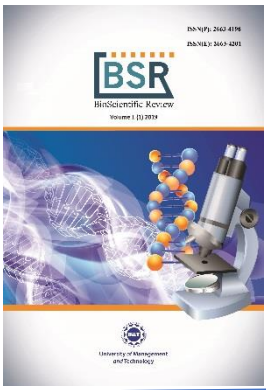

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Molecular Characterization of the Begomovirus Associated Satellites Infecting *Spinacia Oleracea* and *Capsicum Annum* Plants in Kohat, Pakistan

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Abstract

Begomovirus is a major and economically important genus of the *Geminiviridae* family. It comprises a wide range of viruses that infect a number of dicot plants including the horticulture crops, cereal crops, aromatic plants, vegetable crops, medicinal plants and weeds in various regions of the world. This study aims to investigate and correlate the various symptoms of begomovirus / satellites in different plants grown in the vicinity of Kohat, Pakistan. Furthermore, the characterization of the selected virus-associated satellites at the molecular level is also studied. Samples of suspected plants showing begomoviral infection were collected from the Kohat District. Genomic DNA was extracted from the infected plants and subjected to PCR using DNA-1/DNA-2 and Beta01/Beta02 for alpha satellites and beta satellites, respectively. The amplified PCR products were cloned and sequenced commercially. After sequencing, in silico sequence and phylogenetic analysis was also performed. Our study discovered that many plants in the Kohat District display begomovirus and satellite disease symptoms with mild to extreme disease severity. Disease incidence is especially high in okra. Beta satellites were isolated and sequenced from *Spinacia oleracea* and *Capsicum annum* plants and they showed more than 90% sequence similarity with chilli leaf curl and tomato leaf curl beta satellites. The existence of betasatellites in spinach and chilli plants was discovered for the first time in the Kohat region. Moreover, the distribution of these highly pathogenic variants of chilli leaf curl and tomato leaf curl betasatellites in the district Kohat has been reported previously.

Keywords: alphasatellites, begomovirus, betasatellites, geminiviridae, pathogenic variants

1. Introduction

In terms of its economic significance, *Geminiviridae* is the second largest and most important family of plant viruses and it infects the majority of monocotyledonous and dicotyledonous plants on the planet [1]. Gemniviruses are acknowledged as among the most detrimental pathogens of food and other cash crops [2]. These viruses have a circular ssDNA instead of a linear one [3]. They use the rolling circle replication (RCR) mechanism for the replication of the nuclei of the infected host plant cells [4]. It is used as a model for replication and transcription which depends on the machinery of the host DNA.

Begomovirus is the most widespread and economically significant genus in the Geminiviridae family. Bean Golden Mosaic virus and Bean Golden Yellow

Mosaic virus also belong to this family [5]. Begomoviruses have a much wider engulfing ability, destroying the world's most economically valued crops in tropical and sub-tropical areas. Most viruses in this genus are usually transmitted in a persistent, circulatory manner by insect vectorsBegomoviruses are a significant detriment to agricultural productivity in many warmer parts of the world including Pakistan due to a high level of whitefly host species [6]. Previous research showed that begomoviruses only infect dicotyledonous species including bean golden mosaic, cassava mosaic, cotton and tomato leaf curls [7]. Losses of up to several billion dollars a year have been reported [8]. Begomoviruses have emerged as a huge threat to several countries of the world in the form of the reduced productivity in fruits, fibbers, and ornamental crops. These viruses also affect other crops such as tomato, squash, cotton, bean, cucurbits and many more [8, 9, 10, 11].

Begomoviruses of the family *Geminiviridae* are divided into two groups based on their phylogenetic analysis. Viruses from Africa, Asia, Australia, and Europe are known as the old world (OW) viruses, while those from the Americas are known as the new world (NW) viruses [12, 13]. They are also classified as monopartite and bipartite based on their genomic architecture. The genomes of NW begomoviruses are composed of two distinctly encapsulated genomic features referred to as DNA-A or/and DNA-B, which are covalently closed, circular ssDNA molecules with a size of 2.5 to 3.0 kb. [14] Except for a 200-nucleotide fragment of the intergenic region (IR) known as the common region, these components have no sequence similarity [15]. Both molecules share a hairpin stem loop structure with a highly conserved sequence known as nona-nucleotide (TAATATT/AC), which acts as the replication base.

