CABYV paper

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Current Status, Spatiotemporal Dynamics and Genetic Recombination Analysis of *Cucurbit aphid borne yellows virus* (CABYV) infecting cucurbits Punjab, Pakistan

Abstract

Cucurbit is an important field-grown crop of Pakistan which is severely affected by various diseases. A little information regarding the impact of viral diseases is available, therefore, this study was designed to conduct systematic surveys of the tunnel and open field crops being grown in Punjab, Pakistan during 2019 and 2020. The plant samples depicting virus-like symptoms were collected from the field, with no prior knowledge about sanitation and subjected to reverse transcription-polymerase chain reaction (RT-PCR) for Poleroviruses incidence. Mean higher disease incidence was recorded in Rawalpindi (47.42%) and the mean lowest disease incidence was recorded in district Faislabad (30.95%) with an overall disease incidence of 40.48% during both years. Newly detected Pakistani CABY isolates shared 99.6 % nucleotide identities among themselves and 94.7-96.2% with isolates retrieved from GenBank NCBI. Phylogenetic analysis showed that current study isolates (OL828566, OL828567) clustered in separate clades with isolates JQ700305, JF939814 and X7693 from Taiwan, Spain and France, respectively while isolates from Thailand, China, and Korea were grouped separately. It was evident with restriction fragment length polymorphism (RFLP) analysis that the present study CABY isolates were grouped into two types, which seemed to be genetically similar to those identified during 2011-2015 and 2015-2021. Recombination detection analysis showed that newly detected CABY isolates are likely to be a recombinant of Spanish (JF939814) and South Korean isolates (KR231950) having the recombinant breakpoint between nucleotide position number 272 and 830. Comparison and recombination detection of the local isolates might help in devising a breeding program to identify resistant sources against recombinant isolates.

Keywords: Cucurbits, Recombination, *In silico* RFLP, RT-PCR

1. Introduction

The family *Cucurbitaceaceae* includes several economically significant vegetables and fruit crops grown in various regions of Pakistan (Ahsan et al., 2020). Due to the advantage of favourable climatic conditions in Punjab, most of the cucurbits are grown throughout the year as summer and winter crops (Khokhar, 2014). In Punjab, the main cultivated cucurbit species include melon (*Cucumis melo* L.), cucumber (*C. sativus* L.), squash (*Cucurbita sp*.), gourd (*Luffa* sp.), pumpkin (*Cucurbita moschata*), and watermelon (*Citrullus lanatus* L.). The average yield of cucurbit crops in Pakistan is quite low as compared to other countries because of several RNA based viral diseases prevailing in the region. Among these RNA viruses, *Zucchini yellow mosaic virus* (ZYMV; *Potyvirus*), *Papaya ringspot virus* (PRSV; *Potyvirus*), *Watermelon mosaic virus* (WMV; *Potyvirus*), *Cucurbit aphid-borne yellows virus* (CABYV; *Polerovirus*) *Cucumber mosaic virus* (CMV; *Cucumovirus*) are the significant ones which have been reported to infect cucurbits worldwide (Asad et al., 2019; Ahsan et al., 2020a, 2020b; Ashfaq et al., 2021; Asad et al., 2022).

The *Cucurbit aphid-borne yellows virus* (*Polerovirus*: family *Luteoviridae*) was identified for the very first time in France since 1982 (Knierim et al., 2010). Among cucurbits, the single Polerovirus which was familiar for a high duration of time was CABYV (Lecoq et al., 1992). In Pakistan, it was first time reported in 2020 (Ahsan et al, 2020). Poleroviruses are ssRNA(+) with six open reading frames (ORFs) in an approximately 5.7 kb genome (d'Arcy et al., 2005). The three 5' proximal ORFs (ORF 0-2) were separated from ORF 3-5 (three 3' proximal ORFs) by 200 nucleotides (nt) non-coding intergenic region (IR) (Knierim, Tsai, Deng, Green, & Kenyon, 2013; d'Arcy et al., 2005).

