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ORIGINAL ARTICLE

First record of a novel begomovirus and satellites associated with leaf curl disease of passion fruit from India

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Abstract

Passion fruit is an important fruit crop grown in parts of southern and north-eastern states of India. Leaf curl symptoms typical to begomovirus infection were observed on passion fruit plants at three locations of Madikeri District, Karnataka State, India. The disease incidence ranged from 10–20% in all the locations. In order to determine if the begomovirus was associated with leaf curl disease of passion fruit, 20 infected samples collected from different locations were subjected to PCR analysis using primers specific to begomovirus. This resulted in an expected PCR product of ~1.2 kb. Sequence analysis of these products revealed that they have more than 98% similarity among them and have similarity with other begomoviruses. Complete genome sequencing of begomovirus associated with one sample (PF1 collected from CHES, Madikeri) was done using RCA. Further, sequencing of betasatellite and alphasatellite was done after PCR amplification using specific primers. Complete DNA-A sequence of PF-isolate with other begomoviruses revealed that it shared nucleotide (nt) identity of 87.8 to 88.8% with *Ageratum enation virus.* This indicated the association of a novel begomovirus with leaf curl disease of passion fruit in India, for which we propose the name, *Passion fruit leaf curl virus* (PFLCuV) [IN-Kar-18]. PFLCuV associated betasatellite shared 98.3% sequence identity with *Tomato leaf curl Bangladesh betasatellite*, while alphasatellite had 95.7% sequence identity with *Cotton leaf curl Multan alphasatellite*. Recombinant analysis indicated a major component of PFLCuV DNA-A may have originated from a recombination of earlier reported begomoviruses. Recombination as well as GC plot analysis showed that the recombination occurred in the genome regions having low GC content regions of PFLCuV. However, there is no evidence of recombination in alphasatellite and betasatellite associated with leaf curl disease of passion fruit. This is the first record of a novel begomovirus and satellites associated with leaf curl disease of passion fruit from India.

Keywords: begomovirus, passion fruit, phylogenetic analysis, recombination, sequence demarcation tool (SDT)

Introduction

Passion fruit (*Passiflora edulis* Sims) is an important fruit crop originally from Brazil. The crop is grown in different locations of the world. In India, passion fruit is cultivated in Kerala, Tamil Nadu (Nilgiri hills and Kodai Kanal), Karnataka (Coorg) and northeastern states (Manipur Sikkim, Mizoram and Nagaland) on an area of 911 ha with the production of 458 t (Joy 2010).

Passion fruit cultivation was first started in Nilgiris, Coorg and Malabar regions of South India. Its productivity in India is 5.02 t · ha^{-1}, which is very low compared to countries like Colombia, Australia and Brazil, etc. where it is about 32–35 t · ha^{-1}. This may be due to the attack of many pests and diseases, of which viruses are a major threat for passion fruit production. In addition to three different potyviruses associated with passion fruit woodiness disease (Sithole-Niang *et al*. 1996; Iwai *et al*. 2006), begomovirus infecting passion fruit was also reported from Puerto Rico (Brown *et al*. 1993). Later co-infection of both DNA and RNA viruses in passion fruit was recorded from Brazil (Novaes *et al.* 2003). Recently, a passion fruit-infecting novel begomovirus virus was also recorded from Alagoas State (Silva *et al*. 2006), Colombia (Vaca-Vaca *et al*. 2017) and Brazil (Ferreira *et al.* 2010; Fontenele *et al*. 2018).

The family Geminiviridae is classified into 14 genera, based on the genomic structure, vector involved in transmission and their host range, which includes more than 525 species (Walker *et al*. 2021). Of these, genus begomovirus is a highly pathogenic group of plant viruses threatening cultivation of many crops across the world. The members of viruses in the genus are known to be transmitted by whitefly, *Bemisia tabaci* and have been further divided into bipartite (having two genome components, DNA-A and DNA-B) and monopartite (single genome component known as a homologue of the DNA-A of bipartite begomoviruses) based on the presence of one or two genomic components (Zaidi *et al*. 2016). The open reading frames (ORFs) are aligned in both strands overlapping one another. DNA-A harbors two ORFs (AV1 and AV2) in the sense viral strand and five to six ORFs (AC1, AC2, AC3, AC4 and AC5) on the antisense strand required for expression and activation of different genes in the viral genome. DNA-B consists of two ORFs (BV1 and BC1) which are present in the sense and antisense of viral strands, respectively. The coding region of sense and antisense of strands of DNA-A and DNA-B components are separated by a non-coding DNA sequence known as an intergenic/common region. This contains cis-acting elements required for gene expression and a predicted hairpin structure (~200 nts), referred to as the 'common region' (CR) which contains conserved nonanucleotide TAATATTAC sequence, where viral DNA replication is initiated (Hanley-Bowdoin *et al*. 2013).

The majority of begomoviruses are associated with DNA satellites known as alphasatellite (Briddon *et al*. 2004), betasatellite (Briddon *et al*. 2002) and deltasatellite (Fiallo-Olive *et al*. 2016). Beta and deltasatellites are true satellites, dependent on the helper virus for replication, movement and transmission, whereas alphasatellites replicate on their own and are not true satellites. However, they depend on the helper virus for movement and transmission. A literature survey showed that begomoviruses are co-evolving with their host plants, resulting in more genetic diversity via the recombination, pseudo-recombination and exchange of genomes among themselves. This phenomenon has led to the emergence of novel viral strains having more virulence with an extended host range (Seal *et al*. 2006a, b). Leaf curl disease with symptoms typical to begomovirus infection was observed on passion fruit vines at three locations of Madikari, Karnataka State, India. The present study was undertaken to characterize begomovirus and DNA-satellite molecules associated with leaf curl disease of passion fruit in India.

Materials and Methods

Virus infected samples source

During 2017–2018, 20 virus infected passion fruit samples (Fig. 1) and one asymptomatic sample were collected from Kushal Nagar, Gonikoppa and the Central Horticulture Experiment Station (CHES) of Madikeri District, Karnataka State, India. These places are mainly located in Western Ghats (12.5°N latitude; 75.8°E longitude), which receives more than 1,500 mm annual rainfall over a period of 100 days (July to September). Disease incidence assessed by observing 100 plants in each location and the per cent of incidence was calculated by the number of infected plants divided by the number of plants observed multiplied by 100. Due to the creeping growth habit of passion fruit, care was taken not to sample the same plant more than once. The collected samples were brought to the Plant Pathology laboratory, CHES, Chettalli, Madikeri, Karnataka, India and underwent molecular characterization. Twenty infected passion fruit leaf samples collected from different locations were designated as PF1 to 20.

Fig. 1. Passion fruit plants: A – healthy, B – severe leaf curling symptoms under natural conditions

Genomic DNA isolation, polymerase chain reaction and sequencing

Total DNA was isolated from 20 virus infected and one healthy passion fruit leaf samples using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990). Association of begomovirus with DNA isolated from passion fruit samples was assessed by PCR amplification using primers specific to the begomovirus and subsequent sequencing of the PCR product obtained as described by Venkataravanappa *et al.* (2012). The complete genomic DNA of begomovirus was amplified by rolling circle amplification (RCA) from the passion fruit isolate (PF1) sample collected from CHES, Madikeri (Venkataravanappa *et al.* 2016). In order to isolate monomeric circular genome (DNA-A), 2 µl of RCA product was digested with *Bam*H1 and cloned into the *Bam*H1 linearised plasmid (pUC19) (Venkataravanappa *et al.* 2016). The recombinant clones were confirmed by restriction digestion and colony PCR using virus specific primers. To identify satellite genomes in infected passion fruit samples, the total DNA of infected passion fruit was subjected to PCR amplification using DNA satellites (alpha and beta) specific primers (Briddon *et al.* 2002; Kumar *et al.* 2010). The DNA satellites amplified through PCR were cloned into the pTZ57R/T vector as per the manufacturer's instructions (Thermo Fisher Scientific Inc., PA). The transformed recombinant clones were confirmed by PCR using specific primers and restriction endonuclease digestion. Ten selected positive clones were sequenced.

