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Diversity, mutation and recombination analysis of chilli infecting geminiviruses from the Indian Subcontinent

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Abstract

The epidemic of the chilli leaf curl disease complex (ChiLCD) has been of great concern for about a few decades in the Indian subcontinent. A severe epidemic was observed in Jodhpur (India) in 2004. This virus is transmitted by whitefly (*Bemisia tabaci*). The huge population of virus vectors introduces this virus throughout the subcontinent. Analysis of different sequences reported from different parts of the subcontinent showed huge diversity of this *Begomovirus*. Phylogenetic analysis indicates that two sequences (KF312364, KR779820) are ancestors and others are descendants. These two sequences (KF312364, KR779820) evolve and different strains, variants come into being. Total 101 full-length sequences of this *begomovirus* have been analyzed in phylogenetic analysis. Recombination analysis showed that sequences having accession number (JX524173, JN135234) are fully recombinant. Sequence having accession number JX524173 is the only sequence having all positive results in all RDP tests. We determined different substitution rates for different isolates. Our results indicate that *ChiLCV* is a highly virulent strain and could be the most devastating for chilli crops in the Indian subcontinent.

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Introduction

Begomoviruses are the most important and most devastating viruses among all *Geminiviruses*. These are transmitted by *Homopterans* insect, whitefly (*Bemisia tabaci*). For about three decades, these viruses emerged as the most important plant pathogens and started causing severe economic losses in food, fiber and feed crops like cotton. These viruses have two types of genomes either are monopartite or bipartite. Bipartite genome present as DNA-A and DNA-B segment. These are known as new world *Begomoviruses*. Monopartite genome consists of only single segment DNA-A and is also called OW *Begomoviruses*. However, in the old-world, bipartite *Begomoviruses* DNA-A is able to cause disease alone and responsible for further spread. While in the case of new world *Begomoviruses*, DNA-A is dependent on DNA-B for proper functioning. These satellites are associated with disease complexity and involved in the enhancement of disease. These reproduce through *Begomoviruses* and depend on movement and transmission within and between plants. All functions are carried out by βC1 protein. This protein has many functions, like the initiation of pathogenicity and overcoming upon host defense. According to modern studies, these are very diverse in nature (Zulfiqar *et al*., 2012). Three kinds of DNA satellites have been portrayed to be related to begomoviruses, including betasatellites, alphasatellites and deltasatellites (Lozano *et al.,* 2016). The genus deltasatellite contains 11 species (Briddon *et al.,* 2016; Adams *et al.,* 2017), five of them related to bipartite New World begomoviruses. As of not long ago, New World deltasatellites have been found uniquely in the malvaceous plants *Malvastrum coromandelianum* and *Sidastrum micranthum* and just connected with two assistant begomoviruses, *Sida brilliant yellow vein virus* and *tomato yellow leaf bending virus* (Fiallo-Olivé *et al.,* 2012, 2016). As of late, organic proof has been gotten that New World deltasatellites rely upon a restricted scope of begomoviruses for support in *planta* (Fiallo-Olivé *et al.,* 2016). The genome size is 2.8kb in length and has genes across IR region, have promoter and origin of virion-strand replication (v-ori). DNA-A component have 5-6 open reading frames on both sides that encode 10kDa protein. Protein is involved in many functions like replication, silencing and transmission, etc. (Bisaro, 2006). Due to the arrival of a new nomenclature for *Begomoviruses*, these viruses can be classified into several species: *Chilli leaf curl Multan virus* (ChilCMV), *Chilli leaf curl India virus* (ChiLCINV), *Chilli leaf curl Panipat* (ChiLCPP), *Chilli leaf curl Oman virus* (ChiLCOV), *Pepper leaf curl Lahore virus* (PepLCLV). Species such as *PepLCLV* (Accession number JN135234, JN663864, JX524173) have been shown to cause major losses in chilli crops for recent years in India. Some species not only spread in Pakistan but also reported from other parts of the world such as Oman, Bangladesh, and Srilanka, etc. The genome of *Begomoviruses* is ss-DNA and this provides a chance to determine their recombination and mutation rates. In this study, we have provided recombination and evolutionary analysis of chilli and pepper viruses from different parts of subcontinents. We also provided geographical distribution. We had also generated a data bank showing which sequence was isolated, when this sequence was isolated, from where this sequence was isolated and whom this sequence was isolated.