Poleroviruses have effects on the yield of crops but are almost unnoticed due to some reasons because symptoms like leaves become yellow and look like deficiency of any nutrients (Desbiez et al., 2016). CABYV infections are often mixed with infections of other viruses affecting cucurbits (Mnari-Hattab et al., 2009). The initial symptoms due to CABYV on cucumber, watermelon and squash are yellow mottling and patches of interveinal chlorosis which results in coalesce, yellowing, thickening and brittleness of leaves (Lecoq and Desbiez, 2017). Commonly, signs of diseases are retained in the early leaves but significant changes are seen from cultivar to cultivar. In some cultivars, only mild symptoms developed on a few leaves while others showed bright yellowing at the complete plant (Lecoq et al., 1992). Furthermore, a reduction in the number of fruits and dropping of blossoms are often observed (Menzel, 2020). The severity of the

symptoms can change with time, may vary according to the season and are more prominent in the hot season than in cold (Chung et al., 2015). Thickening with yellowing appears on older leaves (Knierim et al., 2010). On young leaves, interveinal chlorosis occurs and dull green veins appear on the earlier leaves. The leaves of infected plants become thicker while the leaf edges roll upwards (Romay and Lecoq, 2014). The transmission of CABYV is possible with the help of particular aphids. The viral transmission is through circulation, persistent and non-propagation manner by specific aphid vectors and there is no possibility of transmission by mechanical ways (Khanal and Ali, 2019). It is demonstrated that transmission of CABYV is possible through *Myzus persicae* and *Aphis gossypii* only (Lecoq et al., 1992).

The data regarding full length and partial sequences of CABY isolates is already being deposited in GenBank from several parts and different geographical regions of the world (Caciagli, 2009; Lecoq and Desbiez, 2012; Romay and Lecoq, 2014, Ahsan et al., 2020). Several Asian countries including Thailand, Philippines, and Taiwan have described another cucurbit infecting polerovirus that emerged as recombinant isolates between melon aphid-borne yellows virus and CABYV (Knierim et al., 2010; 2014). Whereas, the scientists in Brazil recently described a recombinant isolate of CABYV and undetermined polerovirus (Costa et al., 2019). Two major molecular groups; basal Asian and Mediterranean have been defined among non-recombinant isolates and Eastern Asia represented minor highly divergent groups (Kassem et al., 2013; Kwak et al., 2018). In Pakistan, limited research on CABYV has been done and scarce information about its molecular diversity is available. Therefore, this study aimed to characterize the variability of Pakistani CABYV isolates using movement protein (MP), RdRp, and partial coat protein (CP) sequences followed by its relationship with other reported isolates.

2. Materials and Methods

2.1 Field surveys for polerovirus in Punjab.

Surveys were carried out in Punjab (Rawalpindi, Chakwal, Sialkot, Faisalabad, Multan, Vehari, Bahawalpur, Mazafarghar, Khanewal and Lodhran) during 2019 and2020 for the detection of Poleroviruses infecting cucurbits (Fig. 1). In each season, randomly selected cucurbits open fields and tunnels were visited and 650-670 samples depicting suspected Poleroviruses symptoms notably interveinal chlorosis which results in coalesce, yellowness, and thickness and brittleness of leaves (Ahsan et al., 2020) were collected (Fig 2). The coordinates of surveyed sites were recorded using Global Positioning System

(GPS) and visited again for sample collection. Data regarding poleroviruses, varietal response, vector and its control was collected from surveyed farmers using a brief questionnaire.

2.2 Screening of polerovirus using group-specific degenerate primers

In a preliminary screening for the prevalence of Poleroviruses, TRIzol® Reagent (Cat# 15596, Life Technologies, Carlsbad, USA) was used to extract total RNA followed by quantification using Nanodrop (Thermo Scientific Co. USA) as per the instruction manual. Nuclease-free water was used to make a 500 ng/µL RNA working dilution followed by synthesis of first-strand cDNA using Revert Aid Reverse Transcription Kit (K1691, Thermo Fischer Scientific, USA) and PococpR-140 (Xiang, Shang, Han, Li, and Yu (2008) as reverse primer. PCR amplification was done from the resultant cDNA through poleroviruses specific degenerate primers PococpR-140 and PococpF-139 as described by Xiang, Shang, Han, Li, and Yu (2008). A pre-stained 1% (w/v) agarose gel was used to examine the PCR product using electrophoresis followed by visualization under UV trans-illuminator (Vilber Lourmat, S. No. 6532). A sample for poleroviruses was considered positive if a band of 1.4 kb was detected and the below-given formula was used to calculate the disease incidence (Rao et al., 2002).

