Acta virologica **60**: 217 – 223, 2016 doi:10.4149/av_2016_03_217

# Molecular characterization of a first begomovirus associated with lentil (*Lens culinaris*) from India

K. NAIMUDDIN, M. AKRAM, A. K. AGNIHOTRI

Division of Crop Protection, ICAR-Indian Institute of Pulses Research, Kalyanpur, Kanpur 208024, India

*Received October 20, 2015; accepted November 11, 2015*

**Summary. –** A disease of lentil with symptoms of distortion, mottling and chlorosis in the leaves, shortening of internodes and excessive branching was noticed in lentil at Kanpur, India, during 2012–2014. Results of polymerase chain reaction and reverse transcriptase polymerase chain reaction employed to detect suspected RNA and DNA viruses indicated involvement of a geminivirus, which was further characterized by sequencing of full genome amplified by rolling circle amplification. Analysis of full length DNA-A revealed 96.4–96.7% nucleotide similarity with bitter gourd yellow vein virus (BGYVV) isolates and tomato leaf curl New Delhi virus (ToLCNDV) isolate. As per the recent revision of begomovirus species demarcation criteria, if a new virus isolate shares ≥91% nt sequence identity with any other isolate of an existing species, it should be treated as an isolate of that species, even if it is <91% identical to all other isolates from that species. This made BGYVV an isolate of ToLCNDV and resulted in the de-recognizing of the BGGYV. Hence, the present virus has been named as a strain of ToLCNDV and designated as Tomato leaf curl New Delhi virus-Lentil-[India:Kanpur:Lentil:2014] with the acronym as ToLCNDV-Lentil-[IN:Knp:Len:14]. This is the first report of a begomovirus found associated with a disease in lentil from India.

**Keywords:** geminivirus; bitter gourd yellow vein virus; tomato leaf curl New Delhi virus; lentil; rolling circle amplification

## Introduction

Lentil is the second most important winter season legume crop of India. It is mainly grown in the northern and central parts of India. Commonly known as "Masoor", it is a good source of protein and it is used in various ways. The crop residue is also reported to serve as a good livestock feed due to low cellulose content (Erskine *et al.,* 1990). Besides many fungal diseases (Chen *et al.,* 2009), a number of viruses are reported to infect lentil in different parts of the world (Bos *et al.,* 1988, Makkouk *et al.,* 2001, Taylor *et al.,* 2007, Abraham *et al.,* 2006). In fact lentil is known to be the host of thirty different virus species belonging to 16 genera from nine families, but bean leaf roll virus (BLRV), bean yellow mosaic virus (BYMV), beet western yellows virus (BWYV), cucumber mosaic virus (CMV), faba bean necrotic yellows virus (FBNYV), pea seed borne mosaic virus (PSbMV), pea enation mosaic virus 1 (PEMV-1) and pea streak virus (PeSV) are most important ones (Kumari *et al*., 2009). Among geminiviruses, chickpea chlorotic dwarf virus and tomato yellow leaf curl virus are known to infect lentil (Kumari *et al*., 2009).

In India, there appears to be very little information on the occurrence of virus diseases of lentil. Pea seed borne virus was detected in germplasms of lentil in 1991 (Varma *et al.,* 1991). A recent publication (Gautam *et al.,* 2013) has indicated association of flexuous rod shaped particles with lentil plants showing symptoms that appeared to be of a virus infection; an unequivocal evidence for the identity of the virus has, however, not been provided.

During winter seasons of 2012–2013 and 2013–2014, we observed some lentil plants with symptoms such as mottling

E-mail: naimk@rediffmail.com; phone: +91 0512 2580994-277.

**Abbreviations:** BGYVV = bitter gourd yellow vein virus; RCA = rolling circle amplification; ToLCNDV = tomato leaf curl New Delhi virus

in the leaves, shortening of internodes leading to stunting of plants and in some cases reddening of leaves at Main Research Farm of the Indian Institute of Pulses Research, Kanpur. These symptoms were suggestive of viral etiology. Regular occurrence of the disease for last two years and lack of information on the viruses infecting lentil in India, made us to attempt identification of the virus/es associated with such symptomatic plants. Present communication reports characterization of the complete genome of a bipartite begomovirus associated with a stunting disease of lentil on the basis of sequence comparison, phylogenetic relationship and recombination with other closely related viruses.