Sequence analysis

The sequence similarity search for viral genome and satellites was performed by BLASTn Program (http://www.ncbi.nlm.nih.gov/BLAST) and the selected begomovirus sequences and DNA satellites (Tables 1, 2, 3) accessions displaying the highest percentage of nucleotide (nt) identity with the passion fruit infecting begomovirus were retrieved from the NCBI database for analysis. The per cent pairwise nt identities between PF1 and selected begomoviruses were calculated using SDT version 1.2 (Muhire *et al.* 2014). Neighbor-joining method analysis was carried out to determine the evolutionary relationship between begomovirus associated with passion fruit leaf curl using MEGA X software by 1,000 bootstrapped replications (Kumar *et al.* 2018). Recombination break point analysis was carried out between PF1 isolate of begomovirus and other selected begomoviruses using RDP4 with RDP settings with *p* value of 0.05 (Martin *et al.* 2015). The guanine-cytosine (GC) content in the viral genome (DNA-A) of the PF1 isolate and their associated DNA satellites (alphasatellite and betasatellite) with passion fruit leaf curl disease was analyzed using per cent GC-plot graph generated through Artemis DNA plotter analysis tool v18.1.0 (Carver *et al.* 2009).

Results

Per cent incidence of leaf curl disease on passion fruit

The survey for the incidence of leaf curl disease in passion fruit plants in three locations of Madikeri District, Karnataka State, India revealed symptoms *viz.,* severe leaf curl, very brittle vines and fewer flowers, which became unproductive and/or produced shriveled fruits typical of begomovirus infection (Fig. 1). The incidence of passion fruit plants showing these symptoms ranged from 10–20% in the surveyed fields.

Table 1. GenBank accession numbers of selected begomovirus sequences used in this study for analysis

Begomoviruses	Accession numbers	Abbreviation
Ageratum yellow vein Sri Lanka virus [Sri Lanka: 1999]	AF314144	AYVSLV [LK : 99]
Ageratum enation virus – India [India : Pantnagar : TC357 : 2012]	JX436472	AEV-IN [IN : Pan : TC357 : 12]
Ageratum enation virus – [India : Lucknow : Amaranthus : 2011]	JF682242	AEV-IN [IN : Luc : Ama : 11]
Ageratum enation virus – India [India : Palampur : 2011]	HE861940	AEV-IN [IN : Pal : 11]
Ageratum enation virus – India [India : Lucknow : AS–Poppy3 : 2012]	JQ911765	AEV-IN [IN : Luc : AS–P3 : 12]
Ageratum enation virus – India [India : Lucknow : Ageratum : 2012]	JQ911767	AEV-IN [IN : Luc : Age : 12]
Ageratum enation virus – India [India : Mohali : Age10 : 2010]	JF728866	AEV-IN [IN : Moh : Ag10 : 10]
Ageratum enation virus – India [India : Mohali : Age6 : 2010]	JF728864	AEV-IN [IN : Moh : Ag6 : 10]
Ageratum enation virus – India [India : Mohali : Age4 : 2010]	JF728862	AEV-IN [IN : Moh : Ag4 : 10]
Ageratum enation virus – Nepal [India : Gomtinagar : Cleome : 2008]	FJ177031	AEV-NP [IN : Gom : Cle : 08]
Ageratum enation virus – Nepal [India : Gorakhpur : Trichosanthes : 2008]	GQ268327	AEV-NP [IN : Gor : Tri : 08]
Ageratum enation virus – Nepal [India : Lucknow : 2007]	EU867513	AEV-NP [IN : Luc : 07]
Ageratum enation virus – Nepal [Pakistan : Lahore : 2006]	AM698011	AEV-NP [PK : Lah : 06]
Ageratum enation virus – Nepal [Pakistan : Faisalabad : Turnip : 2007]	AM701770	AEV-NP [PK : Fai : Tur : 07]
Ageratum enation virus – India [India : Kangra : 2008]	FN543099	AEV-IN [IN : Kan : 08]
Ageratum enation virus – Nepal [Pakistan : Lahore : 2004]	AM261836	AEV-NP [PK : Lah : 04]
Ageratum enation virus – India [India : Pantnagar : TC364 : 2012]	KC818421	AEV-IN [IN : Pan : TC364 : 12]
Papaya leaf curl virus – Crotonn [India : Bang : Cr2 : Croton : 2007]	JN831446	PaLCuV-Cro [IN : Ban : Cr2 : Cro : 07]
Papaya leaf curl virus – Lucknow [India : Lucknow]	Y15934	PaLCuV-Luc [IN : Luc]
Tobacco curly shoot virus [India : Agartala : 2010]	JN387045	TbCSV [IN : Aga : 10]
Tomato leaf curl Bangalore virus – C [India : Bangalore 4 : 1997]	AF165098	ToLCBaV – C [IN : Ban4 : 97]
Chilli leaf curl virus [India : *Phaseolus aureus* : HJP3 : 2011]	JQ654460	ChiLCV [IN : Pau : HJP3 : 11]
Tomato leaf curl virus – Bangalore [India : Bangalore : 1993]	U38239	ToLCKaV-Ban [IN : Ban : 93]
Tomato leaf curl Kerala virus [India : Kerala 3 : 2007]	EU910141	ToLCKeV [IN : Ker3 : 07]
Papaya leaf curl Guangdong virus [Taiwan : Passiflora : 2011]	KC161184	PaLCuGdV [TW : Pas : 11]
Euphorbia leaf curl virus [Taiwan : PF1 : 2011]	KC161185	EuLCuV [TW : PF1 : 11]
Passion fruit chlorotic mottle virus [Brazil : *Passiflora edulis* : 2014	MG696802	PCMoV [BR : CDSMS : 14]

Table 2. GenBank accession numbers of selected betasatellites sequences used in this study for analysis

Betasatellites	Accession numbers	Abbreviation
Tomato leaf curl Bangladesh betasatellite [India : Ahmedabad : Chilli : 2014]	KM880104	ToLCBDB [IN : Ahm : Chi : 2014]
Tomato leaf curl Bangladesh betasatellite [India : Kanpur : chilli : 08]	HM007107	ToLCBDB [IN : Kanr : Chi : 08]
Tomato leaf curl Bangladesh betasatellite [India : Patna : Chilli : 08]	HM007118	ToLCBDB [IN : Pat : Chi : 08]
Tomato leaf curl Bangladesh betasatellite [India : Jodhpur : Chilli : 09]	HM007105	ToLCBDB [IN : Jodhr : Chi : 09]
Tomato leaf curl Bangladesh betasatellite [India : Vararanasi : 06]	EF190215	ToLCBDB [IN : Var : 06]
Tomato leaf curl Bangladesh betasatellite [India : Ghazipur : Chill : 07]	HM007099	ToLCBDB [IN : Ghazr : Chi : 07]
Tomato leaf curl Bangladesh betasatellite [India : Vellanad : 11]	JN663876	ToLCBDB [IN : Vell : 11]
Tomato leaf curl Bangladesh betasatellite [India : Nar : Chilli : 04]	JF706231	ToLCBDB [IN : Nar : Chi : 04]
Tomato leaf curl Bangladesh betasatellite [India : Noida : chilli : 07]	HM007115	ToLCBDB [IN : Noi : Chi : 07]
Tomato leaf curl Bangladesh betasatellite [India : Lucknow : 2005]	DQ343289	ToLCBDB [IN : Luk : 05]
Tomato leaf curl Bangladesh betasatellite [India : Rajasthan : 03]	AY438558	ToLCBDB [IN : Raj : 03]
Tomato leaf curl Bangladesh betasatellite [India : PUSA3 : 10]	HQ180395	ToLCBDB [IN : PUSA3 : 10]
Tomato leaf curl Bangladesh betasatellite [India : New Delhi : PUSA5 : 10]	HQ180397	ToLCBDB [IN : PUSA5 : 10]
Tomato leaf curl Bangladesh betasatellite [India : Bihar09 : 2010]	HQ257376	ToLCBDB [IN : Bih09 : 10]