$$\%\, D.I.of\ Polerovirus = \frac{No.of\ RT-PCR+ive\ samples}{Total\ no.of\ tested\ Samples} x10$$

2.3 Cloning and sequencing

GeneJET PCR Purification Kit (Thermoscientific, USA) was used to purify the positive amplicons of 1.4 kb and propagated through *Escherichia coli* XL1-Blue as the host of pTZ57R/T TA cloning vector (K1214, Thermo Scientific, USA). To purify recombinant plasmid DNA, GeneJET Plasmid Miniprep Kit (K0503, Thermo Fisher Scientific, USA) was used as per manufacturer directions. The existence of an insert in transformants was validated using restriction digestion with ECoR1 enzyme and positive colonies were sent to Macrogen (South Korea) for sequencing in both directions. NCBI BLAST tool was used to compare the obtained sequences with polerovirus sequences available in GenBank.

2.4 Phylogenetic analysis of Polerovirus

Polerovirus sequences of the present study and sequences submitted from other parts of the world were retrieved from GenBank and aligned with ClustalW embedded in MegaX software (Kumar et al., 2018). After alignment in MegaX software, the Maximum Likelihood method with 1000 bootstrap replicates was used to detect their ancestral lineage and phylogenetic relationship. BioEdit v7.2.6.1 having the Sequence Identity Matrix option was used to determine the nucleotide and amino acid sequences identities (Hall, 1999).

2.5. In silico RFLP and recombination analysis

In silico restriction fragment length polymorphism (RFLP) of CP gene of our isolate from this study and 30 other CABY isolates retrieved from GenBank, was performed using BccI restriction enzyme in CLC Main Workbench 20 (https://www.qiagenbioinformatics.com/). The resulting virtual gel was analysed in PyElph v1.4 (Pavel and Vasile, 2012) for serotyping of isolates and construction of phylograms. Recombinant events in the two new Pakistani and 10 other CABYV isolates were analyzed with RDP4 (Martinl et al., 2017) using all the available methods with default settings viz. MaxChi, BootScan, RDP, 3SEQ, Siscan, GENECONV, PhylPro and Chimaera.

3. Results

3.1.*Polerovirus screening*

During 2019-2020, the incidence of Poleroviruses was tested in 671 symptomatic samples of cucurbits through RT-PCR with group-specific degenerate primers PococpR-140 and PococpF-139 (Xiang, Shang, Han, Li, and Yu, 2008). In 2019, 123 samples of 308 produced 1.4kb RT-PCR product of expected size while 149 of 363 samples produces the expected product in 2020. The production of the expected 1.4kb RT-PCR product implies the poleroviruses incidence. Based on RT-PCR results, the disease incidence percentage was calculated as shown in Table 1. Overall 40.48 % of disease incidence was recorded in Punjab, Pakistan during 2019 and 2020. In 2019, higher disease incidence (45.45%) was recorded in Rawalpindi, followed by Khanewal (44.44), Multan and Lodhran (42.85%), Sialkot (40%), Vehari (37.77%), Muzaffargarh (37.50%), Bahawalpur (35.71%), Chakwal (33.33%) and lowest in Faisalabad (28.57%). A relatively higher disease incidence was recorded in 2020 with the same trend as observed previously in

Rawalpindi (50%) as highest and Faisalabad as lowest (33.33%). This higher incidence may be attributed to the already establishment of aphids as a vector of poleroviruses that are actively transmitted by aphids during the growing season.

3.2. Molecular characterization and phylogenetic analysis

Genus specific primers PoconF-139/PococpR-140 were used to characterize poleroviruses based on RdRp and CP gene and the amplified fragment of 1.4 kb of RdRp-CP gene from maximum number of cucurbits samples (Xiang et al., 2008). BLASTn revealed that the sequence consisted of partial RdRp, entire CP, and overlapping MP genes. Furthermore, all Pakistani Polerovirus isolates show a conserved sequence of eight nucleotides ACAAAAGA immediately upstream of the intergenic NCR, which is identical to the first eight residues of the 5'terminus NCR and has been acknowledged as the transcription beginning site for polerovirus subgenomic RNA1. After careful analysis, two isolates of CABYV, have been submitted to GenBank with accession numbers given in Table 2.