## Materials and Methods

*Samples.* Twenty five samples of lentil plants (15 during 2012–2013 and 10 during 2013–2014) showing symptoms of viral etiology such as mottling, yellowing and overall growth reduction were collected form fields of the Indian Institute of Pulses Research, Kanpur, named as Ln1 to Ln25 and used for mechanical sap inoculations. Part of each sample was also subjected to polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) to detect gemini-, tospo-, poty- and luteo-viruses and DNA Beta satellite using the primers either described earlier or designed for this study (Table 1).

*Nucleic acid isolation and PCR.* Leaves of the twenty five symptomatic samples (Ln1-Ln25) of lentil were taken for RNA and DNA isolation. Total RNA and DNA were extracted using RNeasy plant mini kit and DNeasy plant mini kit (Qiagen Inc., USA), respectively, following the manufacturer's instructions and were used as templates in RT-PCR and PCR. RNA and DNA from one healthy sample were also extracted and used as negative controls. RT-PCR and PCR were used to detect suspected RNA and DNA viruses. Detection of bean leaf roll virus (BLRV, a luteovirus), groundnut bud necrosis virus (GBNV, a tospovirus), chickpea chlorotic stunt virus (CCSV, a polerovirus) and potyviruses was attempted by using the primer pairs BLRVcpfF/ BLRVcpfR, RKJ5/RKJ6, CCSV-CPF/ CCSV-CPR and Potyvirid1/ Potyvirid2, respectively, by TITANIUM One Step RT-PCR kit (Clontech Laboratories Inc., USA). Amplification was performed in an automated Thermocycler (Biometra, Germany) or Mastercycler ProS (Eppendorf AG, Germany) programmed for one cycle of 50°C for 30 min for cDNA synthesis, 5 min initial denaturation at 94°C and 35 cycles involving 30 sec of denatura-

**Table 1. Primer names, nucleotide sequences, annealing temperatures and expected amplicon sizes of primer pairs used in reverse transcriptase-PCR and PCR tests**

| Primer ID | Sequence (5'-3') | Annealing temperature (°C) | Target virus | Expected size of amplicon | Reference/remark |
|---|---|---|---|---|---|
| BLRVcpfF<br>BLRVcpfR | AAGTTCTTAGCTGCGTTTGTG<br>CGCCAGTATAAACGATGAATC | 55 | BLRV | 800 bp | Primers were designed to target coat protein gene of BLRV- AF441393. |
| RKJ5<br>RKJ6 | ATGTCTCGCTTDTCTAAHGTB<br>TTATATTTCAAGAAGATTATC | 48 | GBNV | 900 bp | Akram *et al.* (2004) |
| CCSV-CPF<br>CCSV-CPR | ATGAATACGGTCGTGGTTAGA<br>CTATTTCGGATTCTGAGTTTTG | 54 | CCSV | 800 bp | Primers were designed to target coat protein gene of CCSV- AY956384. |
| CCDV-CPF<br>CCDV-CPR | ATGTCAACTGTGACGTGGG<br>TTATTGATTGCCAACAGACTTG | 54 | CCDV | | Primers were designed to target coat protein gene of CCDV-KC172664. |
| FbLCV-CPF<br>FbLCV-CPR | ATGCCGAAGCGACAAGCAG<br>TTATTTGTGACCGAATCGTAG | 54 | FbLCV | 800 bp | Naimuddin *et al.* (2014) |
| DengA<br>DengB | TAATATTACC(GT)G(AT)G(AGC)CCGC)C<br>GAC(CT)TT(AG)CA(AT)GG(GCT)CCTTCACA | 56 | Geminiviruses | ~500 bp | Deng *et al.* (1994) |
| Potyvirid primer1<br>Potyvirid primer2 | CAC GGA TCC CGG $G(T)_{17}$VGC<br>ACCACAGGATCCGGBAAYAAYAGYGGDCARCC | 60 | Potyviruses | 1600-2100 bp | Briddon *et al.* (2002) |
| Bt01F<br>Bt02R | AGCCTTAGCTACGCCGGAGC<br>GCTGCGTAGCGTAGAGGTTT | 50 | Beta satellite DNA | 1400 bp | Gibbs and Mackenzie (1997) |
| LNA-F<br>LNA-R | CTTCCTTCGACAGAGTTCCT<br>ATGTTGTATATGGCCTGTACT | 56 | ToLCNDV (DNA-A) | 1000 bp | Primers were designed from the DNA-A of ToLCNDV-Lentil (KM190927). |
| LNB-F<br>LNB-R | CAAATGATGCTGCGGAGGT<br>AGTGATGTCTGTGGTTGTA | 55 | ToLCNDV (DNA-B) | 1000 bp | Primers were designed from the DNA-B of ToLCNDV-Lentil (KM190928). |