Materials and methods

Data arrangement

Full-length sequences of *Chilli leaf curl virus* and *Pepper leaf curl virus* were collected from NCBI data bank. A BLAST search was done to determine the homology between other viruses (at specie level) (Muhire *et al*., 2014). The phylogenetic tree of all full-length sequences was also provided (Fig.1).

Phylogenetic analysis

All full-length sequences were aligned in MEGA 6 software by using MUSCLE alignment method. The phylogenetic tree was constructed by using the maximum likelihood method (Kumar *et al*., 2016). By using the tree editor of MEGA 6, similar or identical isolates were converted into a triangle. The chilli leaf curl tree contains 93 sequences and the pepper leaf curl tree contains 8 sequences (Table 3). To calculate pairwise identity, the sequence demarcation tool

(SDT) program was used (Muhire *et al.,* 2014).

Recombination analysis

The recombination detection program (RDP-4) was used to determine the recombination. For this analysis, sequences of *ChiLCV* and *PepLCV* were used. These sequences were first aligned in MEGA 6 software and then exported to RDP-4 program for analysis (Martin *et al.*, 2015). Some events were used in this to determine the recombination, such as GENECONV, MAXchi, SiScan, 3SEQ, BOOTSCAN (Table 5). The *p*-value in this analysis was 0.05. Further, it was confirmed by Meg Align. For betterment and authenticity, these events were confirmed by many methods.

Results

Data bank Analysis

Ten different viruses were reported (n=10) from Pakistan, India, Oman and Sri-Lanka (Table 1). Ninety-three (93) full-length sequences were collected from NCBI. Twenty-two (22) sequences were reported from Pakistan, 35 sequences were reported from India, 34 sequences were reported from Oman, and 2 sequences were reported from Sri-Lanka.

Table 1. Presence of different chilli viruses in different geographical zones.

Serial No.	Virus Name	Pakistan	India	Oman	Sri-Lanka	Source
1	ChiLCV	Yes	Yes	Yes	Yes	NCBI
2	ChiLCMV	Yes	Yes	No	No	NCBI
3	ChiLCOV	No	No	Yes	No	NCBI
4	ChiLCINDV	No	Yes	No	No	NCBI
5	ChiLCPKV	Yes	Yes	No	No	NCBI
6	ChiLCNajV	No	Yes	Yes	Yes	NCBI
7	ChiLCPPV	No	Yes	No	No	NCBI
8	ChiLCNoiV	No	Yes	No	No	NCBI
9	ChiLCV-HD	No	Yes	No	No	NCBI
10	ChiLCV-DU	No	Yes	No	No	NCBI

NCBI= National Center for Biotechnology Information.

The table 1 shows the presence of Chilli viruses in the subcontinent. Different sequences of Chilli viruses were reported from different countries of the subcontinent (NCBI).

Eight viruses of Pepper were reported (n=8) from Pakistan and India. Four (4) sequences were reported from Pakistan and the other four (4) were reported from India (Table 2).

Phylogenetic analysis

Total 93 full-length sequences of *ChiLCV* were analyzed. There were six (6) species, 31 strains and 17 variants were found in these 93 sequences (SDT Analysis). Strain 1 has seven (7) sequences, strain 25 and 30 have two (2) sequences and strain 2 has 25 sequences (SDT analysis data). Fifteen (15) sequences of different strains of *ChiLCV* were reported from Pakistan, 25 from India and the remaining fifteen (15) were reported from Oman (NCBI, n.d.). Total 8 full-length sequences of *PepLCLV* were analyzed.

There were four (4) strains found in these sequences (SDT analysis). These were reported from only two countries, India and Pakistan (NCBI, n.d.). Strain 1, 2, 3, and 4 will be given names as A, B, C, D, respectively.

The first 13 sequences were isolated from Pakistan. These sequences were isolated from chilli crop. Sequences having accession number LN845958 and LN845957 were isolated from the cotton crop (Zaidi *et al.*, 2016). This showed that the virus not only infects chilli crops but also other crops from different families and this also showed the diversity of the

virus. Some sequences were also isolated from families other than *Malvaceae*. Sequences having accession number HM140371 and HM140370 were isolated from papaya crop (NCBI). Sequences having accession number HF968756 and HM968755 were isolated from *petunia spp* (Fig.1 and 2).

Table 2. Presences of different pepper viruses in the subcontinent.

