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Variation of Field Symptoms and Molecular Diversity of the Virus Isolates Associated with Chilli Leaf Curl Complex in Different Agroecological Regions of Sri Lanka

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ABSTRACT. *The present study was conducted to detect and identify the virus isolates and to determine the molecular diversity associated with CLCC-infected chilli plants grown in a wide range of agroecological zones in Sri Lanka over two consecutive cultivation seasons. Randomly-selected chilli plants showing virus-like symptoms were collected from five experimental sites representing different agroecological zones and symptoms were recorded. DNA extracted from the above chilli leaf samples were subjected to PCR amplification using JS35 F and JS36 R primers, which are specific to Chili leaf curl Sri Lanka virus (ChiLCSLV). Amplified PCR products which targeted a region of DNA-A genome of begomovirus were subjected to DNA sequencing. Subsequent DNA homology search identified virus isolates associated with the CLCC-infected chilli plants which gave the highest homology to chilli leaf curl Salem virus-India, chilli leaf curl-Bhavansagar-India, Pepper Leaf curl Bangladesh virus, ChiLCSLV-isolate 14, ChiLCSLV-isolate 15 and Tomato leaf curl geminivirus, but having a variation of percentage identity. Phylogenetic analysis confirmed the genetic divergence of the CL-14 and CL-15 isolates, being them more genetically closer to chilli leaf curl-Bhavansagar-India and chilli leaf curl Salem virus-India, respectively. Eventhough single nucleotide differences were found in different clades of virus isolates, there was no strong relationship with clade separation and the location or season from which the samples were collected. Clade separation also did not show a relationship with a particular type of symptom. Findings of the study clearly revealed the presence of a diverse number of begomovirus isolates associated with the CLCC-infected plants and the genomic variations of them.*

Keywords: *Agroecological regions, Begomovirus, Chilli Leaf curl, diversity, symptom variation*

INTRODUCTION

Chilli (*Capsicum annuum* L.), an economically-important cash crop to Sri Lankan farmers, is grown in a range of agroecological regions of the country during the two major cultivation seasons (*Maha* and *Yala*). Though potential yield is 3 t/ha, the average dry chilli yield in Sri Lanka has been reported to be 1.0 t/ ha (Senanayake *et al*., 2015) and it is mainly due to pest and diseases encountered by the crop. Chilli leaf curl complex (CLCC), one of the major biotic threats to the production of chilli in Sri Lanka, dates back to 1938 (Senanayake *et al*.,

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2012). Most popular chilli varieties grown by the farmers in Sri Lanka, namely MI-1, MI-2, KA-2, Arunalu and MI-hot have found to be susceptible to CLCC (Senanayake *et al*., 2014). Chilli plants infected with CLCC shows virus-like symptoms including severe upward curling, stunted plant growth, leaf thickening, and vein clearing. The severely affected plants are stunted bearing hardly any fruits (Peiris, 1953). The above malformations of the foliage are generally-described as "leaf curl" and they can be caused by viral infections and infestations by mites and thrips (Priyantha *et al*., 2015). Leaf curl in chilli is widespread whenever infestations of *Scirtothrips dorsalis* (chillithrips) are present as evident from previous work in Sri Lanka by Gunawardena (2002). Leaf curl due to thrips results in upward curling of leaves and interveinal buckling. Irregular scraping of epidermis could also be seen (Johnpulle 1939).The wide range of virus-like symptoms could be due to either single or mixed infections by the above probable causes of CLCC. However, mixed infections of chilli due to virus, mites and thrips are difficult to be distinguished purely by field symptoms. Misidentification of the CLCC by the farmers leads in to application of ineffective management methods mainly based on pesticides and it can cause human health and environmental hazards. Association of begomoviurs in CLCC-infected plants in Sri Lanka has been confirmed by molecular methods (Senanayake *et al*., 2013).

Begomoviruses are transmitted by the whitefly, *Bemasiatabaci*, and have circular single stranded one (i.e. monopartite, DNA-A) or two (i.e. bipartite, DNA-A and DNA-B) component genomes ranging in size from ~2.7 Kb (for monopartite species) to ~5.4 Kb (for bipartite species) (Prasanna *et al*., 2010). As described in detailed by Leke*et al*. (2015) six protein-coding genes are universally present in the bipartite genome of DNA-A on the virus sense strand and the antisense strand. DNA-A contains four genes in antisense strand, namely C1 encoding replication-initiation protein (Rep), C2 encoding transcriptional activator protein, C3 encoding replication enhancer protein, and C4 encoding protein required for host range determination, symptom severity, and movement of virus (Rojas *et al*., 2005). The DNA-B component contains two genes, one in virus sense strand, (BV1) and the other in antisense strand (BC1) encoding proteins required for virus movement, host range and pathogenicity (Ingham *et al*., 1995; Sanderfoot and Lazarowitz, 1996).

