



## Original Research

### Molecular characterization of Chickpea chlorotic dwarf virus strain D in Chickpea (*Cicer arietinum*) from District Dera Ismail Khan Khyber Pakhtunkhwa, Pakistan

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**Abstract:** Chickpea chlorotic dwarf virus (CpCDV), a member of genus *Mastrevirus* (family *Geminiviridae*) is an important viral pathogen of chickpea and other legume crops in Middle East, North Africa, India and Pakistan. Among sixteen known strains of CpCDV three are known to infect legume crops in Punjab province of Pakistan. In this study diversity of CpCDV was explored in Khyber Pakhtunkhwa (KP) province of Pakistan. In year 2016, during a survey in the chickpea growing areas of district Dera Ismail Khan, CpCDV infected plants were identified. Leaf samples were collected, and a diagnostic PCR confirmed mastrevirus infection in 4 out of 100 samples. From these samples full-length genome of CpCDV was amplified using specific back-to-back primers. Virus molecules were sequenced to their entirety and sequence analysis of a molecule KRF4 (GenBank accession # KY952837) showed the highest pair wise sequence identity of 97% with a CpCDV molecule (KM229787) isolated from chickpea plant. An SDT analysis revealed it to be the D strain of CpCDV and a recombination detection program (RDP) showed it to be a recombinant between C (KM229780) and L (KT634301) strains of CpCDV. Thus, further supporting the intra-species recombination for CpCDV and presence of the same strain in chickpea growing areas of Pakistan other than Punjab province. This is the first identification of CpCDV (genus *Mastrevirus* family *Geminiviridae*) from chickpea (*Cicer arietinum*) plants in District Dera Ismail Khan, KP province, Pakistan.

**Key words:** Chickpea chlorotic dwarf virus (CpCDV); *Mastrevirus*; *Geminiviridae*; Chickpea; KP.

## Introduction

Chickpea (*Cicer arietinum*), mainly used as a leguminous pulse, is a good source of dietary protein. This crop is reported to be infected by *Mastreviruses*, a diverse genus of family *Geminiviridae* having species with single stranded circular DNA genome of size about 2.6 kb and transmitted by leafhopper. These species infect both monocotyledonous and dicotyledonous plants (1-3). The mastreviruses which infect monocot plants are mostly found in Africa however there are some reports in other countries which are Miscanthus streak virus (MiSV) isolate from Japan (4), Digitaria streak virus (DSV) isolate from Vanuatu (5), Wheat dwarf virus (WDV) isolate from Europe (6), Barley dwarf virus (BDV) originate from Middle East (7), Oat dwarf virus (ODV) isolate from Asia (8) and Chloris striate mosaic virus (CSMV) originate from Australia (9). Six species of mastreviruses, which infect dicotyledonous plants, have also been reported from different countries (10).

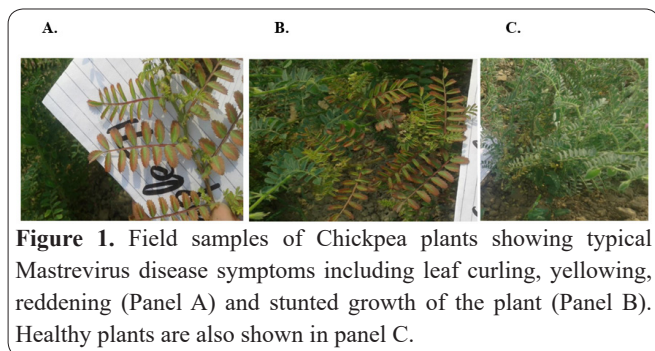
Chickpea chlorotic dwarf virus (CpCDV), a member of *Mastrevirus* genus is economically important viral pathogens infecting chickpea and many other non-legume crops. It was firstly identified in chickpea from India and later it has been reported from other countries i.e. Pakistan, Oman, Iran, Morocco, Turkey, India, Su-

dan and Egypt (10). Currently sixteen strains (A-P) of CpCDV infecting dicots plants have been recognized. CpCDV causes yellowing, reddening, stunting and a reduction in seed production (10). A large number of CpCDV molecules have been isolated and sequenced from the Sudan showed an extensive amount of DNA exchange thus proving an extensive level of recombination both at inter- and intra-species level (11). CpCDV has been isolated from the chickpea plants in Punjab province of Pakistan. This virus has also been reported from many other legume and non-legume crops including the cotton; an economically most important crop of Pakistan (12, 13). This huge diversity of CpCDV at molecular level and presence in such diverse hosts is making this virus very interesting and important for the researchers. This study was planned to further elaborate the presence of CpCDV in chickpea growing areas of country other than Punjab province of Pakistan.