BLASTn analysis revealed that each sequence has 597 nucleotides that encode 198 amino acids (aa) of incomplete RdRp gene, 199 nucleotides of intergenic-NCR, and 600 nucleotides that encode 299 CP gene, and 576 nucleotides that encode 192 aa of MP that overlaps CP gene. All Pakistani CABYV isolates had a conserved sequence of ACAAAAGA immediately upstream of the intergenic NCR and a CP conserved motif "GILKAYHE" at position 94-101.

Sequence Identity Matrix results show that the current study Pakistani CABYV isolates shared 99.6 % of nucleotide identity among themselves while 94.7-96.2% identity with previously reported CABYV isolates In case of amino acid they share 99.7 % similarity with each other and 94.2-96.9% similarity with other isolates reported from world used in this study for analysis. Isolate OL828566 of this study share 96.2% similarity with JQ700305 from Taiwan and 95 and 95.5% similarity with X76931 and JF939814 from France and Spain respectively. Similarly OL828567 isolate share 95.3% similarity with JF939814 from Spain and 95-95.2% with JQ700305 and X76931 from Taiwan and France, respectively. A similarity of 94.2-96.9% was evident between amino acid based sequences of present study isolates and isolates reported from elsewhere (Table 2). Phylogenetic analysis shows that current study isolates form separate clades with isolates JQ700305, JF939814 and X7693 from Taiwan, Spain and France respectively while other isolates from China, Thailand and South Korea form separate

clad (Fig 3). Phylogenetic relationship analysis revealed that CABY Pakistani isolates didn't follow the geographic lineage i.e. Asiatic and Mediterranean.

3.3. In Silico RFLP and spatiotemporal analysis

In silico RFLP simulation of CP gene of CABY by using *BccI* delineates 32 isolates into two different patterns (Pattern I and Pattern II). Pattern I and pattern 2 consisted of approximately 120 bp and 206 bp fragments, respectively (Fig. 4). Of all isolates studied, 9 and 17 isolates corresponded to the pattern I and pattern II, respectively while the other six are considered as others. Interestingly it is observed that both the genetic groups were present in both time periods (2011-2015 and 2015-2021) but all the accession of the CP gene were not equally distributed into two genetic groups however few accession were not found in either group categorized as other. Interestingly it is observed during the study, that in group 1 (Pattern I) only accessions from Asia are present but in group 2 (pattern II) isolates from Asia, Europe and America are present. One noteworthy observation from the spatial and temporal study is that genetic variation and geographical distribution of CABY increased after 2015 than before 2015.

3.4. Genetic recombination analysis

For genetic recombination analysis, all of the methods provided in RADP4 with default settings were used to evaluate recombination breakpoint events in the understudied gene of CABY isolates presented in Table 3, Fig 5. The analysis found that two recombination events were detected, one of which (Event 2) was significant because it was recognized by more than three methods out of nine methods with a significant p-value, while the other was not. Event no 2 revealed that Pakistani isolates OL828566 and OL828567 of CABY virus might be originated from possible recombination between KR231950 as a major parent with 96.9% similarity and an unknown minor parent might be JF939814. This event was significantly detected with nine methods i.e. GENECONV, MaxChi, BootScan, RDP, Chimaera, 3Seq, LARD, Phylprp and SiScan. The recombination breakpoint was ranged between 269-832, 272-830, 272-830, 271-828, 271-830, 272-830, 271-832,272-828 and 269-828 nucleotide in GENCONVE, Chimaera, MaxChi, LARD, Phylpro, SiScan, 3Seq, BootScan and RDP respectively. The P-value for each method is presented in Table 3.