Senanayake*et al.* (2013) have characterized two isolates of begomovirus (i.e. CL-14 and CL-15) using a few number of CLCC-infected plants collected from North Central Province of Sri Lanka and confirmed the presence of a monopartite begomovirus (i.e. DNA-A genome) along with a betasatallite in them. The betasatellites have been shown to be essential for inducing typical disease symptoms in several monopartite begomoviruses (Briddon*et al*., 2001; Guo *et al*., 2007; Tao and Zhou, 2004).

DNA sequence of the two isolates, CL-14 and CL-15 has revealed that the DNA-A genome (2754 bp) of the virus isolates shared 89.5 % sequence identity with each other and 68.80–84.40 % sequence identity with the other begomoviruses occurring in the Indian subcontinent (Senanayake *et al*., 2013). Based on the above results, it is identified as a new species of begomovirus infecting chilli in addition to the already identified species, hence the begomovirus isolates has been named as chilli leaf curl Sri Lanka virus (ChiLCSLV). The betasatellite species identified from CLCC-infected chilli plants of Sri Lanka has been named as ChLCSLB and it also showed distinct phylogenetic relationship with the other betasatellites associated with the leaf curl disease reported from the Indian subcontinent (Senanayake*et al*., 2013).

From the recent past, farmers, extension officers and researchers are claiming of the presence of a wide range of virus-like symptoms in CLCC-infected plants, based on the field

observations. In the Indian subcontinent, more than one begomoviruses are known to affect a single crop and chilli has been reported to be affected by at least four begomovirus species namely, Tomato leaf curl New Delhi virus (ToLCNDV), Chilli leaf curl virus (ChiLCV), Cotton leaf curl Multan virus (CLCuMV), and Tomato leaf curl Joydebpur virus (ToLCJoV) [Khan *et al*., 2006; Senanayake *et al*., 2007; Shih *et al*., 2007; Hussain *et al*., 2004). According to Senanayake *et al*. (2013), the completely-sequenced CL-14 and CL-15 isolates of ChiLCSLV appear to be genetically divergent to each other though they belong to the same begomovirus species. The presence of several begomoviruses in the field, all transmitted by the same vector, likely facilitates frequent mixed infections in which two or more virus species are simultaneously present within individual plants (Rocha *et al*., 2013). This situation increases the probability of recombination and/or pseudorecombination (reassortment of genomic components) among viral genomic components, which could potentially accelerate host adaptation (Duffy and Holmes, 2008; Duffy and Homes, 2009; Ge *et al*., 2007; Isnard *et al*., 1998; Hall *et al*., 2001).

Considering the wide variation of symptoms found in CLCC infected plants and possibilities of having mixed infections by different begomovirus species as well as potential molecular variations among the isolates of the same begomovirus species, the present study was conducted to detect and identify the virus isolates in chilli plants having virus-like symptoms along with the determination of molecular diversity of those virus isolates.

METHODOLOGY

Collection of plant samples and experimental locations

Tender parts of about 20 chilli leaf samples from tender part of the plant showing virus-like symptoms were collected randomly from chilli plants grown at Kilinochchi, Mahailuppallama, Dodangolla, Meewathura and Rahangala during two consecutive cultivation seasons, namely 2013/*Yala* and 2014/*Maha*. The five experimental locations represented different agroecological regions and the environmental details of the locations are given in Table 1. Meteorological data at experimental sites were recorded using an automated weather monitoring station (Watchdog 2000 series Spectrum Tech., USA).

Table 1. Agroecological details of the selected experimental sites

Experimental location	Agro-ecological zone	Elevation from mean sea level (m)	Soil type	Diurnal Mean temperature (^{o}C)		Mean rainfall (mm/day)	
				2013/ Yala	2014/ Maha	2013/ Yala	2014 /Maha
Kilinochchi	DL_3	15	Red yellow latasols	29.0	26.0	0.8	2.2
Mahailuppallama	DL_1	117	Aluthwewa series	28.0	26.2	1.2	2.2
Dodangolla	IM_3	367	Kundasale series	24.4	26.2	4.3	1.4
Meewathura	WM_{2b}	496	Kandy series	24.5	26.2	6.9	2.6

Source: Mapa*et al.* (2010); Punyawardana (2008)

The chilli plants (var. MI-green) were established in the above locations in 5 x 5 m^2 plots. Each plot was replicated three times according to a randomized complete block design. Four weeks old seedlings were replanted and thereafter, maintained according to the recommendations of the Department of Agriculture, Sri Lanka.