Serial No.	Virus Name	Accession No.	Pakistan	India	Source
1	PepLCLV	JN135234	No	Yes	NCBI
2	PepLCLV	JN663864	No	Yes	NCBI
3	PepLCLV	JX524173	No	Yes	NCBI
4	PepLCLV	NC_016984	Yes	No	NCBI
5	PepLCLV	AM691745	Yes	No	NCBI
6	PepLCLV	AM491589	Yes	No	NCBI
7	PepLCLV	AM404179	Yes	No	NCBI
8	PepLCLV	JN880419	Yes	Yes	NCBI

NCBI= National Center for Biotechnology Information.

Total 8 sequences were classified in the phylogenetic tree. Sequence having accession number JN880419 was ancestor and others were descendants (phylogenetic analysis). Sequences having accession number JN663864, JN135234, JN880419 and JX524173 were isolated from India and the remaining sequences were isolated from Pakistan (NCBI data bank). There were four different strains in these sequences. The first strain contains only 1 sequence, which was reported from Nagpur (India), the second strain contain 2 sequences, 3rd contain 4 sequences and 4th strain has only 1 sequence (FASTA file and data bank analysis). In this diversity and distribution, multiple factors could be involved.

Table 3. Showing strain data, their accession no. location and country.

Strain	No of sequences	Accession No	Location	Year of sampling	Country
A	1	JN663864	Nagpur	2009	India
B	2	JX524173	Luckhnow	2012	India
		JN880419		2011	India
C	4	AM691745	Faisalabad		Pakistan
		NC_016984	Punjab	2004	Pakistan
		AM404179	Lahore	2004	Pakistan
		AM491589	Lahore	2004	Pakistan
D	1	JN135234	Luckhnow	2011	India

The table 3 shows the presence of Pepper strains in cities of the subcontinent.

These factors could be the movement of vector species, trade of infected material between these two countries, etc. Some of these viruses were also isolated from different plants other than chilli. These hosts were tomato, tobacco, and winter cherry. This showed that this virus had multiple host plants. There was always a reason for evolution when a pathogen has many numbers host plants. Due to the huge number of host plants, there was always a chance for the pathogen to visit different host plants at the same time. Due to different vector species, viruses were able to evolve according to their vectors and become transmissible (Fig.3 and 4).

Discussion

This study has some unique points as compared to previous studies. This study shows variability in *chilli leaf curl virus* in the form of species and strains.

Table 4. Showing the Recombination Detection Program Analysis.

Overview of RDP analysis of PepLCLV												
Event No	Found in	Recomb.	Major parent	Minor parent	Detection Methods							
						R	G	B	M	C	S	T
1	1	JN135234	Unknown	JX524173	+	+	+	+	+	+	+	
2	1	JN135234	Unknown	JN880419	+	+	+	+	+	+	+	
3	1	JN663864	JX524173	JN135234	-	-	-	+	+	+	-	
4	2	JN880419	AM404179	Unknown	-	+	+	+	+	+	+	
5	2	JN880419	AM691745	JN135234	-	-	+	+	-	+	-	
6	1	JN135234	JX524173	JN663864	-	+	-	-	-	-	-	
7	1	JN135234	Unknown	JN663864	-	-	-	+	+	-	+	
8	1	JN135234	JN880419	Unknown	-	-	-	-	-	+	+	

Sequences having accession number JX524173, JN880419 were found to be positive against every test. Three (3) sequences were found to be having both major and minor parents.

Our results indicate vast diversity of *Begomoviruses* as they were isolated from the Indo-Pak subcontinent. Our results indicate not only mutation and recombination but also major and minor parents of altered sequences. This could be helpful in predicting new hosts of this virus. This study could provide progress for the future in such a way that researchers might isolate *chilli leaf curl virus* from other crops which are not affected by this virus yet but are vulnerable to attack by this pathogen. More progress could be made on the brinjal crop because it is the same family of chilli crops. Genetic variation was important for large population sizes to exist (Biebricher and Eigen, 2006).

Table 5. Showing results of each test applied on each sequence of PepLCLV.

Sr. No	Virus Name	Recombinant	Av.P-value						
			R	G	B	M	C	S	T
1	PepLCLV	JN135234	2.042×10^{-18}	7.067×10^{-23}	1.930×10^{-19}	7.450×10^{-15}	6.068×10^{-16}	5.359×10^{-22}	3.885×10^{-15}
2	PepLCLV	JN135234*	9.190×10^{-13}	7.905×10^{-09}	2.065×10^{-03}	6.498×10^{-10}	1.507×10^{-11}	5.760×10^{-08}	3.885×10^{-15}
3	PepLCLV	JN663864	_	_	_	1.767×10^{-07}	1.732×10^{-02}	2.567×10^{-19}	_
4	PepLCLV	JN880419	_	1.488×10^{-02}	5.431×10^{-03}	8.004×10^{-05}	6.971×10^{-06}	8.104×10^{-23}	1.210×10^{-04}
5	PepLCLV	JN880419	_	_	2.640×10^{-02}	2.200×10^{-04}	_	3.288×10^{-06}	_
6	PepLCLV	JN135234**	_	_	_	_	_	_	_
7	PepLCLV	JN135234	_	_	_	6.526×10^{-03}	4.110×10^{-03}	_	6.268×10^{-03}
8	PepLCLV	JN135234	_	_	_	_	_	1.323×10^{-06}	2.826×10^{-02}