4. Discussion

Pakistan is a major contributor to cucurbits production in Asia with an area of 67360 ha under cucurbit cultivation with 1 million tonnes of yield (Statistics of Pakistan 2019-2020). The cucurbit production is hampered by many biotic and abiotic factors generally and viral diseases particularly. According to our assessment of the current scenario of CABY in cucurbits samples showing yellowing symptoms with an overall 40.48% disease incidence is emerging and a major threat to cucurbits production in major cucurbits producing areas of Punjab, Pakistan as well as all over the world (Juárez et al., 2013; Ahsan et al., 2020; Desbiez et al., 2020). CABY virus reproted to be the most frequent virus damaging cucurbits in temperate, Mediterranean, and subtropical climates (Lecoq et al., 1992). Two aphid species; *M. persicae* and *A. gossypii* were found to be involved in the transmission of CABYV in a persistent and circulative manner. Therefore, the cultivated or wild plant species could be linked with the high prevalence and occurrence of CABYV while the earlier may serve as a source of inoculum for its spread. (Kassem et al., 2013; Khanal and Ali, 2019). Furthermore, CABYV-infected plants have been shown to alter aphid feeding behaviour in such a way that it helps in virus acquisition while healthy plants were preferably used by viruliferous aphids to settle (Carmo-Sousa et al., 2016). This altered feeding behavior of aphid along with overlapping system of cucurbit and existence of wild plants near cultivation fields may lead to the high prevalence of CABYV in those cucurbit crops lacking CABYV-resistant sources. BLASTn revealed that sequence consisted of partial RdRp, entire CP, and overlapping MP gene. Moreover, an intergenic non translated region (NTR) of 199-203 nucleotide region was also observed between RdRp and CP which is in accordance with the finding of Xiang et al. (2008), Kassem et al. (2013), Knierim, Tsai, and Kenyon (2013). Detailed sequence compassion revealed that all Pakistani Polerovirus isolates show a conserved sequence of eight nucleotides ACAAAAGA immediately upstream of the intergenic NCR, which is identical to the first eight residues of the 5'terminus NCR and has been acknowledged as the transcription beginning site for polerovirus subgenomic RNA1 (Beuve et al., 2008; S. Chen et al., 2016; Guilley, Scheibel, Richards, Lecoq, and Jonard, 1994; Krueger, Beckett, Gray, and Miller, 2013; Pazhouhandeh, 2007) and a CP conserved motif “GILKAYHE” (Guilley et al., 1994). Sequence Identity Matrix results show that the Pakistani isolates from the current study shared 99.6% of nucleotide identity among themselves while 94.7-96.2% identity with previously reported CABYV isolates. Phylogenetic analysis shows that the current study

isolates form separate clades with isolates JQ700305, JF939814 and X7693 from Taiwan, Spain and France respectively while other isolates from China, Thailand and South Korea form separate clad. Phylogenetic relationship analysis revealed that CABY Pakistani isolates didn't follow the geographic lineage i.e. Asiatic and Mediterranean as observed by Kassem et al. (2013), Kwak et al. (2018) and Shang, Xiang, Han, Li, and Yu (2009). The key sources of genetic variability in RNA viruses are mutation, reassortment, and recombination (Akinyemi, Wang, Zhou, Qi, and Wu, 2016; Holmes, 2006). This could lead to the encapsulation of discrete sequence components, as well as the repetition, interchange, or obliteration of existing viral elements. All of the methods provided in RADP4 with default settings were used to evaluate recombination breakpoint events. (Martin, Murrell, Golden, Khoosal, and Muhire, 2015) for estimation of recombinant events in the CP+MP+RdPh gene of CABY isolates. The analysis found that two recombination events were detected, one of which (Event 2) was significant because it was recognized by more than three methods out of nine methods with a significant p-value, while the other was not. Currently, various recombinant polerovirus isolates including CABY virus isolates, have been identified as significant (Knierim et al., 2010; Costa et al., 2020), which necessitates extensive studies on the genetic variability of CABYV infecting cucurbit crops. RFLP analysis with BccI suggested that the simulated CABYV isolates may be alienated into three genetic groups i.e. group 1 (Pattern I) consist of nine isolates, group 2 contains 17 isolates with pattern II while remaining six isolates with no definite pattern are considers as others. Interestingly it is observed that both the genetic groups were present in both time periods (2011-2015 and 2015-2021). Similar patterns were also observed by (Garcia-Arenal, Fraile and Malpica, 2001; Kassem et al., 2007). The presence of recombinant isolates and their uneven spatiotemporal dynamics threaten efficient crop production which should be resolved by appropriate viral diagnostic and management strategies.