PCR amplification

Doyle and Doyle method (1990) was used for extraction of DNA from leaf tissues of chilli collected from all the five experimental locations in two consecutive seasons. Extracted DNA samples were subjected to PCR using ChiLCSLV specific primer pair JS35 F (5'TGC CAG AGC GGC ATC AGC GG 3') and JS36 R (5' GTC CCC ATT GTC CCC CAT TCC 3') (Senanayake *et al*., 2015). PCR conditions used were as follows: Initial denaturation at 94 ^{o}C for 5 min, denaturation at 94 ^{o}C for 30 sec, annealing at 58 ^{o}C for 30 sec, extension at 72 ^{o}C at 2 min and final extension at 72 ^{o}C for 5 min. Denaturation, annealing and extension steps were done for 35 cycles. Molecular size of the expected PCR product was 501 bp. PCR products were analyzed on a 1.5 % (w/v) agarose gel and size estimation of was done using 100 bp and 1 kb DNA size markers.

DNA sequencing, homology search and phylogenetic relationships

PCR products amplified by JS35 F and JS36 R primers were sent for sequencing to Macrogen, South Korea. DNA sequence results were subjected to homology search using BLAST (NCBI). Multiple sequence alignment of the DNA sequences and phylogenetic analysis to find the relationship of begomoviruses were performed using Geneious and MEGA (Molecular Evolutionary Genetic Analysis) 6.06 software, respectively by maximum likelihood method at 1000 bootstrap value. Reference sequences of some of the begomovirus (e.g. leaf curl virus Salem, India, chilli leaf curl Bhavanisagar-India, CL-14 and CL-15 isolates of ChiLCSLV) were obtained from NCBI databank. DNA sequence of Sweet potato begomovirus (sweepovirus) was obtained from NCBI databank to be used as a distantly related begomovirusspecies.

RESULTS

Variation of Field Symptoms

A wide array of virus-related symptoms was shown by chilli plants grown at the five locations and collected in two cultivation seasons (Table 2 and Fig. 1). They include leaf narrowing, yellowing, boat shaped leaves, deformed leaves, bunchy appearance of the apical leaves, inter-veinal chlorosis and puckering. To a lesser extent leaf mosaic, motteling and vein clearing/banding were observed in some of the chilli plants collected from the five locations. Majority of the plant samples observed in the present study had mixed symptoms (Table 2 and Fig. 1). A clear relationship could not be drawn with the chilli variety or the location from where the samples were collected with the type of virus-like symptoms shown by them.

Table 2. Details of the field symptoms observed in CLCC-infected chilli samples collected from five experimental locations from two cultivation seasons

Location	Sample code	LY	IC	LC	BS	MS	EB	SG	LP	MT
Dodangolla/2013/	DG1C1	√	√	-	-	-	-	-	-	-
Yala	DG1C2	-	-	√	√	√	-	-	-	-
	DG1C3	√	-	√	√	-	-	-	-	-
	DG1C4	-	-	-	√	√	-	-	√	-
	DG1C5	-	-	√	√	-	√	-	√	-
	DG1C6	-	-	√	√	-	√	√	-	-
	DG1C7	√	-	√	-	-	√	-	-	-
	DG1C8	-	-	√	√	-	√	-	-	-
	DG1C9	-	√	-	-	-	-	-	√	-
	DG1C10	-	√	-	-	-	√	-	√	-
Dodangolla/2014/	DG2C1	-	√	-	-	-	-	-	√	-
Maha	DG2C2	-	-	-	√	-	√	-	√	-
	DG2C3	-	√	-	-	-	-	-	-	-
	DG2C4	√	-	-	-	-	-	√	-	-
	DG2C5	√	-	-	-	-	-	-	√	-
	DG2C6	-	-	-	√	-	√	√	√	-
	DG2C7	-	√	-	√	-	√	-	-	-
	DG2C8	-	-	-	√	-	√	-	√	-
Mahailuppallama/	MI1C1	-	√	-	-	-	-	-	√	-
2013/*Yala*	MI1C2	-	√	-	-	-	-	√	-	-
	MI1C3	-	-	-	-	-	√	-	√	-
	MI1C4	-	-	-	√	-	-	-	√	-
	MI1C5	-	√	-	-	-	-	-	-	-
	MI1C6	-	-	-	√	-	-	-	√	-
	MI1C7	-	-	-	√	-	-	√	√	-
	MI1C8	√	-	-	√	-	-	-	-	-
	MI1C9	-	-	-	√	-	-	-	√	√
	MI1C10	-	-	-	-	-	-	-	√	√
Mahailuppallama/	MI2C1	-	-	-	√	-	√	-	√	-
2014/*Maha*	MI2C2	√	√	-	√	-	√	-	-	-
	MI2C3	-	√	-	√	-	-	√	√	-
	MI2C4	-	-	-	√	-	-	-	√	-
	MI2C5	√	-	-	√	-	-	-	√	-
	MI2C6	-	-	-	-	-	-	√	√	√
Meewathura/2013	MW1C1	-	-	-	√	-	-	-	√	-
/*Yala*	MW1C2	-	-	-	√	-	-	√	√	-
	MW1C3	-	√	-	-	-	-	√	-	-
	MW1C5	-	-	-	√	-	-	-	√	-
	MW1C6	-	√	-	√	-	-	-	√	-
	MW1C7	-	-	-	√	-	-	-	√	-
	MW1C8	√	√	-		-	-	-	√	-
	MW1C9	-	-	-	√	-	-	-	√	-
	MW1C10	-	√	-	√	-	-	-	√	-
	MW1C11	√	-	-	-	-	-	-	-	-
Meewathura/2014	MW2C1	√	-	-	-	-	-	-	√	-