There are 5-6 open reading frames (ORFs) on the complementary sense of the bipartite begomovirus' DNA-A component. There are two ORFs on the virion sense strand which encode coat (CP) and V2 proteins (V2). Sense and complementary strands of DNA-B contains two ORFs encoding movement protein (MP) and nuclear shuttle protein (NSP). OW viruses are distinct from the NW viruses as they lack the V2 gene on DNA-A part of the virion sense strand [16]. This part (DNA-A) also encodes the proteins which are important for the encapsidation and replication of DNA and other key factors responsible for the regulation of plant and viral genes [17]. The other component (DNA-B) is important for the expression of viral proteins needed for the transportation, symptom growth and inter- and intra-cellular movement [18]. In case of the OW bipartite viruses, only DNA is important for systemic infection and movement. However, in most of the NW viruses, both genomic components are necessary for systemic infection and movement [19].

Except for a few bipartite begomoviruses, the majority of the begomoviruses identified from the OW are monopartite, meaning that they lack a DNA-B molecule. Monopartite begomoviruses have a single genomic component that is similar to the bipartite begomoviruses' DNA-A (DNA-A like) molecule. Monopartite genomes, including bipartite begomovirus DNA-A molecules, encode all the necessary proteins for viral DNA replication, transcription, and encapsidation [20]. Monopartite begomoviruses' pre-coat

protein (V2) and coat protein (CP) perform the same transportation activity as the bipartite begomoviruses' DNA-B. Furthermore, the genomes of some monopartite begomoviruses have the power to induce wild type disease symptoms. Among these, tomato yellow leaf curl virus (TYLCV), tomato leaf curl virus (TLCV) and tomato yellow leaf curl Sardina virus (TYLCSV) are the most common [21].

One of the most important satellites associated with the helper begomoviruses is the betasatellite (formerly known as DNA-β). Betasatellite is a true class of satellites that cannot spread without the assistance of a helper virus and plays an important role in disease production [22]. Betasatellites are about 1.4 kb long (approximately half of their helper virus) and belong to the tolecusatellitidae tribe. Encapsidation, transmission by insects, replication, and movement inside the host plants are all dependent on their helper virus. As a result, a helper begomvirus is needed for their survival and spread [12]. Since betasatellites can't make their own Rep protein, they depend on the helper virus's encoded Rep protein [23].

Betasatellites are made of only one βC1 gene that produces the βC1 protein. This multifunctional protein has many functions including acting as the suppressor of the host's defense mechanism and also as a pathogenicity determinant, enhancing virus aggregation, and interacting with a variety of host and virus genes / proteins. Betasatellites have two sections (non-coding) known as satellites typical region (SCR) and A-rich sections in addition to the βC1 coding region. SCR is around 150 nucleotides in length although its function is still unknown. SCR also has a replication origin site for attaching the protein made by the helper begomovirus. As a result, replication begins inside this region by first creating a nick in the replication origin. The other non-coding region is the A-rich region which spans 200 nucleotides and is covered. [14] The aim of this study is to characterize the begomovirus and its associated satellites that cause infections in a variety of crops including medicinal plants, agricultural crops, aromatic plants, herbs, weeds and decorative plants in the Kohat region.

2. Materials and Methods

2.1. Area of Study

Among KP's most important districts is Kohat city. This area is a part of the Kohat Division which is further divided into five districts. There are two tehsils in this district (Kohat and Lachi). Kohat is located at latitudes 72-10 and 72-470 E, and 34-09 and 34-430 N, respectively. The city of Peshawar is situated in the north of Kohat. The hills of Orakzai are situated to the west, while Bannu is situated to the south and the Indus river flows to the east. From the sea level, Kohat is 1768 feet high. However, the elevation from the sea level varies, that is, Jalala Sir is 5,000 feet high in the Cherat range (5,110 feet) and Molaghar is 7,060 feet high. It is a landmark hill in Tira, 12 miles north-west of Kohat. To the west, the Waziri hills are much lower with Kafir Kot, the highest, being only 4,004 feet.