5. Conclusion

Conclusively, this study suggests that CABYV is an emerging threat to cucurbit crops, though the extent of damages posed by yellows disease in cucurbits is unknown. It's important to estimate epidemics caused by plant viruses as they are frequently emerging and transmitted by diverse species of plants as well as varies according to the different agro-ecological zone that can affect viral disease epidemiology. This spatial/temporal flow of viral infections between overlapping crops could play a significant role, as it may obstruct early disease diagnosis. Therefore, the

response of cultivated and wild plant species against genetically diverse CABYV population and its distribution study is need of the hour that would help in the measures related to disease control.

Acknowledgement

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Table 1.Incidence pecrentage of cucrbit infecting Poleroviruses in Punjab, Pakistan

S. No.	Sampling Sites	2019		2020	
		RT-PCR		RT-PCR	
		+Ve/Total	%D.I	+Ve/Total	%D.I
1	**Rawalpindi**	15/33	45.45	15/30	50
2	**Chakwal**	10/30	33.33	15/40	37.50
3	**Faisalabad**	2/7	28.57	5/15	33.33
4	**Vehari**	17/45	37.77	19/45	42.22
5	**Mazafarghar**	15/40	37.50	20/45	44.44
6	**Multan**	15/35	42.85	15/33	45.45
7	**Lodhran**	15/35	42.85	18/40	45
8	**Bahwalpur**	10/28	35.71	20/45	44.44
9	**Khanewal**	20/45	44.44	25/55	45.45
10	**Sailkot**	4/10	40	7/15	46.66
	D.I in Punjab	123/308	39.93	149/363	41.04

Table 2. Nucleotides and Amino acid Identity %ages of Pakistani CABYV isolates with others

Accession No.	Location	Host	Year	NT identity % of Pakistani isolates		AA identity % of Pakistani isolates	
				OL828566	OL828567	OL828566	OL828567
JF939814	Spain	*Cucurbita pepo*	2013	95.5	95.3	96.6	96.9
X76931	France	unknown	1994	95	95.2	94.4	94.7
KF791040	Thailand	*Cucumis sativus*	2015	94.6	93.9	94.7	94.9
KR231947	South Korea	*Cucumis melo*	2015	94.2	94.5	96	96.2
EU636992	China	*C. melo var. cantalupensis*	2010	93	94	95.6	95.8
KR231950	South Korea	*Cucumis melo*	2015	94.2	94.3	94.9	95.1
KF815682	Thailand	*Cucumis sativus*	2015	94.3	94.4	95.1	95.3
EU000535	China	*C. argyrosperma*	2008	94	94	94.9	95.1
JQ700305	Taiwan	*Momordica charantia*	2012	96.2	95	94.2	94.4
GQ221223	China	*Cucurbit asp.*	2012	93.8	93.5	95.1	95.3

Table 3. Recombination events identified in Pakistani isolates of CABYV sequence

Recombinant Isolate	Breaking point		Major Parent	Minor Parent	P-Value								
	Starting	Ending			RDP	GENECONV	Bootscan	MaxChi	Chimaera	SiScan	3SEQ	LARD	PhylPro
OL828566	272	830	KR231950	JF939814	NS	9.850×10^{-2}	NS	1.505×10^{-1}	7.725×10^{-2}	NS	1.889×10^{-2}	NS	NS
OL828567	272	830	KR231950	JF939814	NS	9.850×10^{-2}	NS	1.505×10^{-1}	7.725×10^{-2}	NS	1.889×10^{-2}	NS	NS