/*Maha*	MW2C2	-	√	-	-	-	-	-	√	-
	MW2C3	-	√	-	-	-	-	-	√	-
	MW2C4	-	√	-	√	-	-	-	√	-
	MW2C5	-	-	-	√	-	√	-	-	-
	MW2C6	-	-	-	√	-	-	-	√	-
	MW2C7	-	-	-	√	-	-	-	√	-
	MW2C8	-	-	-	-	-	-	-	-	√
	MW2C10	-	√	-	-	-	-	√	-	-
Kilinochchi/2013/ *Yala*	KN1C1	√	-	-	-	-	-	-	√	-
	KN1C2	-	√	-	-	-	-	√	√	-
	KN1C3	√	-	-	-	-	-	√	√	-
	KN1C4	-	-	-	√	-	√	-	√	-
	KN1C5	√	-	-	-	-	√	-	√	-
	KN1C6	-	-	-	√	-	-	-	√	-
	KN1C7	-	√	-	√	-	√	-	√	-
	KN1C8	-	-	-	√	-	-	-	√	-
Rahangala/2013/*Yala*	RG1C1	√	-	-	√	-	-	√	√	-
	RG1C2	-	√	-	√	-	-	-	√	-
	RG1C3	-	√	-	-	-	√	√	-	-
	RG1C4	-	√	-	√	-	-	-	√	-
Rahangala/2014/ *Maha*	RG2C2	-	-	-	√	-	-	-	√	-
	RG2C3	-	-	-	√	-	-	√	√	-
	RG2C4	-	-	-	√	-	√	√	√	-

LY= Leaf Yellowing, IC= Inter-veinal Chlorosis, LC= Leaf Curl, BS= Boat Shaped, MS= Mosaic, EB= Epical Bunch, SG= Stunted Growth, Leaf Puckering, MT= Mottling

DG1C5- Leaf curl and epical bunch

DG2C2- Boat shaped and epical bunch

MI1C2- Inter-veinal chlorosis and stunted growth

MI1C5- Inter-veinal chlorosis

MW1C1- Boat shaped puckering leaves

MW1C4- Inter-veinal chlorosis and leaf puckering

MW2C2- Inter-veinal chlorosis and leaf puckering

MW2C6- Boat shaped puckering leaves

KN1C3- Leaf yellowing and stunted growth

KN2C8- Yellowing and crinkling of leaves

RG1C1- Boat shaped and stunted growth

RG1C4- Boat shaped puckering leaves

RG2C2- Upward curling, puckering and narrowing of leaves

RG2C3- Boat shaped puckering leaves

Fig. 1. A representative sample of variation of symptoms shown by CLCC-infected plants collected from five experimental locations

DG= Dodangolla, MI= Mahailluppallama, MW= Meewathura, KN= Kilinochchi, RG= Rahangala, 1= growing season 1 (*Yala* 2013), 2= growing season 2 (2013/2014 *Maha*), CN= Sample N of chilli

Detection of ChiLCSLV in CLCC-infected plants

Table 3 summarizes the percentage of chilli leaf samples, with respect to each location and each cultivation season, resulted in the expected PCR product of 501 bp, when amplified with JS35 F and JS36 R primers. Figures 2 and 3 show the amplified products of the samples used for PCR with the above primer pair.

Table 3. Percentage of samples resulted in the expected PCR product when amplified by JS35 F and JS36 R primers

Experimental location	2013/*Yala*	2014/*Maha*
Kilinochchi	100	37.5
Mahailuppallama	70^{+}	50^{+}
Dodangolla	80	50^{+}
Meewathura	100	60^{+}
Rahangala	100	0

$^{+}$Samples showed polymorphism with respect to molecular size of the PCR products.