Table 2. GenBank accession numbers of selected betasatellites sequences used in this study for analysis – *continuation*

Betasatellites	Accession numbers	Abbreviation
Tomato leaf curl Bangladesh betasatellite [India : Bihar9 : 2010]	GU732208	ToLCBB [IN : Bih9 : 10]
Tomato leaf curl Bangladesh betasatellite [India : Bihar12 : 2010]	GU732207	ToLCBDB [IN : Bih12 : 10]
Tomato leaf curl betasatellite [India : Panipat2 : Papaya : 08]	HM143902	ToLCB [IN : Pani2 : Pap : 08]
Tomato leaf curl betasatellite [India : Panipat7 : Papaya : 08]	HM143907	ToLCB [IN : Pani7 : Pap : 08]
Cotton leaf curl Multan betasatellite – CLCuMB [India : Raigunj : 08]	FJ159274	CLCuMB [IN : Rai : 08]
Cotton leaf curl Multan betasatellite [India : Haringhata 19 : Kenaf : 2006]	EF614159	CLCuMB [IN : Har05 : Ken : 06]
Chilli leaf curl betasatellite [India : Panipat4 : Papaya : 08]	HM143904	ChLCB [IN : Pani4 : Pap : 08]
Papaya leaf curl betasatellite [India : New Delhi : Papaya : 07]	EU126826	PaLCuB [IN : ND : Pap : 07]

Table 3. GenBank accession numbers of selected alphasatellites sequences used in this study for analysis

Alphasatellites	Accession numbers	Abbreviation
Cotton leaf curl Multan alphasatellite [Pakistan : cotton : 2011]	HE966423	CLCuMuA [PK : Cot : 11]
Cotton leaf curl Multan alphasatellite [Pakistan : cotton : 2011]	HE966422	CLCuMuA [PK : Cot : 11]
Cotton leaf curl Multan alphasatellite [Pakistan : cotton : Multan : 2015]	LN831970	CLCuMuD1 [PK : Cot : Multan : 15]
Ageratum yellow vein India alphasatellite [India : Luc : *Parthenium hysterophorus* : 2012]	JX570736	AYVIA [IN : Luc : Par : 12]
Cotton leaf curl Multan alphasatellite [India : Punjab : wheat : 2011]	KC305094	CLCuMuA [IN : Pun : wheat : 11]
Ageratum enation alphasatellite [Pakistan : Faisalabd : *Sonchus arvensis* : 2007]	AM930245	AEA [PK : Fai : Son : 07]
Ageratum enation alphasatellite [China : Yunnan : 10]	FN678899	AEA [CN : Yun : 10]
Bhendi yellow vein alphasatellite [India : Haryana : okra : 07]	FN658718	BhYVA [IN : HR : OK : 07]
Bhendi yellow vein alphasatellite [India : Haryana : okra : 09]	FN658716	BhYVA [IN : HR : OK : 09]
Ageratum enation alphasatellite [Japan : tomato : 11]	KC677736	AEA [JP : Tom : 11]
Cotton leaf curl Multan alphasatellite [India : Pan : cotton : 12]	KF584012	CLCuMuA [IN : Pan : Cot : 12]
Ageratum enation alphasatellite [India : Luck : poppy : 12]	JX913532	AEA [IN : Luck : Poppy : 12]
Ageratum enation alphasatellite [India : Luck : Guar : 10]	GU385877	AEA [IN : Luck : Guar : 10]
Gossypium darwinii symptomless alphasatellite [India : Pap : 10]	JQ322970	GDarSLA [IN : Pap : 10]
Tomato yellow leaf curl Thailand alphasatellite [China : Yunnan : Tom : 03]	AJ579357	TYLCuTHA [CN : Yunnan : Tom : 03]
Sunflower leaf curl Karnataka alphasatellite [India : KTK : SnF : 11]	JX569789	SLCuKaA [IN : KTK : SnF : 11]
Malvastrum yellow mosaic alphasatellite [Viet Nam : Thanhhoa : Abutilon indicum : 06]	DQ641717	MaYA [VN : Than : Abu : 06]

Genome structure of begomovirus associated with leaf curl disease of passion fruit

All of the 20 infected leaf samples collected from the different passion fruit growing farmers' fields gave positive PCR amplification for begomovirus using specific primers with the expected amplicon product of 1.2 kb in size. No amplification was noticed in the healthy sample. The analysis of nucleotide sequences obtained from this amplicon revealed that sequences from the 20 samples shared more than 98% identity among them and were closely related to other begomovirus sequences retrieved from the GenBank database which indicated that they belonged to a single species, as per the classification of begomoviruses (Adams *et al*. 2017). Therefore, one sample, PF1 was subjected to amplification of complete genome (2.7 kb) of begomovirus and the nucleotide sequence obtained was submitted to NCBI GenBank.

Sequence identities of DNA-A component with other begomoviruses

The length of the complete genome of begomovirus (PF1 isolate) associated with leaf curl diseases of passion fruit was 2753 nt (Acc. No. MK087124) and exhibited a genomic structure similar to other

monopartite begomoviruses from Old Word (OW), which codes for six conserved ORFs (V2, V2, C1, C2, C3 and C4) required for gene expression and infection. The intergenic region (IR) (was present between ORFV2 and C1 of sense and antisense strands. The Rep gene (encoded by C1) consisted of all conserved motifs described by Vadivukarasi *et al*. (2006) except the GRS motif (RFFDLVSPTRSAHFHPNIQGAKSS), which was identified in this new begomovirus.

Sequence denaturation tool (SDT) tool was used for pairwise sequence comparison between begomovirus PF1 isolate from passion fruit and begomoviruses retrieved from NCBI database. The analysis showed that the DNA-A component of the PF1 isolate from passion fruit showed nt identity of 87.8 to 88.8% with the isolates of *Ageratum enation virus* (AEV) infecting different crops in Indian subcontinents and Sri Lanka, in which sequences are available in the database (Table 4) and only 57.7 to 82.6% identity with *Passion fruit chlorotic mottle virus* (MG696802), *Passion severe leaf distortion virus* (FJ972767), *Papaya leaf curl Guangdong virus* (KC161184), *Passion fruit leaf distortion virus* (KT899302) and *Euphorbia leaf curl virus* (KC161185) identified in infected passion fruit in Sri Lanka, Taiwan and Brazil, respectively. For the classification of begomoviruses, the threshold value was set at 91% nt

Table 4. Pairwise per cent nucleotide sequence identities between DNA-A and intergenic region (IR) of begomovirus associated with leaf curl disease of passion with other selected begomoviruses from the NCBI database