** This event was detected by too few methods to be included in the results.

* Difference in the value due to different major and minor parents.

Eight sequences of *PePLCV* were analyzed under RDP test. Sequence having accession number JN135234 was quite unique and did not show any recombination.

The RNA viruses mutate rapidly as compared to DNA viruses (Holland *et al.*, 1982). This was due to RNA viruses providing more sites for recombination (Eigen *et al.*, 1988; Worobey and Holmes, 1999).

However, in the case of single-stranded DNA viruses, evolution was more likely to be occurred more rapidly as compared to RNA viruses (Drake, 1991; Shackelton *et al.*, 2005; Shackelton and Holmes, 2006; Duffy *et al.*, 2008). Viruses belonging to *Geminiviridae* family show vast genetic diversity. This was due to recombination and mutation (Ge *et al.*, 2007; Grigoras *et al.*, 2010). Studies have been shown that viruses using DNA polymerase show more genetic diversity (Duffy and Holmes, 2009). However, the mutation was the key in genetic variation (Rossink,

1997) (Garcia-Arenal *et al*., 2003; Balol *et al*., 2010). Studies have been shown that the recombination was responsible for evolution in plant viruses, especially in *Geminiviruses* (Bonnet *et al*., 2005; Heath *et al*., 2006; Varsani *et al*., 2006; Fan *et al*., 2007; Garcia-Andres *et al*., 2007). For example, *tomato yellow leaf curl*, *cassava mosaic virus*, *maize streak virus* and *cotton leaf curl virus* seem to be evolved as a result of the recombination (Varsani *et al*., 2008). Actually, this recombination was due to their dependent replication mechanism (Jeske *et al*., 2001). Recombination was occurred due to the contribution of DNA fragments from other viruses (Zhou *et al*., 1997; Barrie *et al*., 2001; Monci *et al*., 2002).