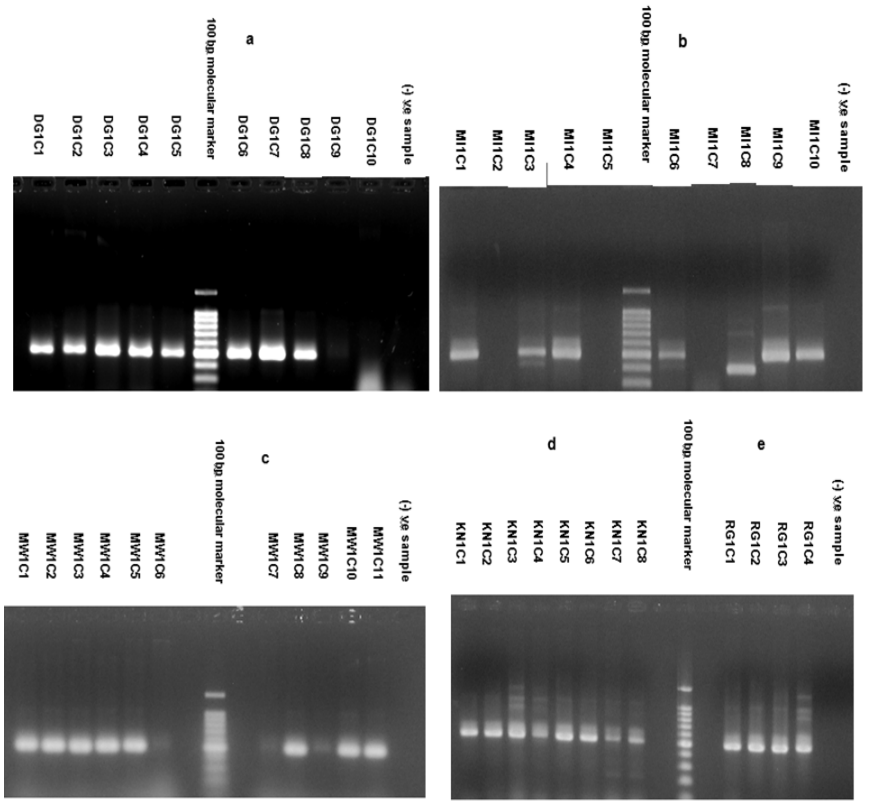

Fig. 2. PCR products given by the chilli leaf samples collected from five different locations in cultivation season 2013/*Yala*. a- samples from Dodangolla, b- samples from Mahailuppallama, c- samples from Meewathura, d- samples from Kilinochchi and e- samples from Rahangala

As shown by Fig. 2 (b), samples collected from Mahailuppallama in 2013/*Yala*, has shown size variations of the PCR product in comparison to the expected size of 501 bp. There was

no polymorphism shown by the samples collected from the other locations with respect to the size of the PCR product expected.