Begomoviruses*	Accession numbers	Crop	Country	Genome	IR	Gene (percentage amino acid sequence identity)					
						AV2	CP (AV1)	Rep (C1)	TrAP (C2)	REn (C3)	C4
AYVSLV	AF314144		Sri Lanka	88.4	86.5	91.3	98.0	83.3	89.5	91.7	49.4
AEV	JX436472	tomato	India	88.8	82.5	78.8	94.1	95.5	82.0	86.5	87.0
AEV	JF682242	amaranthus	India	88.4	80.7	78.8	93.3	93.6	79.8	82.0	85.8
AEV	HE861940	soybean	India	88.4	81.4	79.6	93.7	94.4	82.8	85.8	84.7
AEV	JQ911765	poppy	India	87.5	81.1	79.1	91.7	93.6	82.8	85.8	87.0
AEV	JQ911767	ageratum	India	88.3	81.1	77.1	94.1	93.6	81.3	86.5	87.0
AEV	JF728866	ageratum	India	88.4	81.1	80.8	94.1	94.7	81.3	86.5	88.2
AEV	JF728864	ageratum	India	88.6	81.1	80.1	94.2	95.5	81.3	86.5	88.2
AEV	JF728862	ageratum	India	88.1	81.1	80.8	94.1	94.1	81.3	86.5	88.2
AEV	AM698011	ageratum	India	87.2	74.0	79.1	93.7	93.6	79.8	82.0	84.7
AEV	FJ177031	stinkweed	India	87.1	75.0	79.1	94.1	92.5	80.5	82.8	81.1
AEV	GQ268327	parwal	India	87.1	75.0	80.0	93.7	93.6	79.8	82.0	84.7
AEV	EU867513	amaranthus	India	87.1	75.0	81.7	93.7	92.5	80.5	85.8	83.5
AEV	AM701770	turnip	India	87.0	76.2	78.2	93.7	93.9	80.5	83.5	80.0
AEV	FN543099	zinnia sp	Pakistan	88.7	81.1	79.6	94.1	95.2	82.8	87.3	87.0
AEV	AM261836	milk thistle	India	87.1	73.7	79.1	91.7	94.1	76.9	85.8	83.5
AEV	KC818421	tomato	India	87.8	75.2	77.1	93.7	95.8	82.0	87.3	83.5
PaLCuV	JN831446	croton	India	79.8	80.7	72.8	79.2	78.9	80.5	82.8	42.8
PaLCuV	Y15934	papaya	India	81.5	74.6	76.2	92.9	78.1	80.5	63.0	51.7
TbCSV	JN387045	tomato	India	82.2	79.0	77.1	92.5	85.5	81.3	84.3	32.8
ToLCBaV	AF165098	tomato	India	78.9	68.6	74.7	85.2	80.3	79.1	81.3	46.3
ChiLCV	JQ654460	french bean	India	82.0	85.0	65.2	82.0	84.2	79.8	76.8	45.4
ToLCKaV	U38239	tomato	India	82.9	76.8	81.3	92.6	84.2	81.3	85.8	49.4
ToLCKeV	EU910141	tomato	India	83.0	74.8	79.6	93.3	83.6	82.8	85.8	56.1
PLDV	KT899302	passion fruit	Sri Lanka	70.0	64.9	–	70.0	65.9	49.6	52.5	38.2
PSLDV	FJ972767	passion fruit	Brazil	68.4	68.0	–	69.2	64.9	48.8	51.1	46.3
PaLCuGDV	KC161184	passion fruit	Taiwan	77.2	73.0	77.5	81.3	73.2	68.8	70.8	65.8
EuLCV	KC161185	passion fruit	Taiwan	82.6	78.2	70.3	92.5	85.8	83.5	84.3	61.4
PCMoV	MG696802	passion fruit	Brazil	57.7	58.0	17.7	25.5	35.2	71.0	56.0	46.3

*the species are indicated as: *Ageratum yellow vein Sri Lanka virus* (AYVSLV), *Ageratum enation virus* (AEV), *Papaya leaf curl virus* (PaLCuV), *Tobacco curly shoot virus* (TbSCV), *Tomato leaf curl Bangalore virus* (ToLCBaV), *Chilli leaf curl virus* (ChiLCV), *Tomato leaf curl virus* (ToLCKaV), *Tomato leaf curl Kerala virus* (ToLCKeV), *Euphorbia leaf curl virus* (EuLCV), *Passion fruit leaf distortion virus* (PLDV), *Passion severe leaf distortion virus* (PSLDV), *Papaya leaf curl Guangdong virus* (PaLCuGDV) and *Passion fruit chlorotic mottle virus* (PCMoV). For each column the highest value is underlined