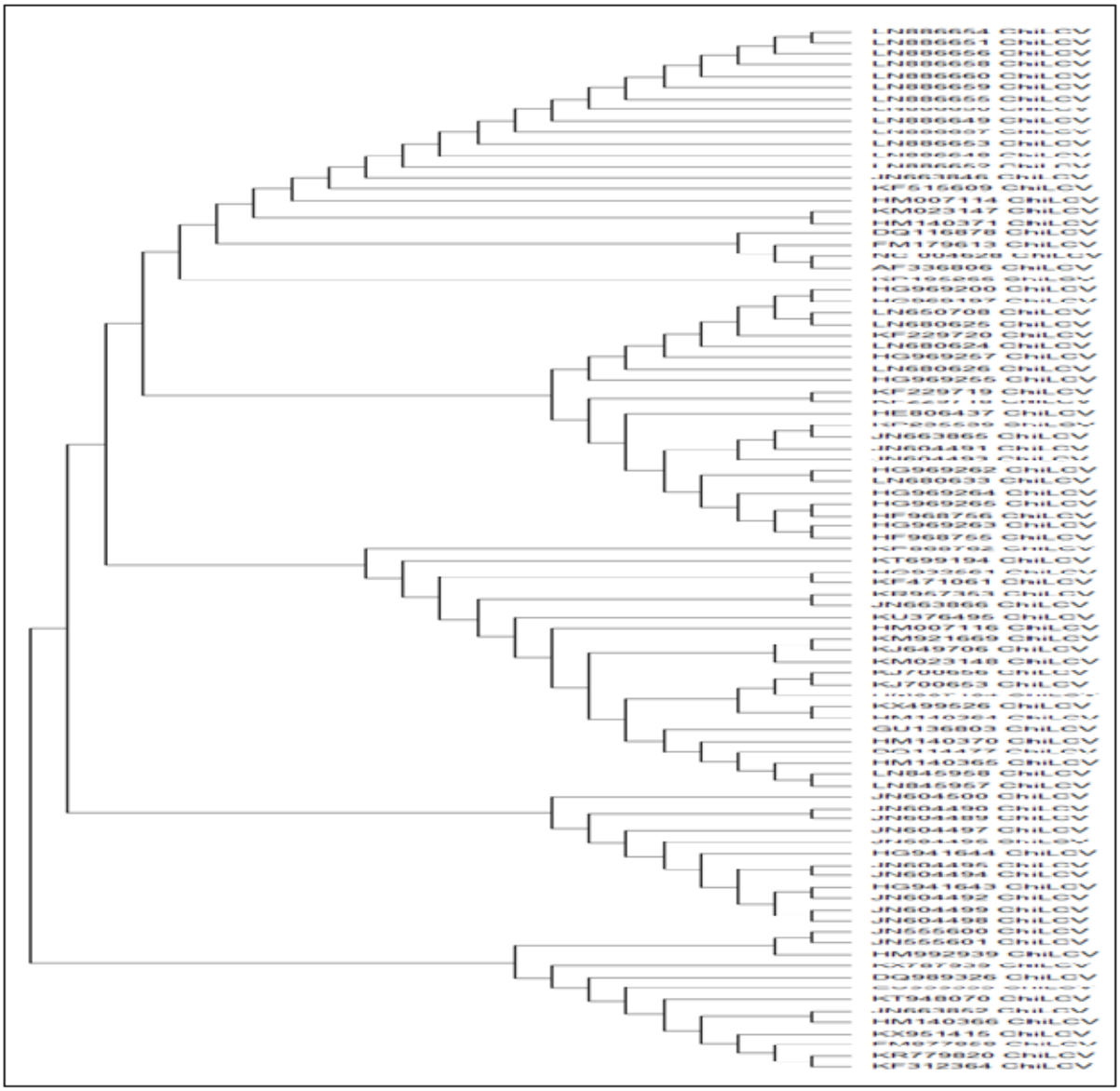

Fig. 1. Phylogenetic tree of *Chilli leaf curl virus*.

This recombination results in genetic variability and diversity (Silva *et al*., 2014). Our results indicate that the exchange of fragments between viruses produces a new strain that contributes to a vast diversity of the viral population. The genetic diversity of *Begomovirus* was due to two factors: their nucleotide substitution at very high rates (Duffy and Holmes, 2008, 2009) and frequent recombination, which results in evolution (Padidam *et al*., 1999; Pita *et al*., 2001). Therefore, mutation and recombination play a vital role in genetic diversity and variability in *Begomoviruses*. Lima *et al*. (Lima *et al*., 2013) analyzed bipartite *Begomovirus tomato severe rugose virus* (ToSRV) add *Macroptilium yellow spot*

(MaYSV). Here we analyzed 93 sequences of *Begomovirus*. For many years, several studies have indicated that *Begomoviruses* recombined at a very high rate (Martin *et al.*, 2011). This was due to the presence of breakpoints that were using a rolling circle replication mechanism for replication and for diversification (Lefurve *et al.*, 2007a; Prasanna and Rai, 2007). For example, in our diversification analysis, results indicated that some sequences (LN845958 and LN845957) were isolated from cotton crops. The cotton hosts of these viruses were only become possible due to the combination of viral sequences. The coat protein structures of *Geminiviruses* provide important information about the genetic variation and recombination (Unseld *et al.*, 2001; Liu *et al.*, 1997).