BLRV = bean leaf roll virus, GBNV = groundnut bud necrosis virus, CCSV = chickpea chlorotic stunt virus, CCDV = chickpea chlorotic dwarf virus, FbLCV = French bean leaf curl virus, ToLCNDV = tomato leaf curl New Delhi virus-Lentil.

tion at 94°C, 1–2 min annealing at the primer defined temperature (Table 1), 1 min for extension at 68°C, followed by one cycle of final extension for 10 min at 68°C. The total volume of the RT-PCR mix was 50 µl for each reaction.

The primer pairs CCDV-CPF/CCDV-CPR, Bt01F/Bt02R, FbLCV-CPF/FbLCV-CPR, LNA-F/LNA-R, LNB-F/LNB-R and degenerate primer pair DengA/DengB were used to amplify the targeted DNA fragments of chickpea chlorotic dwarf virus (CCDV), beta satellite DNA molecule, French bean leaf curl virus (FbCLV), DNA-A and DNA-B of the begomovirus infecting lentil (present study) and geminiviruses, respectively using Green Taq PCR 2X Master mix (Fermentas, Lithuania) according to the manufacturer's instructions. PCR involved one step of initial denaturation for 2.5 min, and 30 cycles of denaturation for 45 sec at 94°C, 1 min annealing at the primer defined temperature (Table 1) and 1 min extension at 72°C followed by a one-step final extension for 10 min at 72°C. The total volume of the PCR mix was 25 µl for each reaction. RT-PCR and PCR products were analyzed by electrophoresis in 1% agarose gel at 60V for 1 hr and stained with ethidium bromide.

*Rolling circle amplification and restriction digestion.* The DNA from one of the symptomatic lentil plant samples (Ln1) which were positive in PCR with Deng A and Deng B primers was subjected to rolling circle amplification (RCA) using REPLI-g mini kit (QIAGEN) following the manufacturer's instructions. The RCA products were digested with restriction enzymes (*Bgl*II, *Drd*I, *Eco*RV, *Eco*RI and *Hind*III) to obtain linearized ~2.7 kb DNA fragments. The restriction digested RCA product was observed in 1% agarose gel to select ~2.7 kb linear DNA. The ~2.7 kb linear DNA fragments obtained from Ln1 sample were purified from the gel using Sure extract spin PCR clean-up/gel extraction kit (Puregene Genetix Brand, India), cloned into pJET/1.2 blunt vector using CloneJET PCR cloning kit (Fermentas) and sequenced commercially (Ist BASE, Malaysia) by primer walking.

*Sequence analysis.* Sequence of DNA molecules of ~2.7 kb released by the restriction digestion of RCA product of Ln1 were assembled with the aid of Bio edit (version 7.2) and subjected to blastn tool against the sequences available at NCBI GenBank data base (www.ncbi.nlm.nih) and to SDTv1.2 software (http://web.cbio.uct.ac.za/SDT) to establish its identity. The open reading frames (ORFs) were determined by subjecting the sequences to the ORF finder available at NCBI site (http://www.ncbi.nlm.nih.Gov/gorf / gorf.html). For phylogenetic relationship, the MEGA6 software was used with bootstrap (1000 replicates) and all positions containing gaps and missing data were eliminated (Tamura *et al.*, 2013). Total seventy seven sequences (full length DNA-A) which include 74 isolates of ToLCNDV (listed at one of the ICTV files- http://talk.ictvonline.org/ictv_wikis/m/files_gemini/5120.aspx) and one isolate each of ToLCNDV2, ToLCNDV3 and ToLCNDV4 were used to analyze phylogenetic relationship.