2.2. Observations and Field Survey

Across the Kohat District, field tours and surveys were organized and observations were recorded for the survey variables. Infected plants with symptoms of classical diseases caused by begomoviruses and associated satellites

were considered and closely observed. The survey form included information about the field site, species / varieties of infected plants, disease symptoms, insect vector activity, prevalence and the severity of the disease. The images of the contaminated plants were taken and the photos were stored on a personal laptop.

2.3. Sample Collection

At various locations in the Kohat District, leaves with symptoms were collected. Leaves which showed typical begomoviral infection in the form of leaf curling, vein yellowing, leaf thickening, vein thickening and leaf yellowing were included in the study. The coordinates were registered using a GPS system. Leaves were initially kept in a zip bag and were immediately moved to the Molecular Biology Laboratory of the Center for Biotechnology and Microbiology, University of Swat. They were carefully tagged with a permanent marker through an ice cooler. The samples were stored in a deep freezer until they were used.

2.4. DNA Extraction from the Infected Plants

Plant DNA was extracted using the CTAB method described by Iram et al. [24] with some minor modifications. Powder was made by crushing 1g of leaf tissues through mortar and pestle. Afterwards, 1000µL of CTAB buffer was applied to the powder. Extract was then transferred to the micro-centrifuge tubes (1.5 ml) and incubated for approximately 30-60 minutes at 60℃ in a dry bath. The vortexing of the tubes was performed after 2-3 minutes while incubating. Then, the extract was kept at room temperature and transferred to 1.5 ml tube. After incubation, 600 µL of chloroform and isoamyl alcohol were introduced to the cooling mixture at a ratio of 24:1 and rendered to centrifugation at 13000 rpm for 10 minutes. Then, the supernatant was carefully transferred to a new 1.5 ml tube. Isopropanol (400µl) and ammonium acetate (7.5 M) were added to the supernatant and incubated for at least 1 hour at -20 °C. After ice chilling for 10 minutes, samples were centrifuged at 13000 rpm. Pellet was rinsed with 500 µL of 70% chilled ethanol followed by centrifugation for 2 minutes. The washing cycle was repeated. Pellet was air dried for 15-20 minutes and then dissolved in 50 µL of double distilled water. Finally, DNA was stored at -20℃ until further use.

2.5. DNA Quantification

To evaluate the quality of the genomic DNA, agarose gel (1%) was used and DNA was loaded into the wells with 6x loading dye. DNA was visualized under UV light in the Gel Doc system.

2.6. PCR Amplification

Abutting primer pairs, DNA-1/DNA-2 for the alpha satellites and Beta01/Beta02 for the beta satellites, were used in the PCR reaction. For viruses and satellites, DNA amplification was expected to be 2.8 kb and 1.4 kb, respectively. A total of 20µl PCR reaction containing 0.25U Taq polymerase, 1.6 µlMgCl2, 0.5 µM of each primer, 2 µL dNTPs, 2µL 1X Taq buffer and 1 µL DNA template was carried out in the PCR machine, as shown in Table 1. Reaction conditions included preheat treatment at 94℃ for 2 min with 35 cycles of 94℃ for 2 min, 55℃ for 1 min and 72℃ for 3 min with a final incubation of 72℃ for 10 min.

Table 1. Primers used for Amplification of Begomovirus and its Associated Satellites

Name of primer	Sequence
Beta-01	5'-GGTACCACTACGCTACGCAGCAGCC-3'
Beta-02	5'-GGTACC TAC CCT CCC AGG GGT ACA-3'
DNA-101	5'-CTGCAGATAATGTAGCTTACCAG-3'
DNA-102	5'-CTGCAGATCCTCCACGTGTATAG-3'
Begomo-F	5'-ACGCGTGCCGTGCTGCTGCCCCCATTGTCC-3'
Begomo-R	5'-ACGCGT ATGGGCTGYCGAAGTTSAGACG-3.