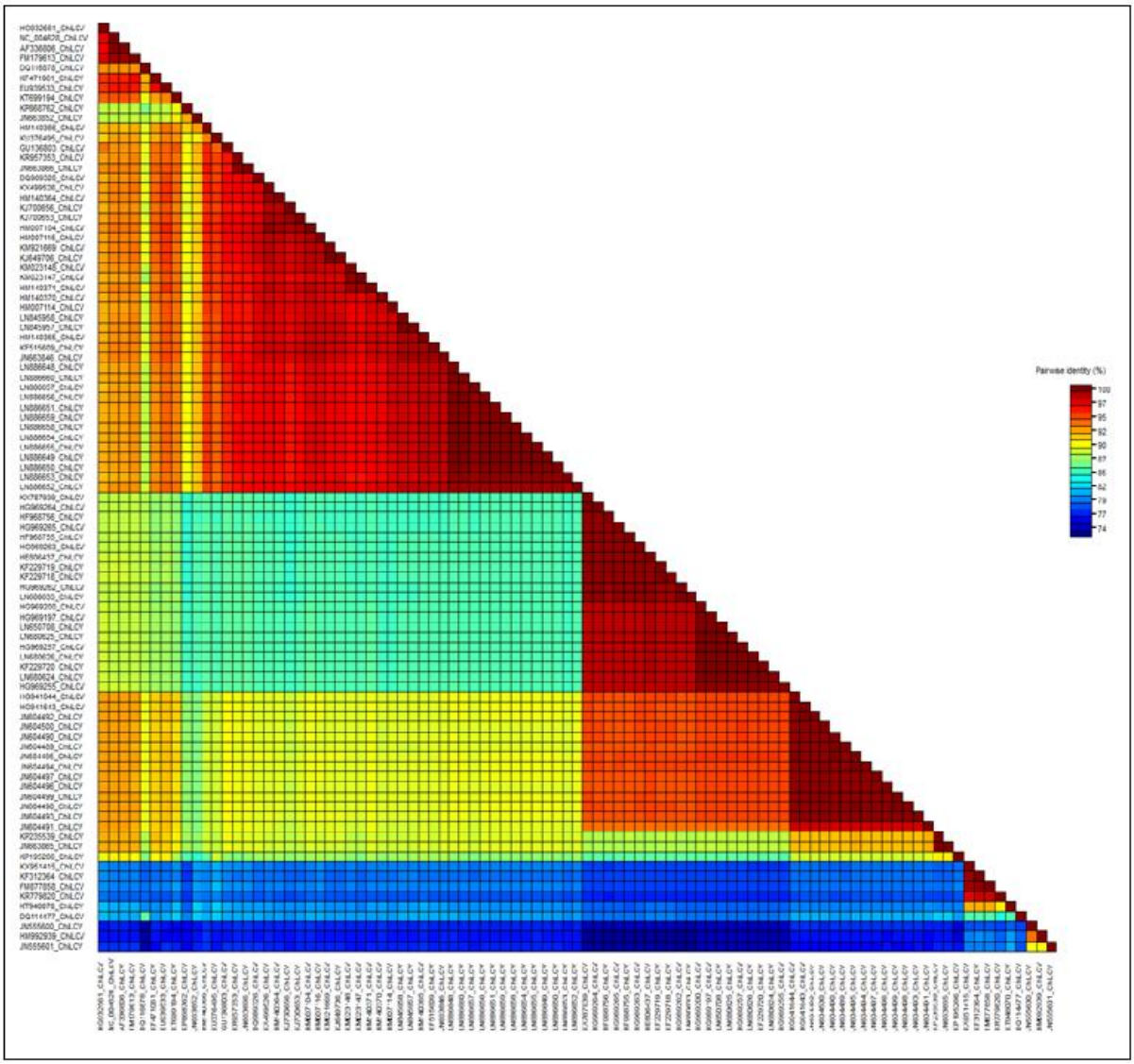

Fig. 2. Matrix showing distance between species of *chilli leaf curl virus*. Matrix reading is kept between 94-91 to demark the species (According to new classification scheme).

The coat protein was required for transmission. Some errors in replication may also cause mutation at certain points in sequences (Zhang *et al.*, 2001; Bottcher *et al.*, 2004). In our recombination analysis of sequences having only minor parents were approximately pure and had a very little amount of mutation as compared to original ones. While sequences having major parents mean that these have adulteration from other sequences of the same viruses (RDP analysis). Recombination was the main an important agency of genetic diversity and variability in *Geminiviruses* found in Brazil (Galvao *et al.*, 2003;

Inoue-Nigata *et al.*, 2006; Ribeiro *et al.*, 2007). This recombination produced new strains, which caused an epidemic on new crops. Studies have shown that weeds were recombination stations for *Begomoviruses*. For example, isolates of *MaYSV* have *BGMV* and *MaYNV* as a parent. This relationship was confirmed by phylogenetic analysis (Castillo-Urquiza *et al.*, 2008). In Central America, *Macroptillium lathyroides* evolved and spread to Jamaica and caused an epidemic (Roye *et al.*, 1999).

