned using CLUSTALX2. The codon usage data for the different hosts were obtained from the codon usage database (available at https://hive.biochemistry.gwu.edu/review/codon) (20).

Nucleotide identity plot was drawn by Sequence Demarcation Tool version1.2 SDTv1.2.

Nucleotide Composition Analysis and Effective Number of Codons (ENc): The overall frequencies of occurrence of nucleotides (A%, U%, C%, and G%), the nucleotide at the third (wobble) position of synonymous codons (A3%, U3%, C3%, and G3%), G+C at the first (GC1), second (GC2), and third (GC3) positions, and G+C at the first and second positions (GC1,2) were calculated for the CP gene sequence of each BCTV and BCTIV isolates using CodonW version 1.4.2 (21).

The ENc values are used to measure the extent of CUB of a gene, and ENc values ranging from 20 to 61 often determine the degree of CUB (22). The ENc value of a gene at or below 35 indicates strong CUB, whereas the gene having an ENc value of 61 indicates that all synony-mous codons are used equally (22).

ENc-GC3 Neutrality Plot: An ENc-GC3 plot was used to investigate the influence of mutation or natural selection on CUB. An ENc-GC3 plot is drawn using the ENc and the GC3 values. If selection is the main force, the ENc values would lie far lower than the standard curve, however, if the mutation is the main force in shaping CUB, the ENc values would lie on or near the standard curve (22).

A neutrality plot (GC12 vs. GC3) is used to decrypt the selection and mutation factors associated with codon usage. GC3 indicates the abundance of G+C at the third codon position and GC12 represents the average of GC1 and GC2. Each dot in the plot represents a CP gene of individual BCTVand BCTIV isolates.

In neutrality plots, if the correlation between GC12 and GC3 is statistically significant and the slope of the regression line is close to 1 (the points positioned on the diagonal line), then mutation pressure is the key factor behind the CUB. However, a lack of correlation between GC12 and GC3 indicates selection pressure is dominant over mutation pressure in codon usage patterns (23).

Results and discussion

PCR and nucleotide diversity: Molecular detection using BCTIV coat protein specific primers resulted in the amplification of a DNA fragment with the expected size of about 600 bp (Fig. 2).

The average evolutionary divergence over all sequence pairs was estimated using Kimura 2-parameter model implemented in MEGAX. Standard error estimate(s) were obtained by a bootstrap procedure (1000 replicates).

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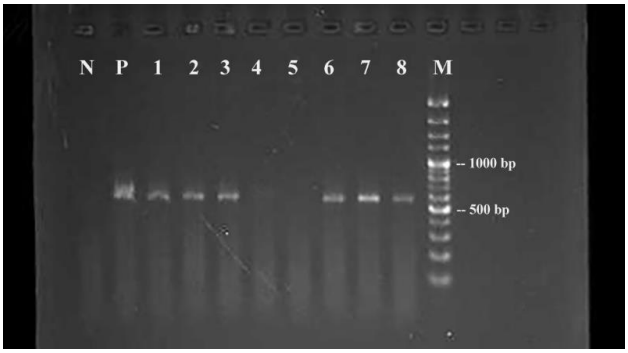


Fig. 2. Agarose gel electrophoresis showing PCR products obtained with primers specific to BCTIV on 8 samples. The presence of the expected 600 bp amplicon indicates the insect was positive for BCTIV. N: negative control, P: positive control, 1 to 8: individual leafhopper samples, M: 100 bp DNA molecular weight marker (GeneRuler, Thermo Scientific, USA).

The highest and lowest of mean nucleotide diversity among BCTV were 0.1 ± 0.01 and 0.04 ± 0.001 for Mexican and USA populations, respectively. Whereas the mean nucleotide diversity was 0.08 ± 0.01 and 0.03 ± 0.01 for Iranian and Turkish BCTIV populations. The pairwise nucleotide diversity between geographical isolation groups was determined. The highest nucleotide diversity was found between BCTV from Mexico and BCTIV from Iran.