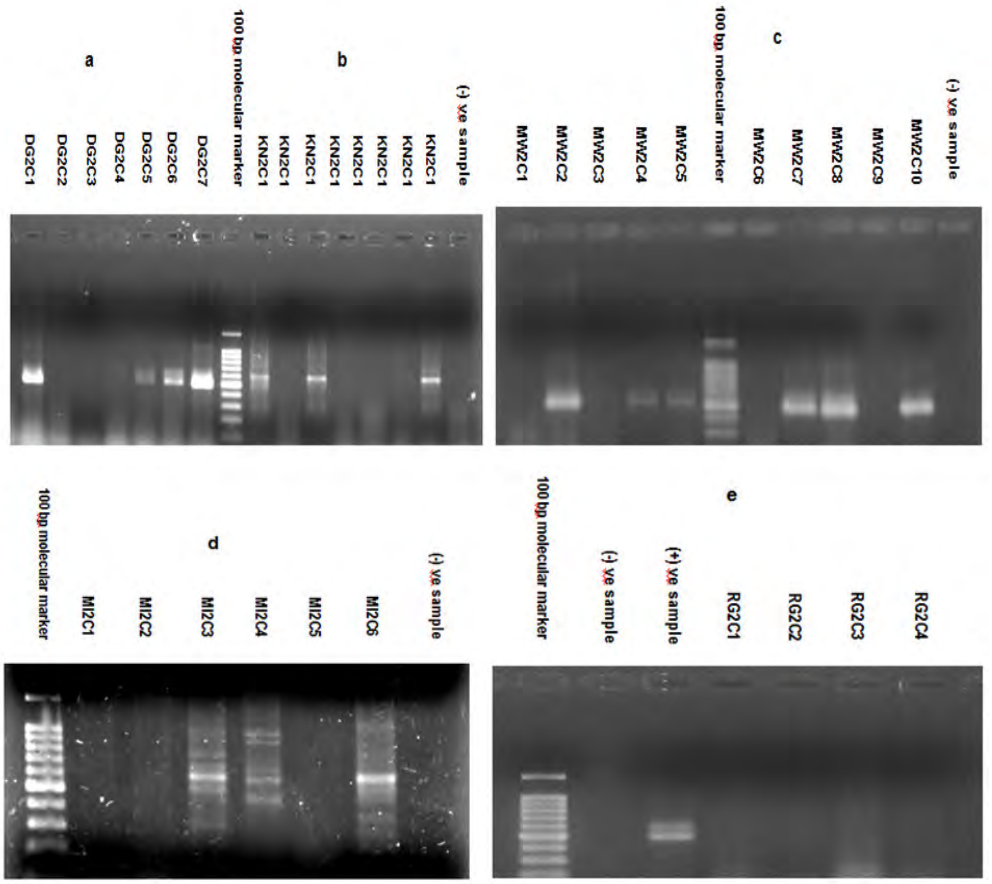

Fig. 3. PCR products given by the chilli leaf samples collected from five different locations in cultivation season 2014/*Maha*. a- samples from Dodangolla, b- samples from kilinochchi, c- samples from Meewathura, d- samples from Mahailuppallama and e- samples from Rahangala

In season 2014/*Maha*, the chilli samples collected from Rahangala did not produce PCR products (Fig. 3e) with the JS35 F and JS36 R primers. In general, the percentages of samples resulted in a PCR product by the above two primers in 2014/*Maha* season were lower than that of the 2013/*Yala* season. Moreover, as shown by Fig. 3 (a), (c) and (d), the PCR products of Dodangolla, Meewathura and Mahailuppallama respectively showed polymorphism with respect to the size of the PCR products resulted in.

Identification of Virus Isolates

Table 4. Details of the homology search of the DNA sequences of the PCR products

Sample Name	Description of the highest homologue	Query cover	E-value	Identity	Accession No.
DG1C1	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	0.0	98%	HM007119.1
DG1C2	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	8e-131	87%	HM007119.1
DG1C3	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	3e-135	87%	HM007119.1
DG1C4	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	1e-139	88%	HM007119.1
DG1C5	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	0.0	97%	HM007119.1
DG1C6	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	8e-141	88%	HM007119.1
DG1C8	Chilli leaf curl virus isolate CL-15 from Sri Lanka, complete genome	88%	4e-138	93%	JN555600.1
MI1C2	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	90%	2e-92	81%	HM007119.1
MI1C6	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	2e-127	87%	HM007119.1
MI1C7	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	2e-137	88%	HM007119.1
MI1C8	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	8e-141	88%	HM007119.1

MI1C11	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	1e-128	88%	HM007119.1
Sample Name	**Description of the highest homologue**	**Query cover**	**E-value**	**Identity**	**Accession No.**
MI1C15	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	0.0	98%	HM007119.1
MW1C1	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	0.0	98%	HM007119.1
MW1C2	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	98%	0.0	98%	HM007119.1
MW1C3	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	1e-139	88%	HM007119.1
MW1C4	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	1e-149	90%	HM007119.1
MW1C5	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	1e-134	87%	HM007119.1
MW1C8	Chilli leaf curl virus isolate CL-15 from Sri Lanka, complete genome	96%	2e-110	99%	JN555600.1
KN1C1	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	0.0	96%	HM007119.1
KN1C2	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	2e-141	88%	HM007119.1
KN1C4	Tomato leaf curl geminivirus AL1 replication protein gene, partial cds	91%	3e-89	84%	L11746.2
KN1C6	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	1e-143	88%	HM007119.1
KN1C7	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone	99%	1e-129	88%	HM007119.1

	pChSalH36 segment DNA-A, complete sequence				
Sample Name	**Description of the highest homologue**	**Query cover**	**E-value**	**Identity**	**Accession No.**
KN1C8	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	1e-118	86%	HM007119.1
RG1C1	Chilli leaf curl virus isolate CL-15 from Sri Lanka, complete genome	96%	2e-132	84%	JN555600.1
RG1C2	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	4e-139	88%	HM007119.1
RG1C3	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	92%	0.0	98%	HM007119.1
RG1C4	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	0.0	98%	HM007119.1
MI2C3	Chilli leaf curl virus isolate CL-14 from Sri Lanka, complete genome	99%	1e-162	96%	JN555601.1
MI2C4	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	92%	6e-153	88%	HM007119.1
MW2C4	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	3e-130	88%	HM007119.1
MW2C5	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	8e-141	88%	HM007119.1
MW2C7	Chilli leaf curl virus-[Bhavanisagar:India:2010] segment DNA A, complete sequence	98%	2e-125	96%	HM992939.1
KN2C1	Chilli leaf curl virus isolate CL-14 from Sri Lanka, complete genome	96%	5e-178	94%	JN555601.1
KN2C7	Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence	99%	3e-140	88%	HM007119.1