## Materials and Methods

### Sample collection and DNA isolation

In March 2016, leaf samples from the symptomatic chickpea plants with yellowing, reddening, stunting and curling (Fig.1, Panel A & B) were collected from District Dera Ismail Khan, KP province of Pakistan. Total



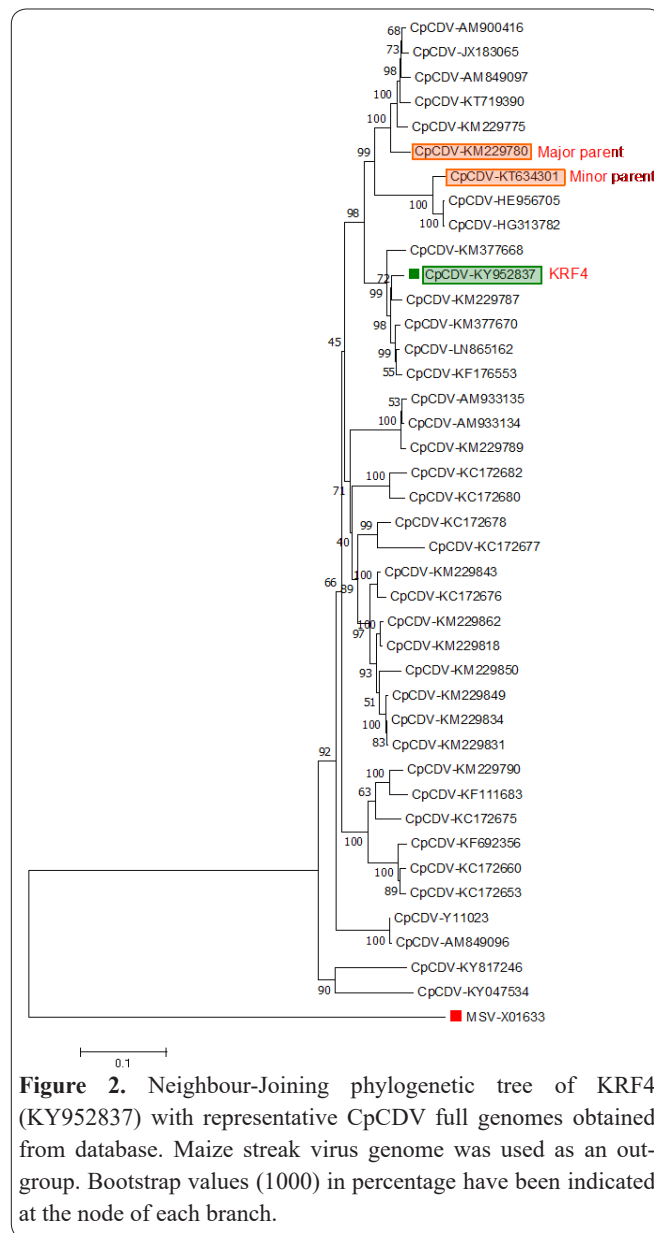
genomic DNA was extracted using the CTAB method and circular viral genomes were amplified by rolling circular amplification using Phi29 DNA polymerase as described by Akram *et al.*, 2017 (14).

**Amplification of virus molecules**

CpCDV genomes were diagnosed using primer CCDV6-F (5'-TAAAGGCGCACTAATGGGTAGACCGTAGA-3') and ChpUni-R (5'-GAAGTACTCGGAATAACCATTACATA-3') (13). After sequencing of the diagnostic PCR product, primers were designed, and full-length virus molecules were amplified by PCR using back-to-back primers CCDVK-F (5'-GAATTCCCAGGGATCCTCT-3') and CCDVK-R (5'-GAATTCGTATTCACAGGAGG-3'). The full-length genome of approximately 2.6 Kb was amplified, purified and ligated into pTZ57R/T vector using TA cloning kit. The ligated products were cloned into Escherichia coli strain Top10. Clones were confirmed by restriction analysis using EcoRI endonuclease enzyme. Confirmed clones were sequenced through primer walking (Macrogen Inc., Korea). Contigs were assembled using Lasergene package (DNASTAR and Seqman).

**Sequence analysis**

Nucleotide sequences were subjected to Basic local alignment search tool (BLAST) for identification of clones. Multiple sequence alignments were performed; phylogenetic tree was constructed using MEGA7 and sequence pairwise identity was identified using Clustal W algorithm in MegAlign software (DNA star). Species Demarcation Tool (SDT) (15) was used to determine the pair-wise sequence identity for our clones and the other mastreviruses. Recombination pattern for the CpCDV molecules cloned during this study was determined using the RDP4 (16). Every possible CpCDV sequence was used to determine the recombination pattern for the isolate cloned from KP, Pakistan. A recombination pattern was accepted as a reliable one that was supported by at least two methods out of the seven methods (RDP, Siscan, GENECONV, Maxchi, Bootscan, 3Seq and Chimera) of RDP4.



**Figure 2.** Neighbour-Joining phylogenetic tree of KRF4 (KY952837) with representative CpCDV full genomes obtained from database. Maize streak virus genome was used as an out-group. Bootstrap values (1000) in percentage have been indicated at the node of each branch.