2.7. Agarose Gel Electrophoresis for PCR Amplified Products

Agarose gel (1%) was prepared using 1g agarose and 100 ml of TAE buffer. For visualization, ethidium bromide was added to the heated gel. When the gel was dried at room temperature, it was dipped in 1% of the TAE buffer in a gel tray. DNA was mixed with the loading dye and loaded onto the wells with a reference DNA ladder. DNA was separated at 80 volts and visualized with the help of UV trans-illuminator

2.8. Cloning

The amplified PCR products were cloned using the InsTAclonE PCR Cloning Package prepared by Fermentas into a cloning vector named pTZ57RT (Fermentas / Thermo Fisher Scientific) as guided by the manufacturer. Clones were digested with the limiting endonuclease enzymes Apa1, HindIII and BamH1, with their subsequent buffers in compliance with the guidelines of the supplier (Fermentas) to confirm the cloned beta-satellite products for the begomovirus portion. Cloned PCR products were then sent for sequencing (Macrogen Korea). BLAST was used for comparing the sequences with the other begomovirus sequences.

2.9. Sequence and Phylogenetic Analysis

The similarity of our sequences was evaluated by comparing them with related sequences using BLAST. To locate ORFs, ORF Finder and Gene Quest were used. Sequences were eventually sent to the EMBL database. For a full-length sequence study, MEGA6.0 was used. CLUSTAL X program was used for multiple sequence alignment. To construct the phylogenetic tree, MUSCLE and neighbour-joining methods were used. By using Clustal X, MEGA (7.0) and Tree viewer, phylogenetic trees were constructed and the sequences were aligned. To generate the adjacent tree, the aligned sequences present in the database were used with 1000 bootstrap replicates. The neighbor-joining method was used to establish the evolutionary history of the sequenced organisms. The evolutionary distance was calculated using the formula of maximum probability.

3. Results

3.1. Field Survey and Personal Observations

From April 2018 to August 2018, contaminated plants with suspected begomovirus / satellite symptoms were collected from various locations in Kohat. In the selected plants, leaf curling, vein thickening, enations on leaves, chlorosis and yellowing of leaves were observed. The observed symptoms varied from plant to plant as moderate to extreme. To screen symptoms, various regions of Kohat including Lachi, Billitang, Chambai, Shadikhail and

Table 2. Samples Collected in this Study, Showing Typical Symptoms of Begomoviruses/Satelllites from Kohat Region

S. No	Common Names	Botonical Names	Symptoms	Severity	Incidence (%)	Location	Vector	Figure3.1 (Panel)
1	Okra	*Abelmoschus esculentum*	Curling and yellowing of leaves, retarded growth and small fruits,	Severe	75%	33°28°25°N 71°29°36°E	White fly	A
2	Pumpkin	*Cucurbitace* and, C.*moschata*,	Chlorosis (dotted) and leaf curling	Mild	15%	33°29°15°N 71°29°32°E	White fly	B
3	Biter gourd	*Momordica charintia*	Chlorosis, vein related alteration and leaf modification	Mild	30%	33°29°39°N 71°29°49°E	White fly	C
4	Tomato	*Solanum lycopersicum*.	Swelling of veins, chlorosis, and thickening of veins	Severe	60%	33°30°32°N 71°25°39°E	White flies and Aphids	D
5	Spinach	*Spinaciaoleracae*	Leaf yellowing and curling, vein thickening and enations	Severe	70%	33°31°07°N 71°33°32°E	White fly	E
6	Bell pepper	*Capsicum anum*	Curling and yellowing of leaves, vein thickening	Mild	65%	33°28°40°N 71°29°57°E	White fly	F
7	Mentha	*Mentha spicatta*	Halted growth, chlorosis and curling of leaves	Mild	25%	33°30°58°N 71°26°51°E	White fly	G
8	Pea	*Pisumsativum*	Curling of leaves, distortion of veins, thickening of veins and chlorosis	Severe	55%	33°28°50°N 71°25°06°E	White fly	H
9	Bean	*Phaseolus voulgaris*	Leave enation, curling of leaves, and chlorosis	Mild	35%	33°26°15°N 71°31°24°E	White flies and Aphids	I
10	Rose	*Rosa kordesii*	Curling of leaves, leaves yellowing and thickening of veins	Severe	10%	33°31°22°N 71°26°48°E	White flies and Aphids	J
11	Black nightshade	*Solanum negrum*	Reduced growth, curling of leaves, and chlorosis	Severe	20%	33°30°27°N 71°25°37°E	White flies	K
12	Luffa	*Luffa egyptica*	Distortion of veins, enation of leaves and chlorosis	Severe	22%	33°26°10°N 71°31°25°E	White flies	L