## Results and Discussion

### *Symptoms and transmission*

Affected plants showed some degree of distortion in the leaves, shortening of internodes and excessive branching leading to stunted appearance of the plant (Fig. 1a). Chlorosis and reddening in the leaves were also noticed. None of the sap inoculated plants of the lentil developed any symptom indicating that the causal virus was not sap transmissible through mechanical sap inoculation from field affected plants to healthy plants of lentil.

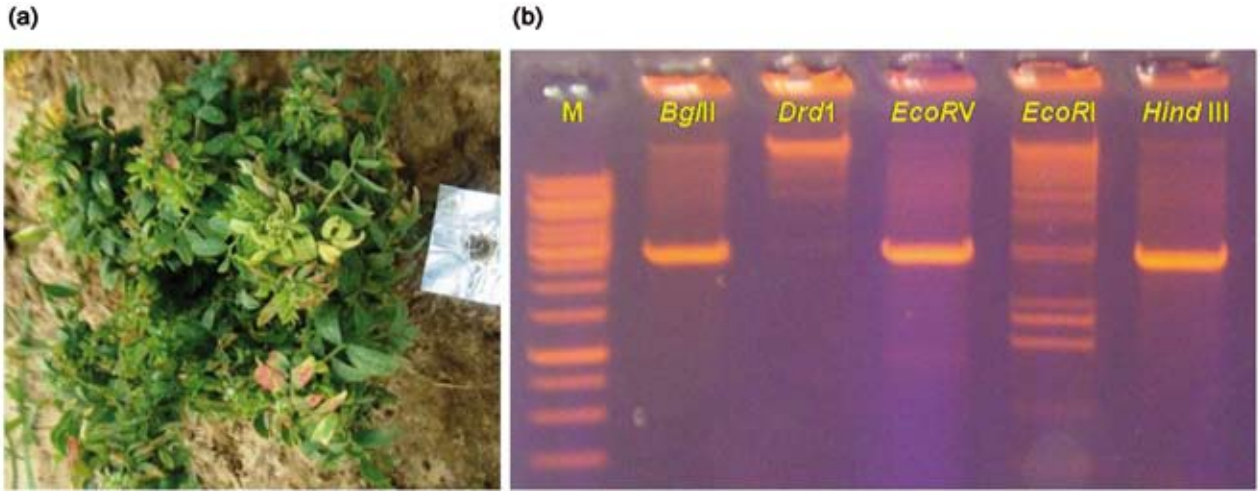



**Fig. 1**

**Field affected lentil plant showing stunting (a), gel photograph of restriction digested rolling circle amplification products (b), (M = 1kb DNA ladder, *Bgl*II, *Eco*RV and *Hind*IIIb gave ~2.7 kb DNA fragments)**

*Detection of the suspected viruses*

Results of RT-PCR using primers targeting potyviruses, BLRV, CCSV and GBNV were negative, indicating that the field affected lentil samples did not carry any of these viruses. In PCR, Deng A and Deng B primers amplified the product of expected size i.e. ~530 bp in seven (Ln1, Ln2, Ln9, Ln12, Ln12, Ln22 and Ln25) of the 25 samples tested. None of the samples gave positive amplification with primer pairs CCDV-CPF/CCDV-CPR, Bt01F/Bt02R and FbLCV-CPF/FbLCV-CPR. Of all the 25 samples subjected to PCR with primer pairs LNA-F/LNA-R and LNB-F/LNB-R designed using the sequences of DNA-A (KM190927) and DNA-B (KM190928) of the begomovirus infecting lentil, only the samples positive with Deng A and Deng B primers revealed presence of DNA bands of expected size (~1000 bp). This confirmed that these samples carried the same begomovirus.