However, the lowest nucleotide diversity indicated between BCTV from USA and Mexico, in comparison with two BCTIV from Iran and Turkey populations (Table 1).

Table 1 Nucleotide diversity was estimated between different populations of BCTV and BCTIV based on geographical isolation.

Country	Turkey	Iran	USA
Turkey			
Iran	0.124		
USA	0.308	0.300	
Mexico	0.320	0.315	0.112

The higher mean nucleotide diversity indicates that Iranian and Mexican isolates are older than Turkish and USA populations.

The two dimensional pairwise identity plot indicated two main clusters BCTV and BCTIV. The highest pairwise identity was (81 to 1005) for BCTV and the lowest identity (73 to 81%) was for BCTIV (Fig. 3).

Base composition analysis: To determine the potential influence of compositional constraints on codon usage, the nucleotide compositions of the BCT CP coding sequences were determined (Table S1). The mean values of A% (31.23 ± 1.31) and U% (28.53 ± 0.463) were highest, followed by G% (25.36 ± 1.03) and C% (14.86 ± 0.66). The mean values of AU% and GC% were 59.77 ± 1.47 and 40.22 ± 1.47, respectively, whereas the mean values of AU3% and GC3% were 55.55 ± 2.19 and 44.44 ± 2.97, respectively (Table S1).

According to the nucleotide occurrence frequencies, the BCT CP gene is AU-rich.

Therefore, A and U seem to be found more commonly than G and C at the third (wobble) position of CP gene sequences. However, the nucleotides at third positions of synonymous codons (A3, U3, G3, and C3) show that the mean values of U3% (43.12 ± 1.21) and G3% (32.96 ± 1.47) were higher than the mean values of A3% (12.43 ± 1.27) and C3% (11.48 ± 1.24). The uneven usage of A3/U3 and G3/C3 nucleotides in the AU-rich CP gene in this study shows that the compositional patterns of the BCT CP gene sequences are more complex than the commonly observed GC- and/or AU-rich compositions of most virus genes. For instance, a GC- or AU-rich genome tends to contain codons preferentially ending with either G/C or A/U. Such trends, when observed, support the influence of mutation pressure.

This unequal use of nucleotides indicates the overlapping influences of mutational pressure and natural selection on the codon preferences in the present CP gene sequences.

BCT CP gene shows higher genomic stability and low CUB: The magnitude of CUB of the CP gene of 164 BCT isolates was measured using the effective number of codons (ENc). The ENc values among the present BCT isolates are high and ranged from 42.83 to 46.56 with a mean of 44.10 ± 1.71 (Supplementary Table S1). The higher ENc values indicate low CUB, resulting in higher genomic stability. The mean ENc values were 46.56, 45.32, 44.14, and 42.83 for Mexico, Turkey, the USA, and Iran respectively. The low CUB might be beneficial to BCTIV on its fitness to the host species with potentially distinct codon preferences.