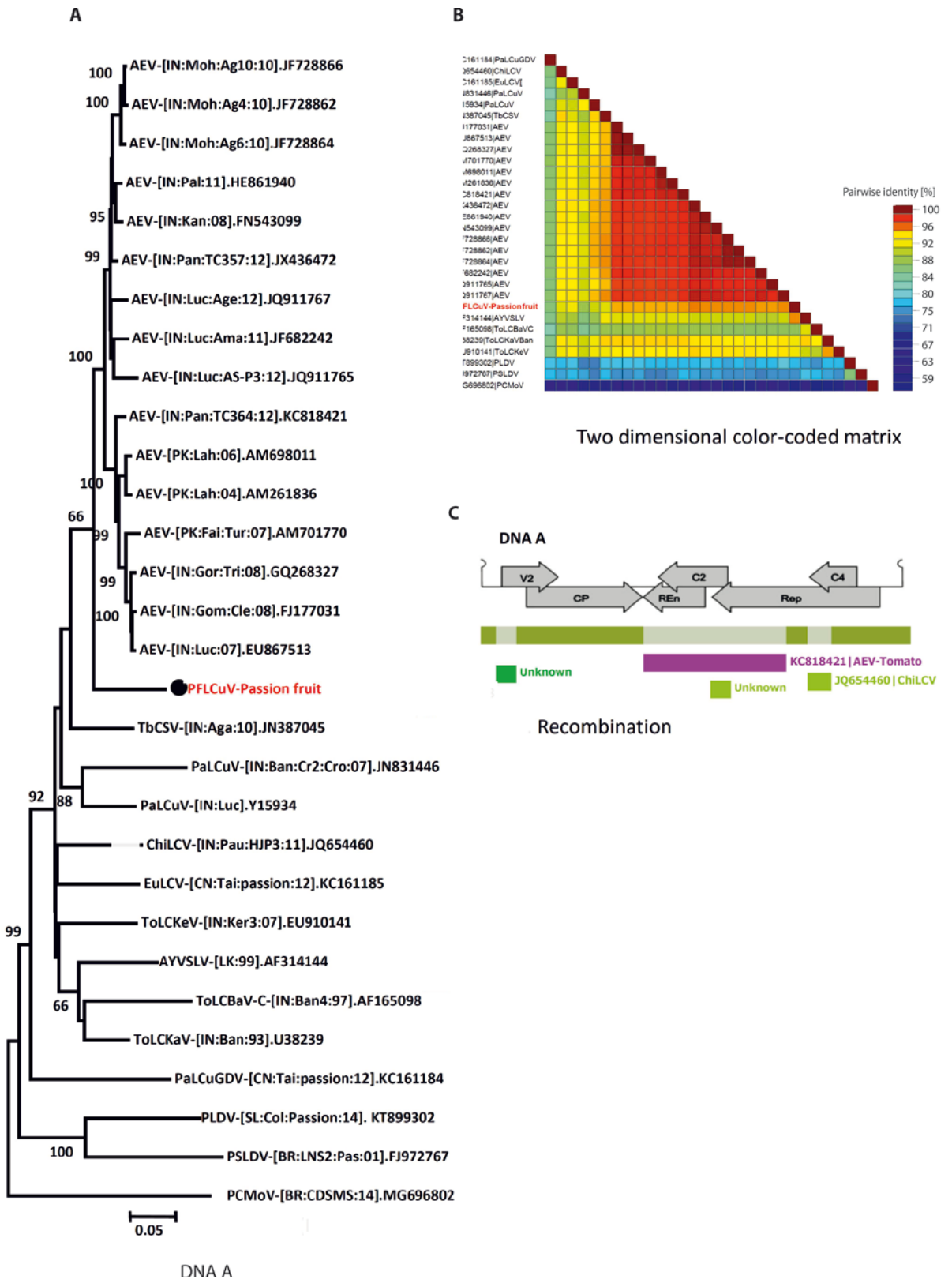

Fig. 2. Phylogenetic analysis of the begomovirus associated with leaf curl disease of passion fruit under study (MK087124) (A) with selected begomoviruses. The phylogeny was drawn using the neighbor-joining method by employing the MEGA7 with 1,000 bootstrap replicates. The pairwise identity for begomovirus associated with leaf curl disease of passion fruit (B) with other selected begomoviruses under study was calculated using Sequence Demarcation Tool. The putative recombination events of begomovirus (MK087124) associated with leaf curl disease of passion fruit were identified by RDP analysis (C). A genomic map of begomovirus and arrangement of genes along with their coding direction nucleotide scale (1 to 2753). The acronyms of begomoviruses were given as *Ageratum enation virus* (AEV) and *Chilli leaf curl virus* (ChiLCV). The indeterminate sequence origin indicated as "unknown". The recombination position in the genome of the begomovirus indicated as a box below, at the top of the diagram. The details of the sequences used for this study are listed in Table 1. The abbreviations indicate an intergenic region (IR), AV2-Pre-coat protein, CP/AV1 – coat protein, Rep/AC1 – replication-associated protein, REn/AC3 – replication enhancer protein, TrAP/AC2 – transcriptional activator protein

identity for demarcation of species (Adams *et al.* 2017). PF1 isolate from passion fruit from the Karnataka State, India showed less than 91% nt sequence identity with other known viruses and can be considered as a new species, for which we propose the name, *Passion fruit leaf curl virus* (PFLCuV) [IN-Kar-18]. This result was also supported by phylogenetic analyses showing that PF1isolate from passion fruit closely clustered with AEV infecting diverse crops on Indian subcontinents and Sri Lanka (Figs. 2A, B).

The amino acid (aa) sequence identities of different ORF were compared with other closely related begomviruses. The results revealed that PFLCuV associated with the passion fruit shared maximum aa identities in AV2, CP, C2 and C3 regions with ORFs of *Ageratum yellow vein Sri Lanka virus* (AYVSLV) and in Rep (C1) and C4 regions with AEV infecting tomato and *Ageratum conyzoides* (Table 4). The nt identity of the IR region of PFLCuV from passion fruit (isolate PF1) had more than 86.5% identity with IR of reported AYVSLV (Table 4). The length of IR in PFLCuV was 287 nt, which is similar to other begomoviruses reported so far.

Attempts were made to amplify a second component (DNA-B) in symptomatic passion fruit plant samples using primers specific to the DNA-B molecule. No amplification was detected in any of the samples indicating that the PFLCuV under study is probably a monopartite begomovirus.

Genome organization of DNA satellites of passion fruit

Since OW begomoviruses are commonly encountered with satellite molecules, the PCR assay was performed using universal primers specific for alpha and betasatellites (Briddon *et al.* 2002; Kumar *et al.* 2010). The PCR product of 1.3 kb and 1.2 kb size products specific to beta and alpha satellites, respectively, were obtained. This indicates the presence of alphasatellite (DNA D1) and betasatellite (DNA β1) in leaf curl disease affected passion fruit plants. The length of DNA D1 and DNA β1 amplified from the infected passion fruit were 1375 nt (Acc No. MK087126) and 1369 nt (Acc. No. MK087125) and were submitted to the NCBI, GenBank. The alphasatellites have a one single ORF in sense (coordinates 89–1036) strand with a coding capacity of 315 aa. The DNA D1 sequence associated with the begomovirus isolate from passion fruit showed high nt identity (95.7%) with *Cotton*

Table 5. Comparison of nucleotide or amino acid sequence identities between alphasatellite with *Passion fruit leaf curl virus* (PFLCuV) from passion fruit with other selected alphasatellite sequences retrieved from NCBI database

Alphasatellites*	Accession numbers	Crop	Complete sequence of DNAD1 (percentage NSI)	Percentage amino acid sequence identity of Rep gene
CLCuMuA	HE966423	cotton	95.7	97.1
CLCuMuA	HE966422	cotton	95.4	96.1
CLCuMuA	LN831970	cotton	78.9	85.9
AYVIA	JX570736	parthenium	82.9	90.1
CLCuMuA	KC305094	wheat	84.4	92.6
AEA	AM930245	milk thistle	83.9	91.7
AEA	FN678899	–	83.5	92.3
BhYVA	FN658718	okra	80.3	88.5
BhYVA	FN658716	okra	80.3	88.5
AEA	KC677736	tomato	85.2	90.1
CLCuMuA	KF584012	cotton	79.9	84.4
AEA	JX913532	pepper	84.9	91.1
AEA	GU385877	cluster bean	85.0	91.7
GDarSLA	JQ322970	papaya	78.9	93.3
TYLCuTHA	AJ579357	tomato	81.6	87.3
SLCuKaA	JX569789	sunflower	81.2	87.9
MaYA	DQ641717	monkey Bush	78.8	86.9

*the species are indicated as: Cotton leaf curl Multan alphasatellite (CLCuMuA), Ageratum yellow vein India alphasatellite (AYVIA), Ageratum enation alphasatellite (AEA), Bendhi yellow vein mosaic alphasatellite (BhYVA), Gossypium darwinii symptomless alphasatellite (GDarSLA), Tomato yellow leaf curl Thailand alphasatellite (TYLCuTHA), Sunflower leaf curl Karnataka alphasatellite (SLCuKaA), Malvastrum yellow mosaic alphasatellite (MaYA). For each column the highest value is underlined

leaf curl Multan alphasatellite (HE966423) (Table 5) isolates originating from the Indian subcontinent infecting cotton. As per the recent classification, the threshold level of alphasatellites was set at 88% (Briddon *et al.* 2018) and the identified satellite was similar to an isolate of *Cotton leaf curl Multan alphasatellite* infecting cotton which belongs to family *Alphasatellitidae*, subfamily *Geminialphasatellitinae* and genus *Colecusatellite.* These results were well supported in phylogenetic analysis (Figs. 3A, B).