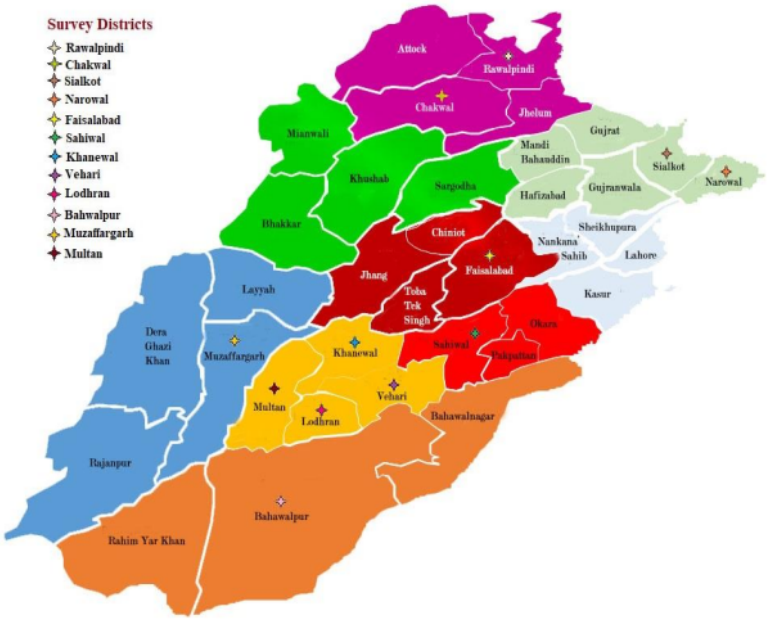

Fig 1. Map of Survey Districts

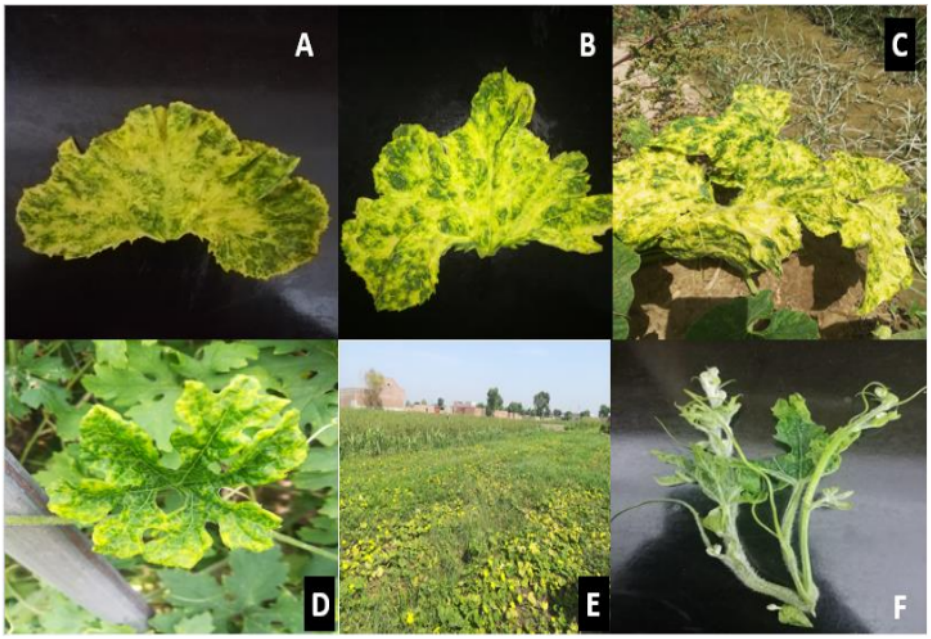

Fig 2. Typical symptoms of CABY infecting cucurbits; **A** (Round gourd), **B** (Pumpkin) **C** (Squash), **D** (Bitter gourd), **E** (Ridge gourd field) and **F** (Watermelon)