*Rolling circle amplification and restriction digestion*

The RCA product of Ln1 viewed in 1% agarose gel showed presence of high molecular weight DNA indicating amplification of single stranded circular DNA molecules present in the sample. Restriction digestion of RCA product with the *Eco*RI resulted in the release of multiple bands. The *Drd*I did not release any band and the *Bgl*II, *Eco*RV and *Hind*III released similar bands of ~2.7 kb (Fig. 1b). Linearized ~2.7 kb DNA fragments were further pursued and successfully cloned, named Ln1A (digested with *Bgl*II), Ln1B (digested with *Eco*RV) and Ln1C (digested with *Hind*III) and sequenced.

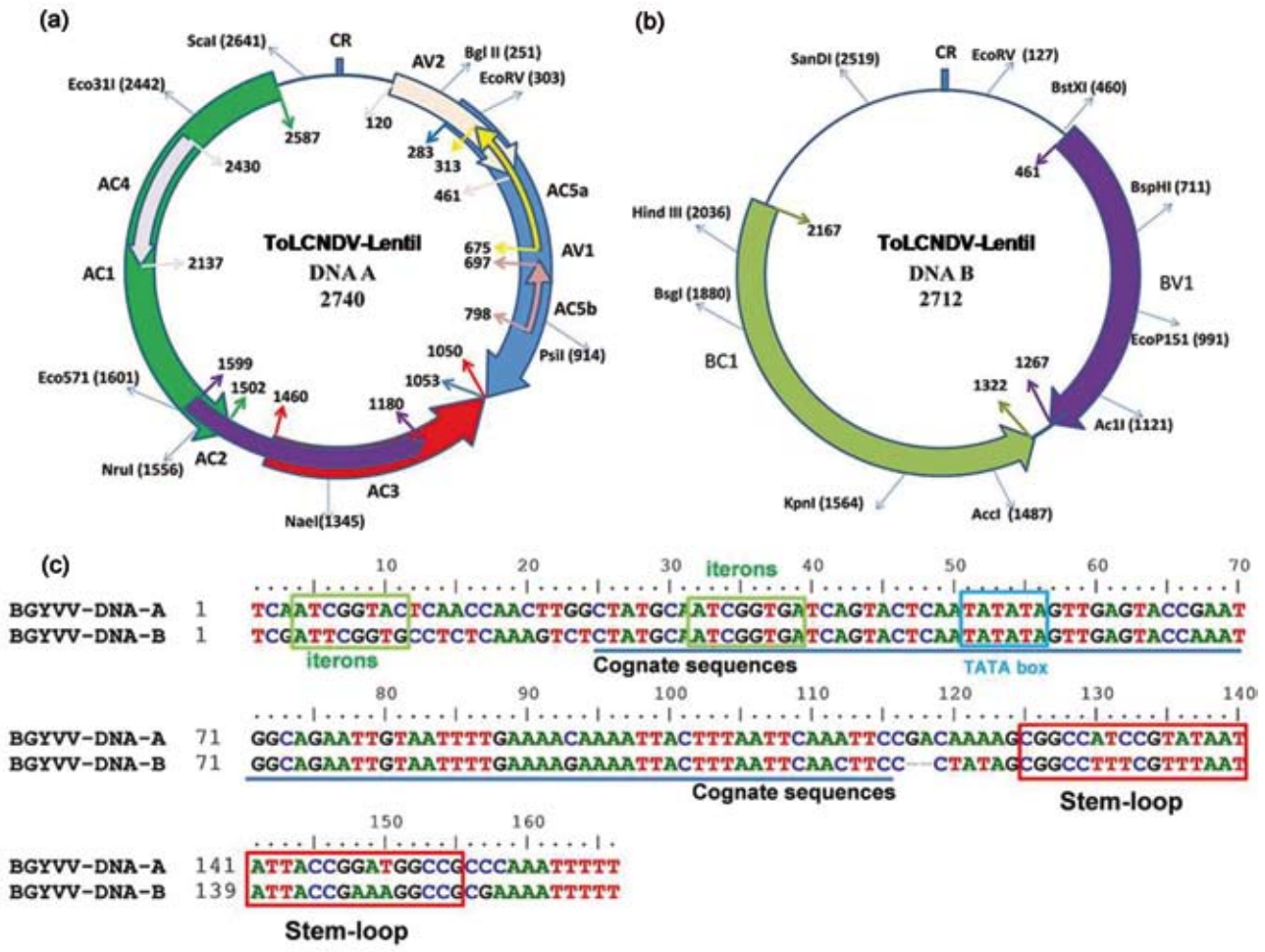



**Fig. 2**

**Genome map of tomato leaf curl New Delhi virus-Lentil (ToLCNDV-Lentil) DNA-A (a), DNA-B (b), alignment of common region sequences of DNA-A and DNA-B of ToLCNDV-Lentil, iterons, TATA box and stem-loop are shown with different color boxes and cognate sequences are underlined (c).**