Thirty six PCR products amplified by JS35 F and JS36 R primer pair in the present study were DNA sequenced and subjected to homology search (Table 4). Majority of PCR products (80.55 %) showed the highest homology with chilli leaf curl Salem virus – India with an identity ranging from 90-98 % and a query cover of a range of 99-90 %. Less number of PCR products showed the highest homology with chilli leaf curl virus isolate CL-15 (8.3 % of the PCR products), isolate CL-14 (5.55 % of the PCR products), Chilli leaf curl virus-(Bhavanisagar-India) (2.77 % of the PCR products) and tomato leaf curl geminivirus (2.77 % of the PCR productsThe results revealed the possible sequence variations present in begomovirus isolates associated with CLCC-infected chilli plants investigated in the present study in comparison to already characterized isolates of Sri Lanka (i.e. CL-14 and CL-15 of ChiLCSLV) and isolates reported from the Indian subcontinent (i.e. chilli leaf curl Salem virus – India, Chilli leaf curl virus-(Bhavanisagar-India) and tomato leaf curl geminivirus).

Phylogenetic Analysis

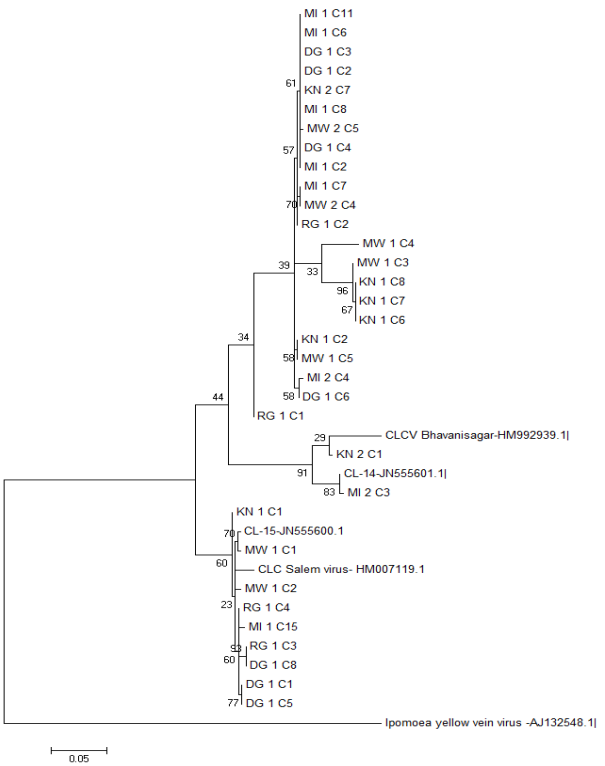

Fig. 4. Phylogenetic tree drawn to show the evolutionary relationship of the amplified region of the DNA-A genome of different isolates of ChiLCSLV with the other begomoviurs species reported from the Indian subcontinent

The phylogenetic tree was developed to analyze the DNA sequences of thechilli leaf curl virus isolates used in this study in comparison with other reference begomovirus isolates reported from different geographical areas of Sri Lanka and from neighbouring India (Fig. 4). Based on the phylogenetic analysis of the present study it was re-confirmed that chilli leaf curl virus CL-14 and CL-15 isolates of Sri Lanka are distantly related isolates and they

belong to two different monphylectic clades. CL-14 isolate was closely related to chilli leaf curl Bhavanisagar isolate of India whereas CL-15 isolate was more related to chilli leaf curl Salem virus isolate. It was also clear that the separation into clades has no relationship with the location and the cropping season from which those isolates were obtained.

DISCUSSION

The present study revealed the presence of chilli leaf curl virus isolates in CLCC-infected chilli plants though they exhibited a wide variation of field symptoms. It was evident by the present study that majority of the virus isolates associated with CLCC-infected plants representing a range of locations of Sri Lanka showed the highest homology with begomovirus species (e.g. Chilli Leaf curl Salem virus-India, Chilli leaf curl virus-[Bhavanisagar:India]) other than CL-14 and CL-15 of ChiLCSLV, indicating the potential association of diverse number of begomovirus species with CLCC-infected chilli in Sri Lanka. All these begomovirus species have a South Indian origin. The possibility of having such begomovirus species with a South Indian origin has been supported by the finding by Senanayake*et al*. (2013), because, the recombination analysis by Senanayake*et al*. (2013) has revealed that ChiLCSLV is a recombinant begomovirus potentially emerged from several begomovirus prevalent in Southern India and Sri Lanka.