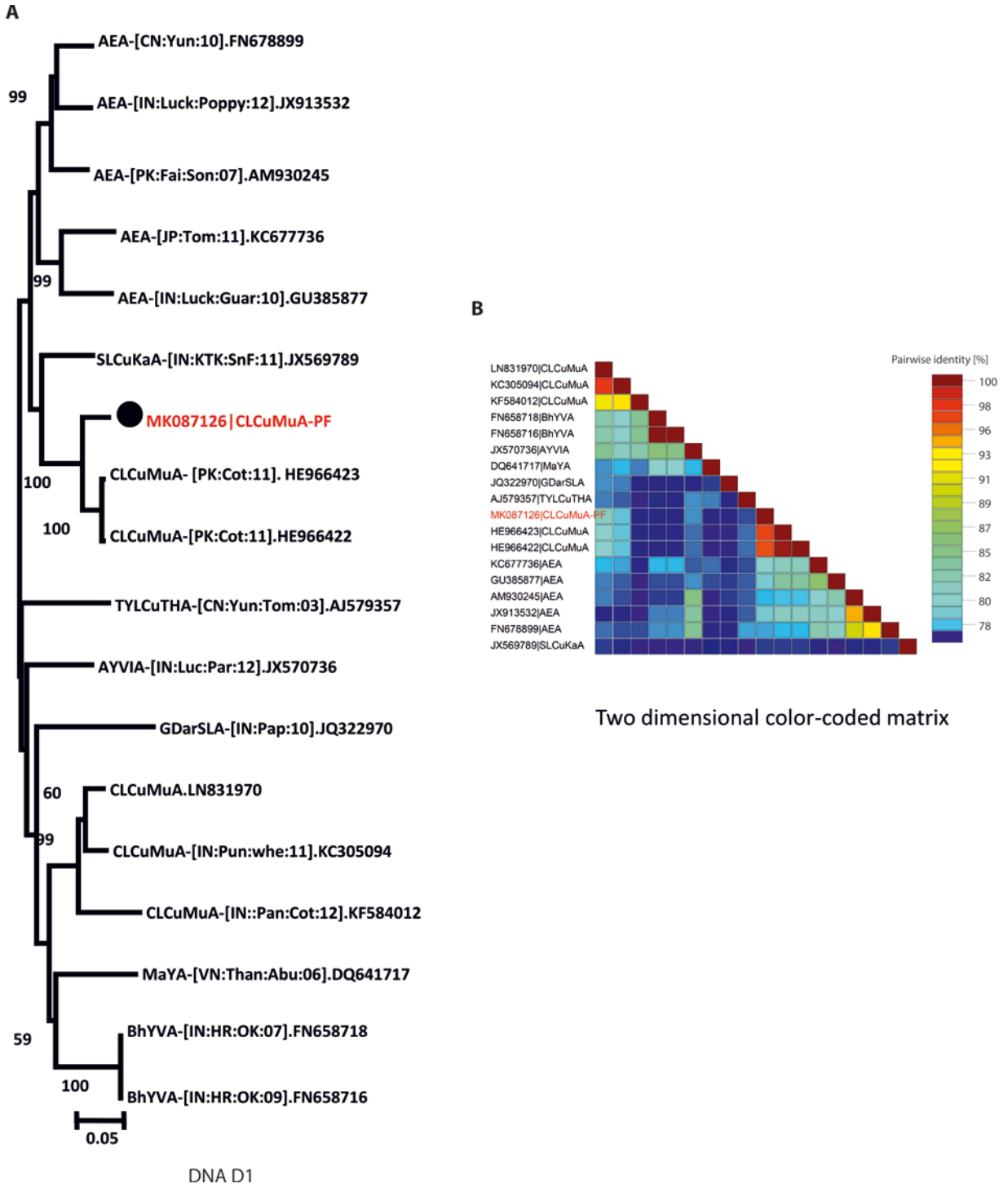

Fig. 3. Phylogenetic relationships of the alphasatellite (MK087126) (A) associated with *Passion fruit leaf curl virus* (PFLCuV) isolated from passion fruit with selected alphasatellites. The phylogeny was drawn using neighbor-joining method by employing the MEGA7 with 1,000 bootstrap replicates. The pairwise identity scores of the alphasatellite were calculated (B) using sequence demarcation tool (SDT). The details of the sequences used for this study are listed in Table 2. The abbreviation indicates a Rep/AC1 – replication--associated protein

Betasatellite isolated from the passion fruit sample associated with begomovirus had characteristics similar to other betasatellites reported so far (Briddon *et al.* 2002; Venkataravanappa *et al.* 2011) and showed maximum nt identity (98.3%) with *Tomato leaf curl Bangladesh betasatellite* (ToLCBDB) infecting chilli crops on the Indian subcontinent (Table 6). As per the classification of betasatellites (Adams *et al.* 2017), the identified betasatellite is closely related to ToLCBDB infecting chilli, which is supported by a phylogenetic tree (Figs. 4A, B).

Recombination analysis

The recombination analysis using RDP4 based on the alignment of PFLCuV DNA-A sequence from passion fruit and other begomviruses indicates an intra specific of recombination in DNA-A like sequence of PFLCuV associated with passion fruit. A recombination break point of 226 nts was identified in the DNA-A molecule of PFLCuV associated with passion fruit shown to be derived from *Passion severe leaf distortion virus* (FJ972767) and *Passion fruit leaf distortion virus* (KT899302) as major and minor parents, respectively. The recombinations were determined at nucleotide positions of 160 and 386 with the probability value of 1.243×10^{-6}. Another recombination breakpoint of 636 nts was identified in the DNA-A molecule of PFLCuV and may be derived from *Ageratum yellow vein Sri Lanka virus* (AF314144), *Ageratum enation virus* (KC818421) as major and minor parents, respectively. Recombination break point was predicted at nucleotide, 1,733 and 2,369 with the *p*-value of 1.208×10^{-52}. The recombination fragment of 140 nts was identified in the DNA-A molecule of PFLCuV with the minor and major parent resembling *Chilli leaf curl virus* (JQ654460) and *Papaya leaf curl virus* (Y15934), respectively. Similarly, another breakpoint of 112 nts was detected with parents resembling *Ageratum yellow vein Sri Lanka virus* (AF314144), *Chilli leaf curl virus* (JQ654460). The breakpoints were also detected between 2,552 and 2,692 nts with

Table 6. Comparisons of nucleotide or amino acid sequence identities between betasatellite (PF1β) with *Passion fruit leaf curl virus* (PFLCuV) from passion fruit with other selected betasatellites sequences retrieved from NCBI data base

Betasatellites*	Accession numbers	Crop	Complete sequence of DNAβ (percentage NSI)	Percentage amino acid sequence identity of βC1 gene
ToLCBDB	KM880104	chilli	98.3	97.5
ToLCBDB	HM007107	chilli	86.9	83.8
ToLCBDB	HM007118	chilli	88.5	87.5
ToLCBDB	HM007105	chilli	89.3	85.0
ToLCBDB	EF190215	chilli	86.9	85.0
ToLCBDB	HM007099	chilli	88.1	86.6
ToLCBDB	JN663876	chilli	89.1	86.6
ToLCBDB	JF706231	chilli	89.0	86.6
ToLCBDB	HM007115	chilli	88.6	82.5
ToLCBDB	DQ343289	chilli	88.9	85.8
ToLCBDB	AY438558	tomato	89.9	86.6
ToLCBDB	HQ180395	tobacco	88.2	88.1
ToLCBDB	HQ180397	tobacco	88.4	86.6
ToLCBDB	HQ257376	okra	87.3	61.3
ToLCBDB	GU732208	okra	87.1	61.2
ToLCBDB	GU732207	okra	88.0	62.0
ToLCB	HM143902	papaya	89.2	85.5
ToLCB	HM143907	papaya	86.7	83.8
CLCuMuB	FJ159274	hibiscus	63.7	27.9
CLCuMuB	EF614159	hibiscus	65.5	27.8
ChLCB	HM143904	chilli	91.8	87.2
PaLCuB	EU126826	papaya	88.0	83.3

*the species are indicated as *Tomato leaf curl Bangladesh betasatellite* (ToLCBDB), *Tomato leaf curl betasatellite* (ToLCB), *Cotton leaf curl Multan betasatellite* (CLCuMuB), *Chilli leaf curl betasatellite* (ChLCB), *Papaya leaf curl betasatellite* (PaLCuB). For each column the highest value is underlined