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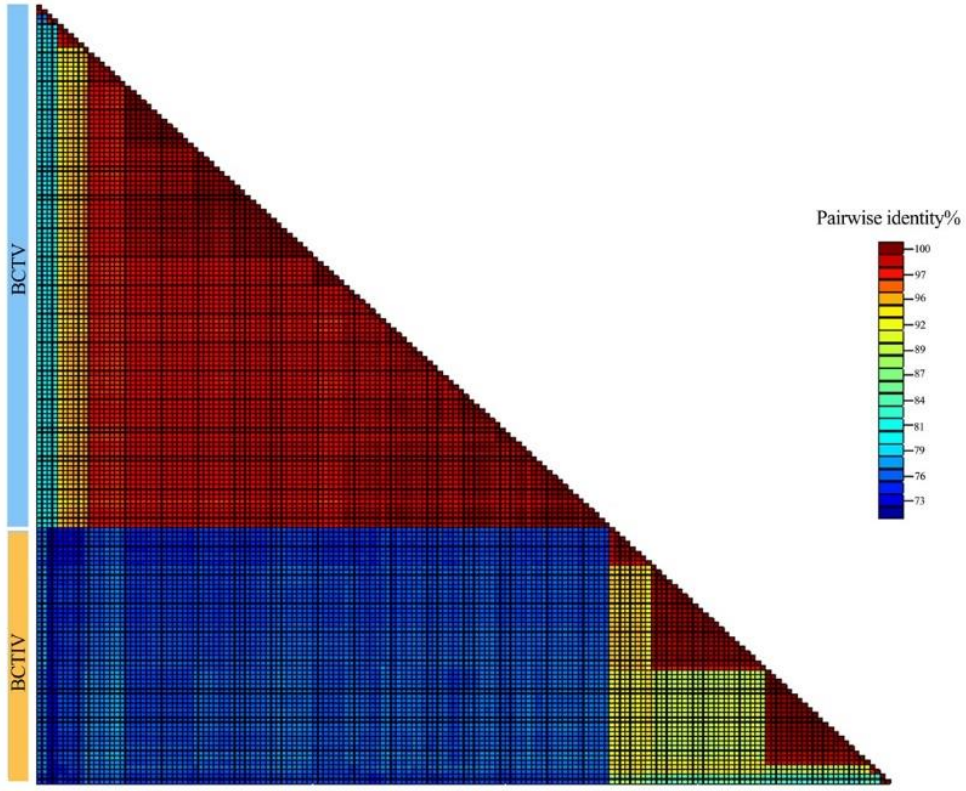


Fig. 3. Two-dimensional identity plot was drawn by SDT v 1.2 using 164 CP sequences of BCTV and BCTIV isolates.

Low CUB was also observed in several viruses, such as Begomoviruses (14), *Papaya ringspot virus*-PRSV (15), *Rice stripe virus*-RSV (16), and *Potato virus M*-PVM (17), *Citrus tristeza virus*-CTV (18), as well.

A lower replication rate increases the fidelity, which leads to better fitness of the virus population. Thus, a low CUB has an advantage for efficient replication in the host cells by reducing the competition between the virus and host in using the synthesis machinery (24).

Natural selection and mutation pressure both play roles in CUB of BCT CP gene: The ENc values of BCT isolates ranged from 42.83 to 46.56, and at GC3 values of 0.339 to 0.449. All of the BCT and BCTI isolates fall slightly below on the left side of the expected curve, indicating that selection pressure is dominant over mutation pressure in codon usage patterns of BCT isolates (Fig. 4) as previously reported for Begomoviruses (14).

Natural selection plays a key role in shaping the CUB of BCT CP gene: The magnitude of natural selection and mutation pressure in CUB was investigated by constructing a neutrality plot (GC12 vs. GC3) (Fig. 5). The neutrality plot shows a significant positive correlation (r^2 = 0.5314) between the GC1,2s and GC3s values, which indicates selection pressure is dominant over mutation in shaping codon usage bias of CP gene.

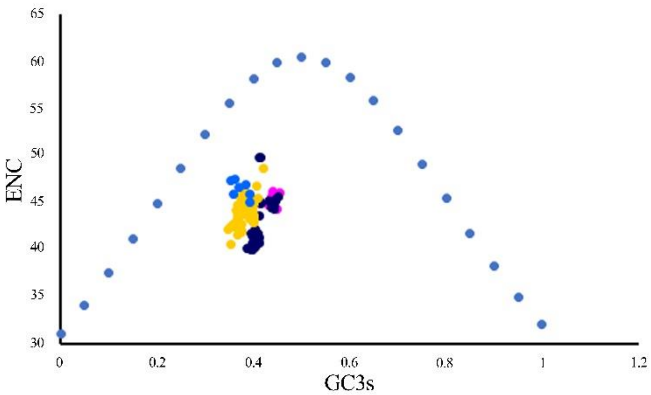


Fig. 4. ENc-GC3 plot analysis of the coat protein (CP) gene sequences of BCT CP gene sequences. The standard curve plotted while using the codon usage bias (calculated by the GC3s composition only) indicated by blue points.