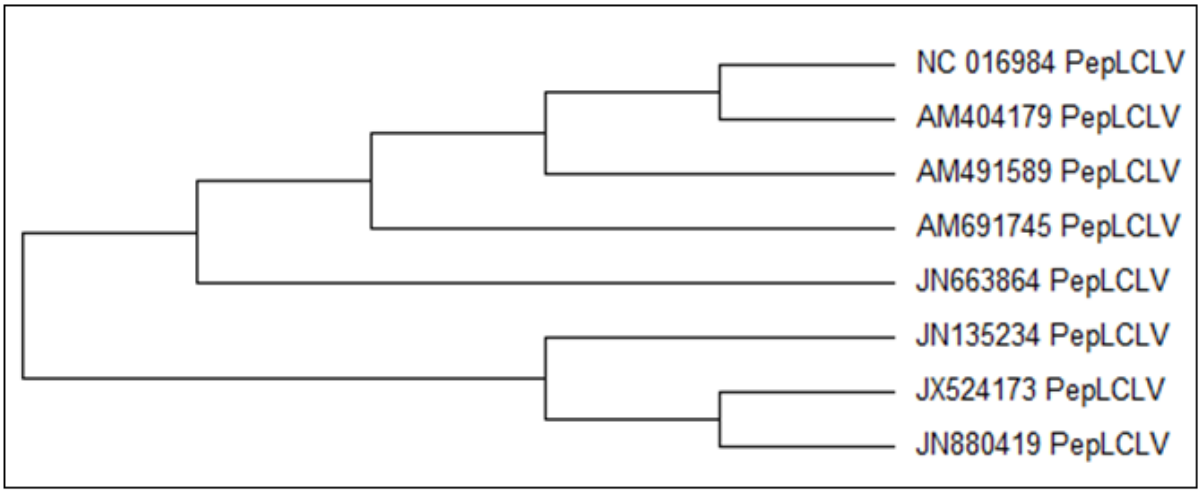

Fig. 3. Phylogenetic tree of *Pepper Leaf Curl Lahore Virus*.

Later it was confirmed that weeds provide a platform for combination. *Begomoviruses* become evolved, diverse and then spread to Jamaica (Paprotka *et al.*, 2010b). Some evidence shows that parts of ssDNA of *Geminiviruses* provide mutation to genetic material (Harkins *et al.*, 2009). Some viruses in Brazil, such as *BGMV,* show a low level of genetic variability (Faria and Maxwell, 1999). However, this virus showed greater variability within a species when experiments were conducted by using the RCA method (Ramos-Sobrhino *et al.*, 2010).

Phylogenetic analysis of *cotton leaf curl Geminivirus* reveals that *cotton leaf curl Multan virus* and *cotton leaf curl Kokhran* virus are responsible for the arrival of several new and virulent strains in cotton crop (Ahmed *et al.*, 2010). Their recombination in nature evolves many species in nature. Evolutionary study indicates that *CLcuMuV* provides Rep protein and *CLcuKoV* provides coat protein during the recombination process (Ahmad *et al.*, 2011). Recombination analysis of available sequences on NCBI data bank reveals that *CLcuMuV* has a substitution rate that was ($4.96x^{10-4}$ /site) and *CLcuKoV* has a substitution rate ($2.706x^{10-4}$ /site) (Briddon, 2003). Diversity analysis indicates that *cotton leaf curl Geminiviruses* not only reported from Pakistan but also from China, Africa and India. The diversity of eleven different types of *Geminiviruses* viruses was studied. *CLcuMuV* isolated from China shows 98-99.3% similarity with *CLcuMuV* isolated from the Philippines. *CLcuMuV* isolated from Pakistan shows 89.4-99.7% homology with *CLcuMuV* isolated from India. This study indicated that *Geminiviruses* had huge diversity and rate of evolution. The mutation rate of *CLcuMuV* in Rep protein was ($0.821gene/10^7$ genes). The mutation rate of *CLcuMuV* was coat protein is ($1.64gene/10^7$ genes). Mutation rate *CLcuMuB* was ($0.85gene/10^7$ genes) (Ahmad *et al.*, 2011). These very high rates of mutation enable *Begomoviruses* to evolve very rapidly and form new strains, variants and species (Ahmad *et al.,* 2011). Diversity analysis indicated that *CLcuMuB* was more prominent in the Indo-Pak sub-continent. This diversity was due to the persistent transmission of this virus by whiteflies (Bosco *et al.*, 2004). However, the factor of trade between the two countries cannot be ignored. The presence of *Begomoviruses* in some parts of Rajasthan (India) was due to recombination between two genera of