Muslimabad were surveyed. The highest disease incident was of okra plant (75%) followed by spinach (70%). Bell pepper plant incidence was 60%. We also discovered the whitefly that acts as a vector for begomoviruses / satellites in the same study area (Table 2).

3.2. PCR Amplification and Sequencing

Universal primers indicated the existence of viral and betasatellite infections in the samples. Using primers against begomovirus and betasatellite, DNA fragments of 2.8 kb and 1.4 kb were amplified. The alphasatellite portion was not amplified. Genomic DNA was used as a template in the PCR, as shown in Figure 1. A. 1.4 kilobase DNA fragment was amplified through PCR. 1Kb DNA ladder shown in line 1 (from the left side) was used as a scale estimate marker, as shown in Figure 2. Afterwards, the amplified betasatellite DNA was commercially sequenced yielding the sequences of 500 nucleotides (partial sequence) and 1378 bp (complete sequence). Sequences were entered into a database and analyzed further. Then, the sequences of our clones were submitted to GenBank under the accession numbers MK737916 and MK862460. The clones were isolated from spinach and chilli plants collected in the Billitang and Duda Sharif regions of Kohat.

3.3. Diversity of Begomoviruses Betasatellites in Kohat

To determine the diversity of betasatellites infecting crops in the Kohat area, we analyzed the isolates of betasatellites with a 1378 bp complete sequence and another 500 bp partial sequence. The sequences were compared to similar sequences already submitted to the NCBI database using BLAST. The tomato leaf curl alphasatellite (ToLCuA) was used as an outgroup. Our betasatellite sequences were very similar to the tomato leaf curl betasatelliites and chilli leaf curl betasatelliites already isolated and sequenced in the other parts of the world, according to BLAST. For the first time, begomovirus components were isolated and sequenced in the Kohat area.

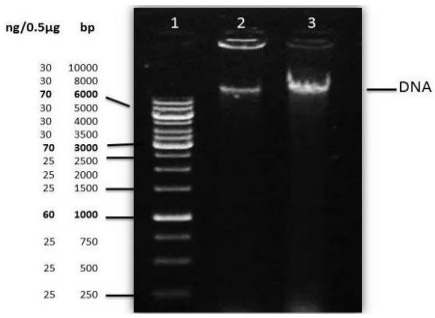

Figure 1. Gel that represents DNA extraction in our study: In this figure, lane 1 represents standard while lane 2 and lane 3 represent the DNA extracted from chili and spinach plants respectively

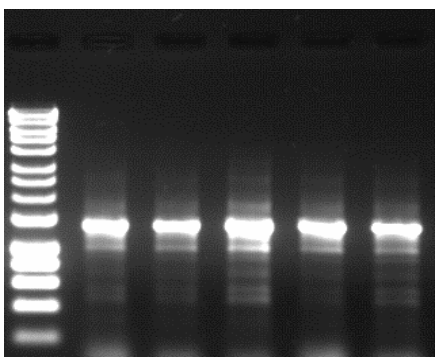

Figure 2. Lane 1 represents a standard 1 Kb DNA ladder, while lanes 2-6 depict PCR amplified items (1.4 Kb) with Beta01/02 universal primers