**Results and Discussion**

Chickpea crop in the District Dera Ismail Khan, KP, Pakistan showed leaf yellowing and stunted growth. These symptoms were attributed to be the CpCDV infection. Diagnostic PCR performed on the DNA extracted from the infected leaf samples was positive for 4 out of 100 DNA samples. Full-length virus molecules amplified from these samples using specific back-to-back primers was of the same size (~2.6kb) of the CpCDV as expected. The sequence analysis of a clone KRF4 showed a typical mastrevirus genome having four open reading frames (ORFs) coat protein (CP), movement protein (MP), replication associated protein (RepA) and full-length replication associated protein (Rep) (Table 1). We have also observed non-nucleotides sequence TAATGTTAC, which is conserved

**Table 1.** Positions and coding capacity of predicted genes for the KR4 virus isolated from chickpea

Gene	Coordinates	Predicted coding capacity (aa) [kDa]
Coat protein (CP)	424----1056	210 [23.1]
Full length replication associative protein (Rep)	2492-1320	305 [33.55]
Movement protein (MP)	133-411	92 [10.12]
Replication associative protein (RepA)	2371-1532	279 [30.69]

signature sequence in all geminiviruses. The pair-wise sequence similarity determined by MegaAlign showed a highest similarity of 97% with CpCDV (GenBank accession # KM229787) isolated from infected chickpea plant. Thus, clone KRF4 was believed to be a strain of CpCDV. Since, the KM229787 isolate belongs to the D strain of CpCDV, KRF4 was believed to be the representative of D strain too.

A phylogenetic analysis of KRF4 (GenBank accession # KY952837) with selected CpCDV genomes indicated that KR4 is closely related to all CpCDV viral genomes including CpCDV reported from Sudan (KM229787: Fig. 2) to which it showed highest similarity of 97% (Fig. 3). Pair wise sequence identity showed the highest sequence identity 97% with isolates of CpCDV viral genomes D and minimum sequence identity 82% with KY047534 (Fig. 3). In phylogenetic analysis the output control group i.e. Maize streak virus -A [Nigeria1] (Accession # X01633.1) showed 30.2% identity with our isolated viral genome KRF4.

A recombination analysis revealed KRF4 to be a recombinant (Fig. 4) between CpCDV-C and CpCDV-L isolates (KM229780 and KT634301 as major and minor parents, respectively). This recombination between the strains of CpCDV supported the previous finding of the Kraberger *et al.* 2015 about the inter-strain recombination. Previously, the D strain of CpCDV has been reported, infecting both chickpea and lentils from the Punjab province. A few isolates of CpCDV belonged to six strains B, C, D, H, F and L have been reported from Pakistan. All of these strains were isolated from different legume and non-legume host plants from Punjab province. There is a need to explore the diversity of CpCDV in the all chickpea growing areas to elaborate the molecular diversity of the virus. To the best of our knowledge, this is first report of CpCDV-D isolate from Dera Ismail Khan, Khyber Pakhtunkhwa, Pakistan.

**Acknowledgements**

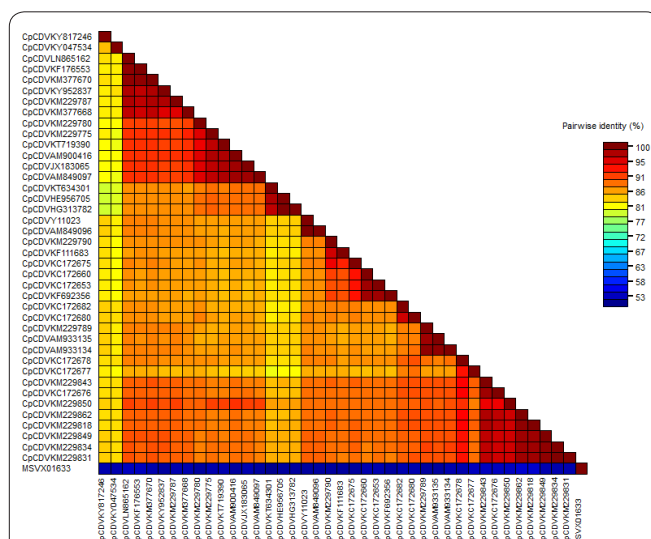
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**Conflict of Interest**

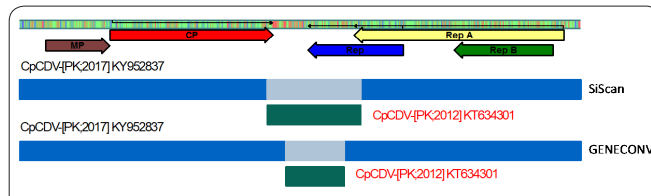
All authors declare no conflict of interest.

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**Figure 3.** Pairwise nucleotide comparison percentage of KRF4 (KY952837) compared with different strains of Chickpea chlorotic dwarf virus (CpCDV) genomes using sequence demarcation tool (SDT).



**Figure 4.** The recombination pattern of CpCDV isolated in the study was found using all strains of CpCDV. KRF4 (GenBank accession # KY952837) showed its Rep and small intergenic region (SIR) recombinant between CpCDV-C and CpCDV-L strains (KM229780 and KT634301 as major and minor parents, respectively), but the rest of molecule does not show significant homology with CpCDV-C and CpCDV-L isolates.

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