Geminiviruses. Pedilanthus leaf curl virus (PeLCV) was a *Begomovirus* known to infect 15 different host plants. The majority of these hosts were from Pakistan. Some studies have shown that it was mutated and spread from Pakistan (Moriones and Navas-Castillo, 2000; Zaidi *et al*., 2016). Some RNA viruses, like a *cucumber mosaic virus* (CMV) shown to infect several host plants (Palukaitis and García-Arenal, 2003). *Pepper leaf curl virus* (PeLCV) was one of the best viruses among *Geminivirus* to infect a broad range of plants (Shakir *et al*., 2018). Phylogenetic tree of CP and Rep genes of *RaLCuV,* which isolated from India, shows closeness to *cotton leaf curl khokharan virus* (CLCuKoV). *CLCuKoV* was responsible for the evolution and emergence of cotton leaf curl disease (Saleem *et al*., 2016). Studies have shown that Rep genes of *Begomoviruses* were more recombinant and variant than Cp genes (Lima *et al*., 2017). However, in PeLCV genetic variability is equal between CP and Rep genes. *Cassava mosaic Begomovirus* (CMB) was also a potential pathogen in African countries when recombined with other viruses (Zhou *et al*., 1997; Fondong *et al*., 2000; Patil and Fauquet, 2009).

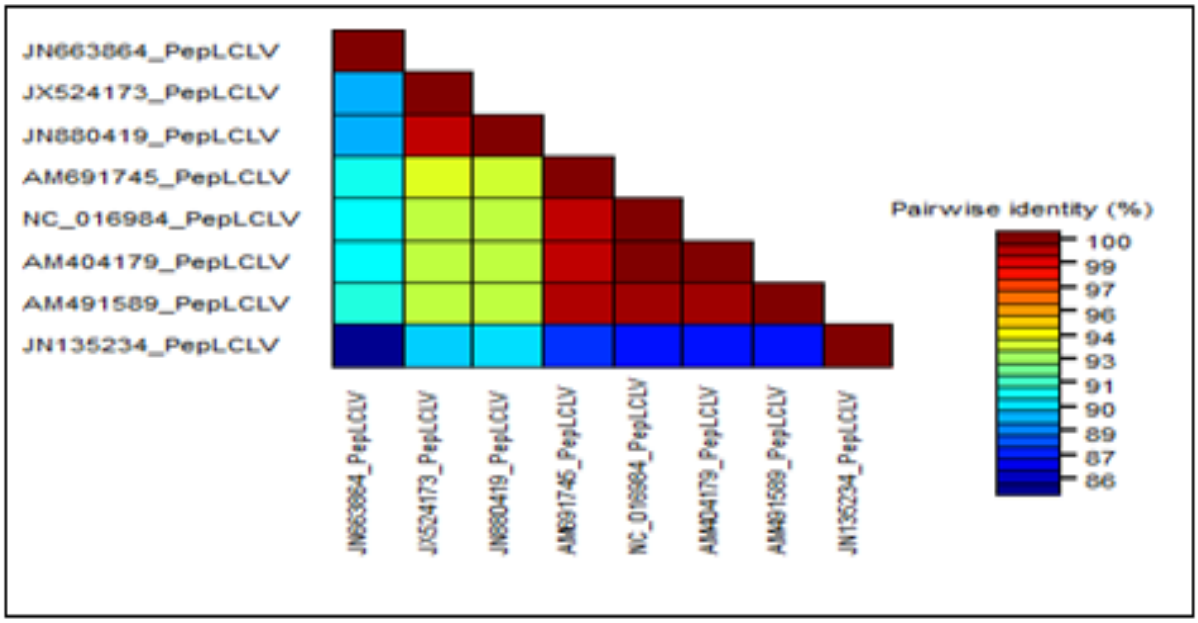

Fig. 4. Matrix showing the pairwise identity of different sequences of *PepLCV* isolated from various places. Pairwise identity is labeled with colors—dark red color indicating 100% identity between described sequences.

In South Africa and Angola, *EACMV* and *ACMV* combined together, which results in a new variant of *Begomovirus* in these countries (Kumar *et al*., 2009; Bisimwa *et al*., 2012). *Chilli leaf curl virus (ChiLCV)* was a *Begomovirus* and was responsible for chilli leaf curl disease (ChiLCD) across India after *tomato leaf curl new Delhi virus (ToLCNDV)* and *Pepper leaf curl Bangladesh virus (PepLCBV)* (Kumarvinoth *et al*., 2015). Synergistic effects of *ChiLCV* and *PepLCBV* had also been observed in eastern parts of India (Kumarvinoth *et al*., 2015). Synergistic effects of these *Begomoviruses* were found at six various locations in India (Palampur, Nagpur, Salem, Ghazipur, New Delhi and Chapra). These monopartite *(ChiLCV)* and bipartite *(ToLCNDV) Begomoviruses* were a source of evolution of new species (Kumarvinoth *et al*., 2015).

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