3.4. Phylogenetic Analysis

With the sequences of 34 closely related species, a phylogenetic tree was

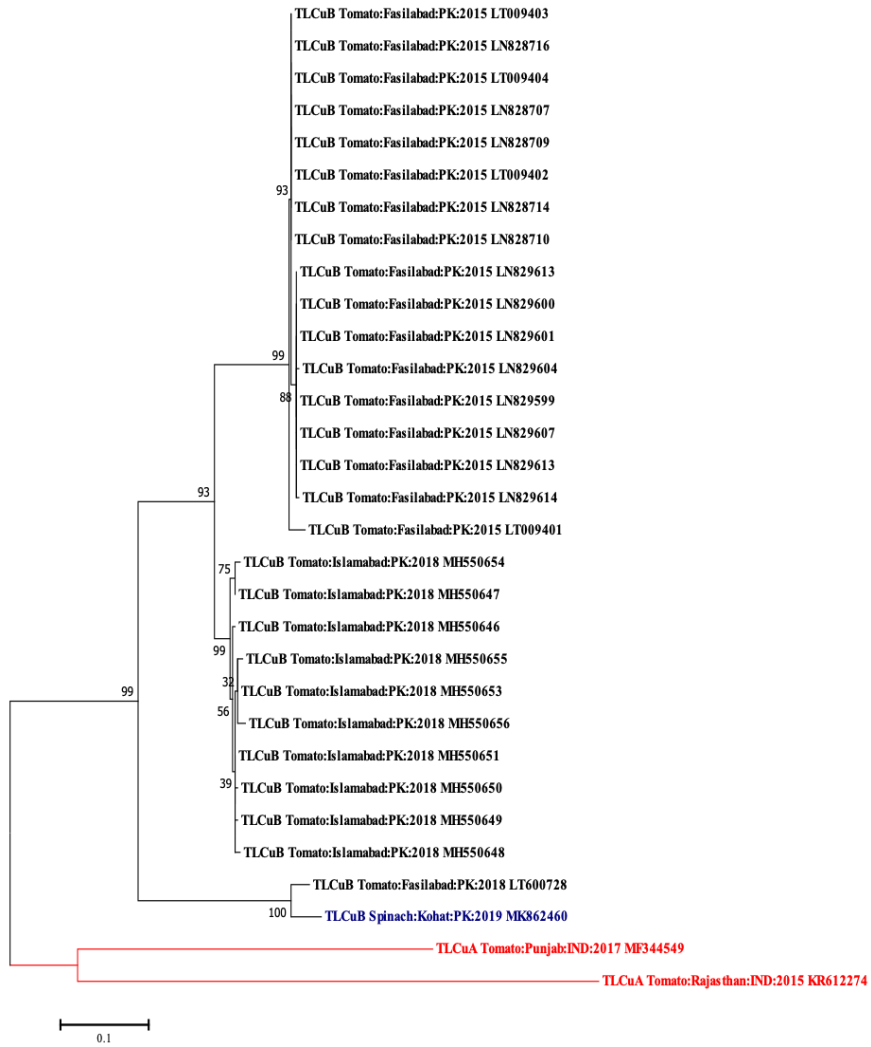

Figure 3. Phylogenetic dendogram generated on the basis of alignment of some genomic sequences of the selected betasatellites

established. The tree was built using a neighbor-joining algorithm that had a high boot strap value (1000). The bootstrap value (100 replicates) for each node value is shown in figures 3 and 4. Outgroups for the betasatellite isolated from the spinach plant included tomato leaf curl alphasatellite (accession no MF344549) and tomato leaf curl alphasatellite (accession no KR612274). We used the cotton leaf curl Multan alphasatellite with the accession no KR816014. The phylogenetic dendogram revealed that the sequences of chilli leaf curl betasatellite and tomato leaf curl betasatellite isolates were mostly separated. It further showed that the betasatellite exhibited the maximum sequence similarity (100%) with that of the tomato leaf curl betasatellite, which