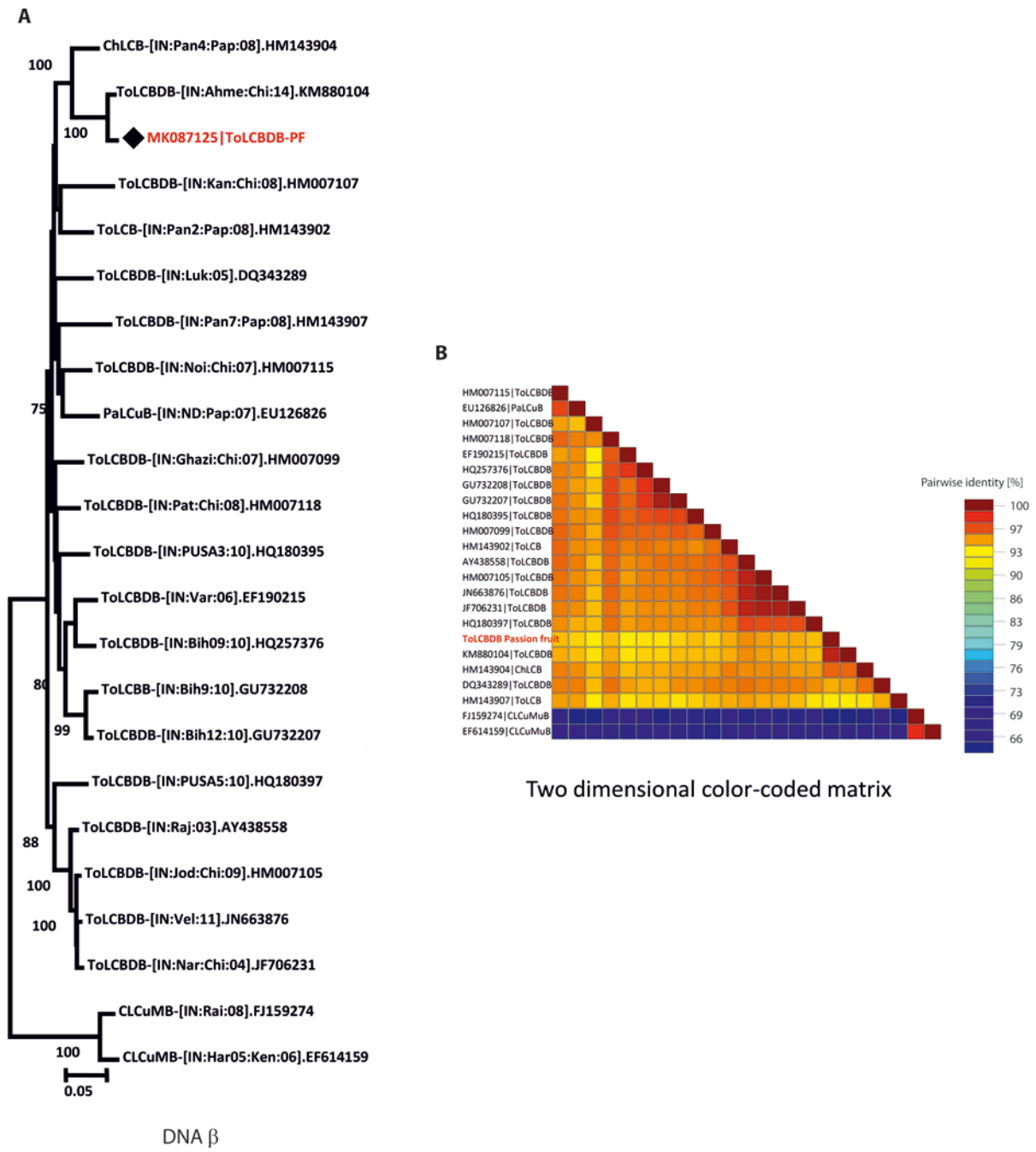

Fig. 4. Phylogenetic relationships of the betasatellite (MK087125) (A) associated with *Passion fruit leaf curl virus* (PFLCuV) from passion fruit with selected betasatellites. The phylogeny was drawn using neighbor-joining method by employing the MEGA7 with 1,000 bootstrap replicates. The pairwise identity scores of the betasatellite were calculated (B) using Sequence Demarcation Tool. The details of the sequences used for this study are listed in Table 3. The abbreviation indicates a satellite conserved region (SCR) and Adenine-rich (A-rich) region

the probability value of 2.853×10^{-2} and at 257 and 2683 nts with the average probability value of 6.723×10^{-3} (Fig. 2C). Further RDP analysis of betasatellite and alphasatellite showed that there is no evidence of recombination in betasatellite and alphasatellite associated with begomovirus infecting passion fruit.

GC plot analysis

Guanine-cytosine (GC) content refers to the proportion of guanine (G) and cytosine (C) in a given stretch fragment of the genome. The GC content of DNA-A of PFLCuV from passion fruit and associated DNA satellites was analyzed using Artemis DNA plotter version 18.1.0 (Figs. 5A, B, C). The GC analysis showed

variation in GC content at different stretches of the DNA-A nucleotide sequence. The innermost circle and bar represent above-average (green) and below average (red) of the GC content in the genome of PFLCuV associated with leaf curl disease of passion fruit, with a window size of 100. However, there was no variation with respect to the GC content across the genome, except in stretches, which fell in the overlapping region of genes encoding DNA-A (AV2-AV1, AC3-AC2 and AC4-AC1). All the genes had stretches of GC rich and GC low regions in the viral genome, except the IR region, which had completely above average GC content (Fig. 5A). Similarly, in alphastellite, the innermost circle and bar represented above-average (black) and below average (dark green) of the GC content. GC plot analysis showed that high GC content was present in the non-coding region of SCR and Rep gene coding region and low GC content was present in the A-rich region (Fig. 5C). However, in the case of betasatellite, the innermost circle and bar represent above-average