Some of the chilli leaf samples collected from some of the locations (e.g. Mahailuppallama 2013/*Yala* and Rahangala 2014/*Maha*) did not produce PCR products as the way it was expected, when amplified by JS35 F and JS36 R primers. This could be due to the actual absence of the targeted begomovirus in the leaf samples or most probably the presence of another begomovirus, though they showed virus-like symptoms. As virus-like symptoms could be caused by thrips and mites infestation this is a possibility. If not, these samples could have been infected with some other viruses. According to Senanayake*et al.* (2013), the same primers were not been able to amplify some of the begomovirus namely, *Tomato leaf curl New Delhi virus* (ToLCNDV), *Tomato leaf curl Bangalore virus* (ToLCBaV), *Tomato leaf curl Sri Lanka virus* (ToLCLKV) and *chilli leaf curl virus* (ChiLCV).

Phylogenetic analysis of the present study re-confirmed the finding by Senanayake*et al*. (2013) that the two isolates of ChiLCSLV, namely CL-14 and CL-15 from chilli plants infected with chilli leaf curl disease, which are genetically divergent but belong to the same begomovirus species. It has also been claimed by Senanayake*et al*. (2013) that the presence of slight symptom variations in the two chilli plants from which CL-14 and CL-15 were obtained, though both infected with the chilli leaf curl disease. Accordingly, the plant of CL-14 sample showed chlorotic, motteling and upward curling of leaves whereas the plant of CL-15 sample showed puckering and curling of dark greenish small leaves. However, the sample MI2C3 of the present study which gave the closest relationship with CL-14 isolate showed boat shaped leaves, puckering of leaves, inter-veinal chlorosis and stunted growth (Fig. 2 and Table 2), indicating no identical symptoms of the two plants. MW1C1 sample of the present study which had the closest relationship with CL-15 isolate showed boat shaped leaves and puckering of leaves and it is not matching exactly with the symptoms shown by the plant associated with CL-15 isolate.

Betasatellites have been shown to be essential for inducing typical disease symptoms in several monopartite begomoviruses (Briddon *et al*. 2001; Guo *et al*., 2008; Tao and Shou, 2008). In addition, C4 encoding protein of the DNA-A genome of monopartite genomes is required for host range determination, symptom severity and movement of virus (Rojas *et*

al., 2005). In the present study, the PCR products used for DNA sequence analyses were amplified by JS primers and they targeted a region covering C1 and C4 of the DNA-A genome (Personal communication by J. Senanayake). Therefore, the DNA region used for the present study can be considered as a useful region when determining the variation of symptoms developed by begomovirus in infected plants. However, to find a strong relationship of symptom variation, a detailed study on betasatellites associated with the individual isolates (including complete sequencing) would be more informative.

Emergence of genomic variation of the begomovirus genome is happening in a rapid rate and it is possible due to recombination, mutations and genetic drift (Rojas *et al*., 2005; Varsani *et al*., 2008). Mutation frequencies and nucleotide substitution rates have been estimated for the begomoviruses TYLCV, Tomato yellow leaf curl China virus (TYLCCNV), and East African cassava mosaic virus (EACMV) and for the Maize streak virus (MSV) and have been found to be similar to those estimated for RNA viruses (~10−4 substitutions per site per year) (Duffy and Holmes, 2008; Duffy and Homes, 2009; Ge *et al*., 2007; Isnard *et al*., 1998; Hall *et al*., 2001). In the present study, analyses of DNA sequences of the virus isolates clearly provide information on point mutations present on the amplified region by JS primers. However, those point mutations cannot be correlated with the clade differentiation (data not shown).The present study considered a region in DNA-A genome which is covering C1 and C4 genes region (i.e. over 2163-2665 bp positions of the virus covering C4 gene, a portion of the AC1 gene and 55 nt in intergenic region). In order to identify genetic variations in a more informative manner, complete DNA sequencing of the monopartite genome and the associated betasatellites would be needed. However, the findings of the present study shed light on the possibility of having a wide variation of begomovirus species and the genetically-divergent isolates of a given begomovirus species associated with CLCC-infected chilli. It highlights the challenges ahead the plant breeders when developing resistant germplasm to combat CLCC.

CONCLUSIONS

Field symptoms of CLCC-infested chilli are highly diverse and those plants are associated with begomovirus isolates of high molecular diversity. These plants are associated with virus isolates showing closer relationships to chilli leaf curl Salem virus-India, chilli leaf curl-Bhavansagar-India, ChiLCSLV-isolate 14, ChiLCSLV-isolate 15 and Tomato leaf curl geminivirus.

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