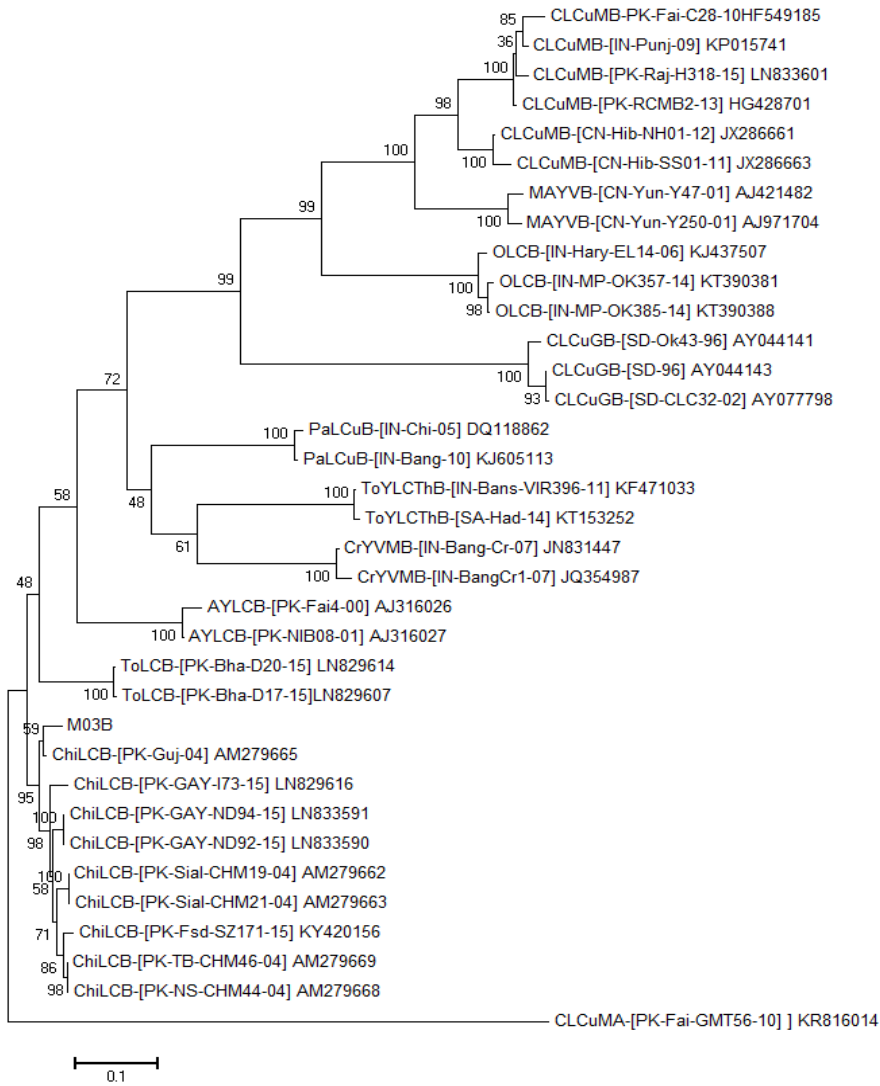

Figure 4. Phylogenetic dendogram generated on the basis of alignment of the 34 begomoviruses betasatellites. For this, we used MUSCLE and MEGA 6.0 softwares

is an isolate of the tomato plant from the Faisalabad area of Punjab, Pakistan. The isolates of the chilli leaf curl betasatellite showed the second highest similarity that is around 93%. Some other tomato leaf curl betasatellites which were isolated from tomato and chilli plants respectively also showed more than 90% sequence similarities.

It was also noted that the sequence with accession no MK737916 carried one ORF which encoded a protein with 29 amino acids. However, the sequence with accession no MK862460 had one ORF that encoded a protein with 120 amino acids. To measure the identity percentage of the newly discovered spinach leaf curl, betasatellite sequences

were compared with other betasattelites in the databank. Spinach and chilli leaf curl alphasatellites revealed the lowest percentage. Tomato leaf curl betasattelites showed the highest nucleotide identity of around 97%, while chilli leaf curl showed 98% identity. Table 3 shows the nucleotide sequences of our clone at the bottom in blue shade.