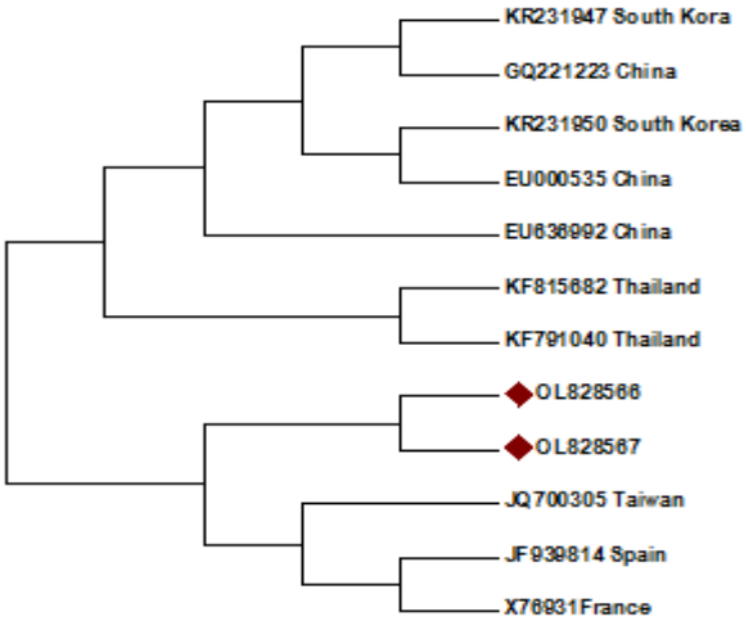

Figure 3. Evolutionary relationships based on all the understudied genes of nucleotides sequences of Pakistani CABYV isolates with already reported sequences

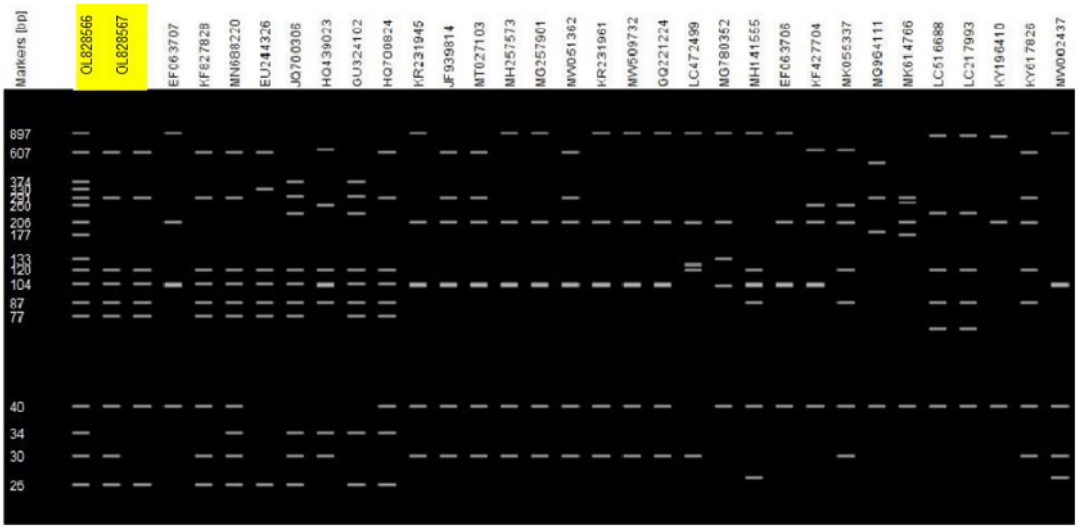

Figure 4. In silico RFLP analysis and genotyping of 32 isolates of CABY

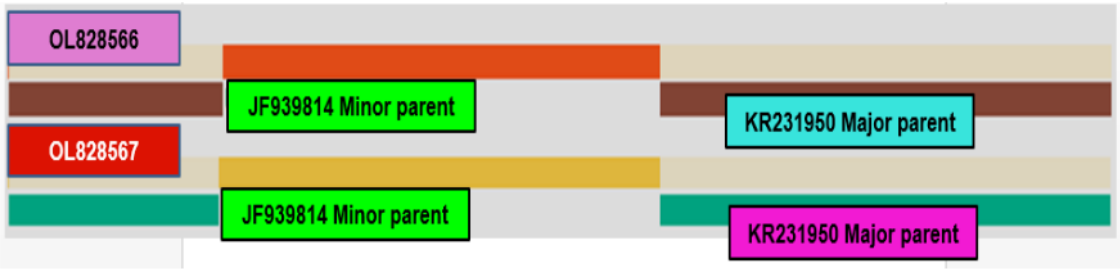

Fig 5. Recombination events identified in Pakistani CABYV isolates employing available methods in RDP4 with major and minor parents

CABYV paper

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