*Sequence analysis and characterization of genome*

Sequences of Ln1B and Ln1C were identical and hence only one (Ln1B) was used. Ln1A and Ln1B were found to be 2740 and 2712 nucleotides long and have been submitted to the NCBI database under the Acc. Nos. KM190927 and KM190928, respectively. These sequences subjected to similarity searches using BLAST program available at the NCBI site revealed that Ln1A has 96.6% similarity with the sequence Acc. No. FN645905 of tomato leaf curl New Delhi virus (ToLCNDV) (Zaffalon *et al*., 2012) and 96.2% with bitter gourd yellow vein virus (BGYVV-AM491590). Ln1A has 96.3% similarity with another sequence KJ862841, available in data base under the name of BGYVV from Bangladesh. Interestingly, Tahir *et al.* (2010) while analyzing the complete nt sequence of DNA-A (AM491590) of the begomovirus infecting bitter gourd (*Momordica charantia*), found it to have 94.4% similarity with ToLCNDV (FN645905) and named it along with latter as BGYVV. The existence of BGYVV as a distinct begomovirus species has however been questioned and deleted from the list of begomovirus species since it had 91% nt identity with ToLCNDV (AM747291) (http://talk.ictvonline.org/files/proposals/taxonomy_proposals_plant1/m/plant01/5307.aspx).

Ln1A sequence along with the ToLCNDV isolates listed at ICTV website (http://talk.ictvonline.org/ictv_wikis/m/files_gemini/5120.aspx) and newly created begomovirus species ToLCNDV2, ToLCNDV3, ToLCNDV4 (Brown *et al*., 2015) subjected to SDTv1.2 (Muhir *et al*., 2014) revealed that it had 96.4–96.7% nt similarity with ToLCNDV (FN645905) and BGYYV (AM491590 and KJ862841) and 90.5% nt similarity with an isolate of ToLCNDV (AM747291) (supplementary Fig. 1 and Table 1). Following the guidelines given by Brown *et al.* (2015), the pair wise nt similarity of 90.5% is rounded to the nearest full percentile i.e. 91%. Hence, the present virus (Ln1A and Ln1B) has been named as tomato leaf curl New Delhi virus-Lentil-[India:Kanpur:Lentil:2014] with the acronym ToLCNDV-Lentil-[IN:Knp:Len:14].