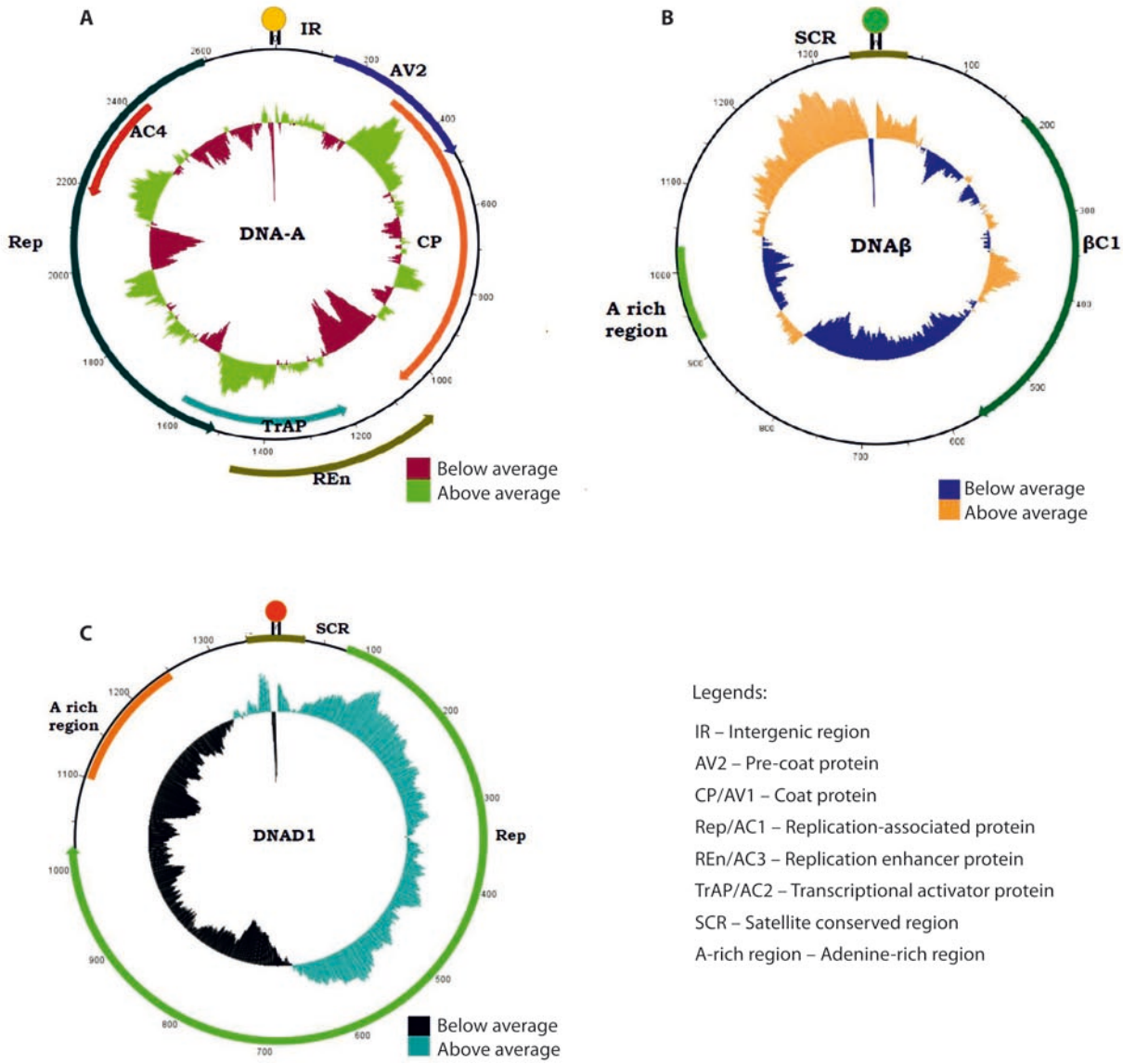

Fig. 5. Guanine-cytosine (GC) analysis *Passion fruit leaf curl virus* (PFLCuV) associated with passion fruit from the passion fruit plants showing leaf curl disease. The outermost ring represents the nucleotide position in DNA-A (A) of the viral genome. Inner color code represents the respective coding genes (AV2, AV1) and AC1, AC2, AC3 and AC4 encoded by the DNA-A of PFLCuV, the innermost circle and bar represent the GC-plot with above average (green) and below average (red) GC content of the genome showing the highest and lowest possible regions of recombination sites. Above-average (orange) and below average (violet) GC content of the betasatellite (B), and above-average (orange) and below average (blue) GC content of the alphasatellite (C) with window size of 100. This analysis was performed using Artemis DNA plotter version 18.1.0, (http://www.sange r.ac. uk/Softw are/Artem is)

(orange) and below average (blue) of the GC content. GC plot analysis showed that the moderate to high GC content was present in the non-coding region of sattelite conserved region (SCR) and the coding region of βC1 gene and low GC content was present in the A-rich region (Fig. 5B).

Discussion

Passion fruit is an important fruit crop for small and marginal farmers that can help increase their productivity and double their incomes (TechnoServe 2010). However, diseases caused by viruses are a major constraint for passion fruit production worldwide (Moreira 2008). Important viral diseases in passion fruit are caused by potyviruses (Brand *et al.* 1993; Sithole-Niang *et al.* 1996; Iwai *et al.* 2006; Nascimento *et al.* 2006). These viral diseases are potentially major threats to passion fruit production and reduce the orchard life span to only a year resulting in 100% yield loss (Trevisan *et al.* 2006). In this study, passion fruit plants showing typical leaf curl symptoms were observed in the orchards and they indicated a possible association of begomovirus infection. The disease was prevalent in all the orchards surveyed indicating its economic importance for passion fruit. Complete genome sequence of begomovirus and associated satellites from passion fruit samples with leaf curl symptoms revealed the association of a novel species, *Passion fruit leaf curl virus* ((PFLCuV) [IN-Kar-18]) with previously reported alphasatellite (CLCuMuA) and betasatellite (ToLCBB) from other crops. Novel begomovirus associated with leaf curl disease of passion fruit fulfilled the criteria of less than 91 nt identity with other known begomoviruses reported so far.

The complete nt sequence of alphasatellite associated with PFLCuV isolated from passion fruit belongs to CLCuMuA infecting cotton in Pakistan. The exact role of this satellite has not been completely shown. However, it was suggested that it has a role in attenuating disease symptoms and maintaining the low level of accumulation of betasatellite in the host (Wu and Zhou 2005). Furthermore, it was reported that rep protein of satellites plays a role in RNAi silencing of begomovirus disease complexes (Nawaz-ul-Rehman *et al.* 2009). However, the role of alphasatellites in passion fruit needs to be established. *Tomato leaf curl Bangladesh betasatellite* isolated from passion fruit associated with PFLCuV showing leaf curl symptoms is a more frequently encountered satellite molecule and has been observed with begomoviruses in tomato and chilli from India (Chattopadhyay *et al.* 2008; Sivalingham *et al.* 2010). The relationship between begomoviruses and betasatellites may be facultative or obligate, in which a few of the begomoviruses required satellites to express the symptoms and in some cases satellite molecules were not required for begomovirus to cause the disease on a particular host (Briddon *et al.* 2001; Sattar *et al.* 2013).

The diverse methods used in the recombination analysis strongly indicated past recombination in the viral genome. The overlapping recombination in the PFLCuV genome associated with leaf curl disease of passion fruit with other begomoviruses is interesting and needs to be resolved. Such intra and inter-species recombinations play major roles in the evolution of begomoviruses (Lefeuvre *et al.* 2007), and lead to the appearance of a new virus species in the agricultural system (Garcia-Andres *et al.* 2007).

The low GC region in DNA-A of the PFLCuV associated leaf curl disease of passion fruit might serve as potential recombination sites for facilitating the evolution of a virus as documented in many viruses infecting plants and animals (Yogindran *et al.* 2021; Robinson *et al.* 2013). The sequence with high GC content has more stability due to a triple hydrogen bond, stacking interactions between the bases and is also linked with topology and orientation of DNA strands (Ninh 2013; Yogindran *et al.* 2021). A higher number of bonds between bases in a DNA strand, generally requires more energy to break the strand. Similarly, high GC content was found in the intergenic region of *Herpes simplex virus* (HSV) genome, linked with a possible role in viral evolution and pathogenesis (Brown 2007).

In the present study, GC content analysis indicates the possible recombination break points was detected in DNA-A of the PFLCuV at below average GC content. The regions of DNA-A, in which recombination is occur were identified as intergenic region (160 and 386 nt recombinations occur), replication-associated protein and AC4 protein (1,733–2,369; 2,552–2,692; 2,571–2,683 nts) respectively. Therefore, the GC plot analysis suggests that PFLCuV associated with leaf curl disease of passion fruit may undergo recombination at below average GC content regions of its genome The literature surveyed also showed similar results in regions having low GC content as potential recombination sites in plant infecting begomovirus (Yogindran *et al.* 2021) and human adenovirus, which may facilitate virus molecules to evolve and also allow a species to increase its host range in a new environmental niche (Robinson *et al.* 2013).

Passion fruit is mainly propagated through seed, grafting as well as stem cutting. However, propagation through seed may not be the preferred method due to lot of variability. Most growers depend on stem cutting and grafted seedlings for their commercial cultivation. The occurrence of leaf curl disease on passion fruit is alarming, signaling the need to utilize planting material free from it. The PCR based detection method

developed in this study will be useful in tackling virus infection early in clonally propagated passion fruit samples. To our knowledge, this is the first record of the novel begomovirus affecting passion fruit in India.

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