Trends in codon usage variation: To determine the trends in codon usage variation among coding sequences of different beet soil-

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borne virus isolates, we plotted principal axes according to geographical isolation for each virus. Principal component analysis (PCA), combined with the correlation analysis effectively demonstrated the factors influencing codon usage bias (25). A plot of the 1st axis and the 2nd axis of the isolated strains according to the geographical isolation (Fig. 5) were drawn.

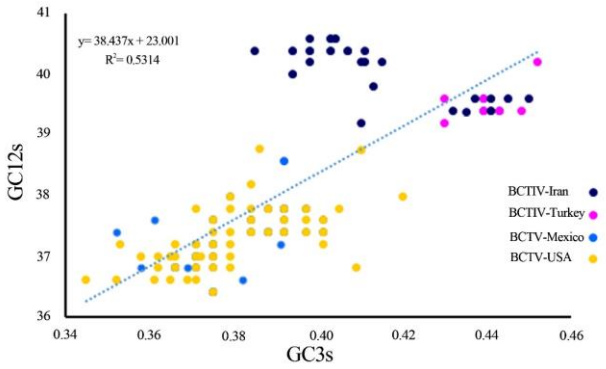


Fig. 5. Neutrality plot analysis (GC12 vs. GC3) for the coat protein sequences of BCT isolates was analyzed based on geographical isolation. GC12 stands for the average value of GC contents at the first and second positions of the codons (GC1 and GC2), while GC3 refers to the GC contents at the third position of the codons. The blue line is the linear regression of GC12 against GC3. Different BCTIV and BCTV isolates are indicated with different color markers Tuykish isolates (Pink), Iranian isolates (Black), USA isolates (Yellow), and Mexican isolates (Blue).

The PCA analysis demonstrated that American and Mexican BCTV clustered near to each other, which showed the common ancestor for these isolates, however, BCTIV separated into two groups (Fig. 6).

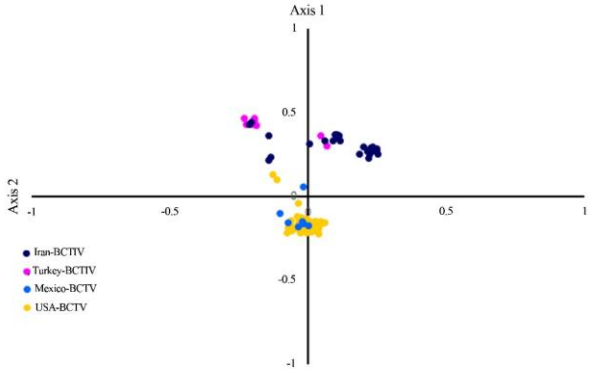


Fig. 6. Correspondence analysis of codon usage patterns in BCT CP sequences based on region of isolation.

Conclusion

Monitoring the occurrence and incidence of BCTIV in vector leafhoppers provides useful information on the potential risk to other economically important hosts including tomato, pepper, bean, spinach, and a number of cucurbit species (26). Although it has been known that *C. haematoceps* transmits BCTV in Iran (27, 28), relatively no work had been done related to its detection in individual insect vectors (29, 30, 31, 32) before this work. In this study, we developed and used a PCR method (30, 33, 34), to detect BCTIV in the individual insect vector body. *C. haematoceps* is a highly polyphagous leafhopper and feeds on a wide range of economically important cultivated plants and weeds. This leafhopper is a widespread insect and has been reported as an important vector of plant pathogens in numerous countries. Our PCR method would be useful in monitoring and detection of BCTV in this important insect vector, and the data regarding viruliferous vectors can be applied in disease forecasting and management. Research on the genetic diversity of viruses provided critical information for understanding virus evolution, geographical origin, virulence variations, and the occurrence of emerging new epidemics. Based on our findings, this study showed that overall codon usage bias within BCT CP gene sequences is slightly biased. The evolution of BCT perhaps reflects a dynamic process of mutation and natural selection to adapt their codon usage to different environments and hosts. This research makes an essential contribution to the understanding of plant virus evolution and reveals novel information about their evolutionary fitness.

Acknowledgment

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Conflict of interest

No conflict of interest is declared.

Funding

None.

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