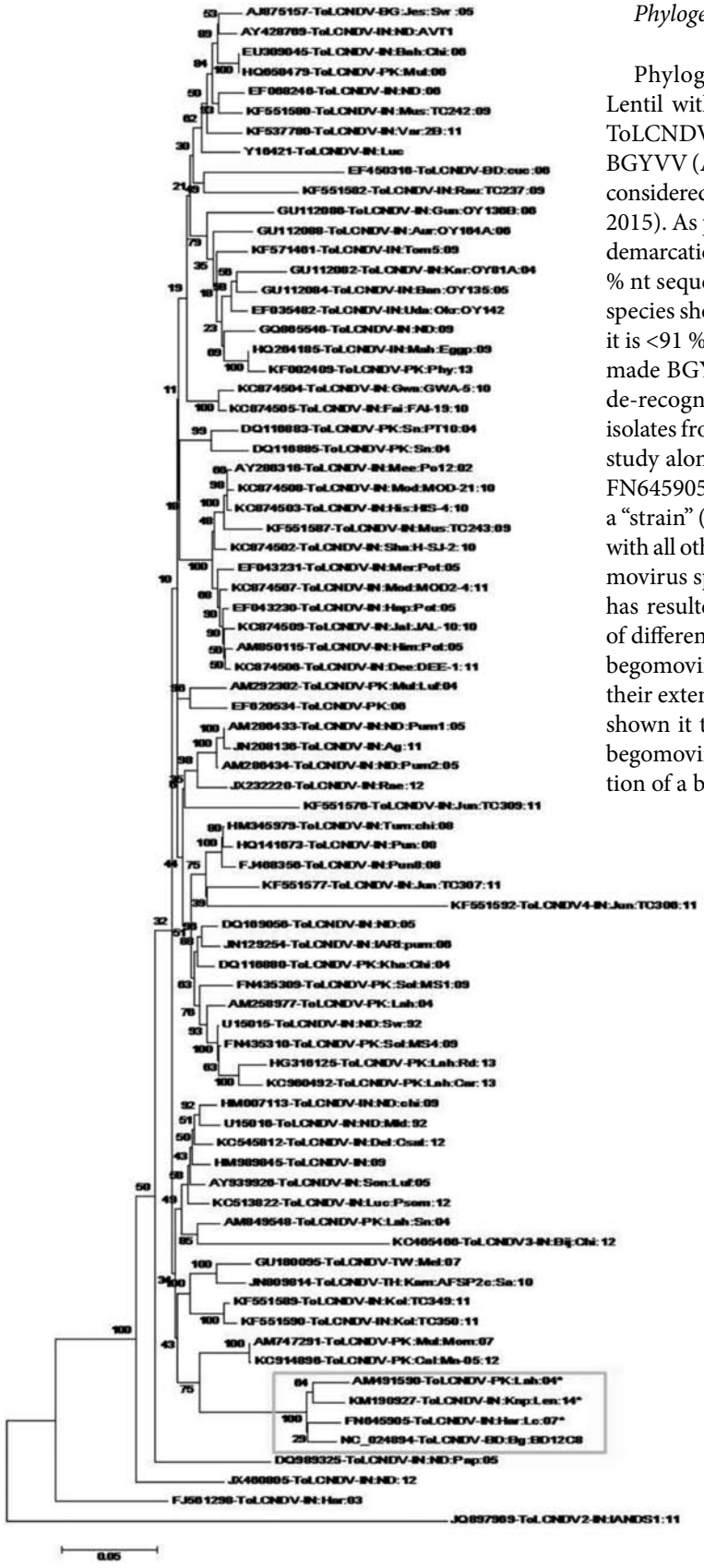
The ToLCNDV-Lentil has a genome organization typical of DNA-A of old world begomoviruses with two open reading frames (ORFs) [AV1 and AV2] in positive or virion sense and five ORFs (AC1, AC2, AC3, AC4 and AC5) in negative or complementary sense encoding for proteins having putative conserved domains of Gemini super family (Fig. 2a). One additional ORF, V3 in virion sense as reported in some begomoviruses (FN645905, AM292302, FN435310) was also found. However, the protein encoded by this ORF has no putative conserved domain. ORFs are separated by an inter-genic region (IR) which contains all the features of begomoviruses such as a putative stem loop structure with the conserved nonanucleotide sequence (TAATATTAC), iteron sequences, ATCGGTA at nt 2601–2607 and ATCGGTG at nt 2629–2635 upstream to the 5' side of the TATA motif at nt 2648–2651.

The DNA-B of ToLCNDV-Lentil was 2712 nt long and has a genome organization typical of DNA-B of begomoviruses with two ORFs, BV1 in virion sense and BC1 in complementary sense and two intergenic regions-large and small (Fig. 2b). The large IR has a putative stem loop structure with the conserved nonanucleotide sequence, iteron sequences, AATCGGTG at nt 2575–2582 and ATTCGGTG at nt 2603–2610 upstream to the 5' side of the TATA motif at nt 2622–2625. The DNA-B showed highest nt similarity (92.4%) with a sequence available at NCBI data base as DNA-B of BGYVV (KJ862842) which has been merged with ToLCNDV.