Table 3. Typical Feature of an Isolated Betasatellite Collected from Chilli and Spinach Plants

Isolate	Sample name (OF-1)
Open reading Frames (ORFs)	1
Codon (Start)	563
Codon (Stop)	201
Size	120 amino acids
Molecular weight (KDa)	13.7

3.5. Chilli Leaf Curl Betasatellite and its Multiple Alignment and the Study of Nucleotide Sequence Variations

Chilli leaf curl was taken and the percentage identity of the difference in the nucleotide sequence was calculated. The sequence deviation in our clone (MK862460) was compared with other related sequences retrieved from the databases. Our cloned sequences had the most variation with chilli betasatellite sequences, followed by the tomato leaf curl betasatellites. Cotton leaf curl betasatellites had the least differences with our cloned sequences.

4. Discussion

In Pakistan, geminiviruses (particularly begomoviruses / satellites) have been extensively studied. However, these studies are restricted to only cotton relevant begomoviruses / satellites in the Punjab and Sindh provinces [25, 26]. Economic conditions, globalization and other parameters have been changing in the world which may cause an increase in the non-cotton host crop infections of the begomoviruses and associated satellites [27]. Okra, pumpkins, bitter gourd, tomato, spinach, bell pepper, mintha, pea, bean, rose, black nightshade, and luffa areamong the plants that exhibit the usual disease symptoms of begomoviruses / satellites. These plants show a wide range of symptoms, from severe to mild. Conditions such as leaf curling, internodal distance and leaf size reduction were observed in chilli plants. In Pakistan and other tropical and subtropical regions of the world, these crops serve as the backbone of the local economies. The chilli infecting begomoviruses are a serious threat in various countries due to the fact that they target several valuable crops such as bitter gourds, tobacco, papaya and potato [11]. The pharmaceutical industries in these countries are also affected when such viruses reduce the productivity of the various medicinal and aromatic plants [7]. In this study, we reported the molecular and symptomatic evidence of begomovirus / satellites in the Kohat area for the first time.

From an economic perspective, the genus begomovirus is responsible for the majority of diseases, with over 100 species [11]. In our study, disease severity was around 10-70% and the majority of the plants infected were okra, tomato, chilli, and spinach plants, among others. The whitefly (*Bemisia tabaci*), which was also seen during the field survey, was responsible for the majority of the begomovirus transmissions. It is the main cause of begomovirus spreading across borders and infecting economically significant plants at any

time [28]. Since there is no published information on begomovirus / satellite incidence in the KP province, we are unable to immediately equate our findings with those of the previous studies. Most of the previous research was based on the characterization of begomoviruses and satellites in Pakistan's Punjab and Sindh provinces. Characterizing the sections of begomovirus in the research area was the second component of our research.

The recent outbreaks of diseases such as chilli and spinach leaf curl diseases in Pakistan have highlighted the need to fully comprehend the diversity and spread of the begomovirus / satellite species in Pakistan. The first betasatellite was discovered in 1997 [24]. Following this finding, a large number of satellites and begomoviruses have been recognized and their respective sequences have been added to the GenBank [29]. It has been found to be the most significant in the field of begomoviruses and satellites. [30].

5. Conclusion

Since interactions among different begomoviruses and satellite DNAs may lead to the emergence of damaging diseases, it is therefore pertinent to monitor begomovirus / satellite complexes infecting field crops and the native vegetation. Leaf sample is an unbiased sampling approach for detecting begomoviruses and associated DNA satellite molecules in different cultivated and non-cultivated crops, providing an important glimpse into the reservoir of the viral genetic diversity. In this study, suspected leaf samples were collected in the Kohat region from which betasatellites infecting chilli and spinach were isolated. More detailed research is needed to better understand the diversity of viruses and satellites in the field, as well as to discover their biodiversity. This study will help in the development of potential control strategies to prevent the spread of the begomoviruses in Pakistan.

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