*Comparison of ORFs sequences*

The ORFs AV1, AC1, AC2, AC3 of ToLCNDV-Lentil were 771, 1086, 420, 411 nt long respectively and encoded protein products consisting of 256, 361, 139, 136 animo acids respectively, similar to the corresponding ORFs in all the viruses used for comparison (Fig. 2a). The ORFs AC4 and AC5 however, presented some interesting comparisons. The AC4 ORF was 294 nt long and coded a putative protein of 97 aa similar to that in ToLCNDV (FN645905) and BGYVV (KJ862841), but it was only 177 nt long and encoded a product of 58 aa in all other viruses used for comparison (data not shown) except BGYVV (AM491590) in which it is truncated being only 126 nt long and encoding a protein with only 41 aa (Tahir *et al.,* 2010).

The AC5 ORF in ToLCNDV-Lentil was present but split in two parts *viz*. AC5a and AC5b which were 363 and 120 nt long encoding for the proteins consisting of 120 and 33 aa, respectively similar to the corresponding ORF in isolates of BGYVV (AM491590, KJ862841) and ToLCNDV (AM286434, KC914896). ToLCNDV isolate (FN645905) however, differed from other isolates of BGYVV including ToLCNDV-Lentil in having AC5a of only 225 nt (74 aa) long. The AC5 ORF is not present in all the begomoviruses but in some cases such as many isolates of ToLCNDV (KC513822, DQ116880 and KC874503) where it is present, it encodes for a product of 161 aa. AC5a of ToLCNDV-Lentil is located at nt 313–675 and AC5b at nt 697–798 of the DNA-A. A look into the sequence between 798–313 revealed presence of a stop codon TTA (complementary of TAA) after 696$^{th}$ nt. Changing of this stop codon into a functional one yielded a product of 161 aa (nt 798–313) much like the AC5 product of ToLCNDV isolates (KC13822, DQ116880, KC874503, HM007120 and AM292302). Presence of a stop codon in the AC5 resulting in its splitting into two parts might be caused by PCR amplification or due to a natural mutation. However, since the splitting of AC5 is present in BGYVV isolates from Bangladesh and Pakistan and in many isolates of ToLCNDV, it is more likely to have been effected due to a naturally occurring mutation during the course of evolution rather than caused by PCR.




*Phylogenetic relationship and recombination analysis*

Phylogenetic relationship of DNA-A of ToLCNDV-Lentil with ToLCNDV isolates and three more species of ToLCNDV 2, 3 and 4 showed it to cluster with isolates of BGYVV (AM491590 and KJ862841) (Fig. 3). BGYVV is now considered as isolate of ToLCNDV (FN645905) (Brown *et al.*, 2015). As per the revised guidelines of begomovirus species demarcation by Brown *et al.* (2015), a new isolate sharing ≥91 % nt sequence identity with any other isolate of an existing species should be treated as an isolate of that species, even if it is <91 % identical to all other isolates of that species. This made BGYVV an isolate of ToLCNDV and resulted in the de-recognizing of the BGGYV. Therefore, the begomovirus isolates from lentil (KM190927) characterized in the present study along with three isolates (AM491590, KJ862841 and FN645905) of ToLCNDV should be treated as members of a "strain" (of ToLCNDV) because they had <94 nt similarity with all other known isolates of ToLCNDV. Revision of begomovirus species demarcation criterion (Brown *et al.*, 2015) has resulted in the regrouping/renaming of many isolates of different species. BGYVV (Tahir *et al.,* 2010) is one such begomovirus species which Brown *et al.* (2015) based on their extensive analysis using different statistical tools, have shown it to be an isolate of ToLCNDV rather than a new begomovirus species. This is the first report of the association of a begomovirus with lentil from India.

**Fig. 3**

**Phylogenetic relationships between tomato leaf curl New Delhi virus (ToLCNDV) isolates and three new species (ToLCNDV2, ToLCNDV3 and ToLCNDV4) based on alignment of full length DNA-A**

The evolutionary history was inferred using the Neighbor-Joining method with bootstrap test (1000 replicates). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 77 nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. There was a total of 2595 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

**Supplementary information** is available in the online version of the paper.

**Acknowledgements.** We thank Council of Science and Technology, Uttar Pradesh for providing financial assistance. We also thank Dr. N.P. Singh, Director, Indian Institute of Pulses Research, Kanpur for providing necessary support and constant encouragement.

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