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Original article

Natural occurrence of mesta yellow vein mosaic virus and DNA-satellites in ornamental sunflower (*Helianthus* spp.) in Pakistan

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ABSTRACT

Weeds and ornamental plants serve as a reservoir for geminiviruses and contribute to their dissemination, genome recombination and/or satellite capture. Ornamental sunflower (*Helianthus* spp.) plants exhibiting mild leaf curl symptoms were subjected to begomovirus and DNA-satellites isolation. The full-length genome of the isolated begomovirus clone (Od1-A) showed 96.8% nucleotide (nt) sequence identity with mesta yellow vein mosaic virus (MeYVMV; accession no. FR772081) whereas, alphasatellite (Od1-a) and betasatellite (Od1-b) clones showed their highest nt sequence identities at 97.4% and 98.2% with ageratum enation alphasatellite (AEA; accession no. FR772085) and papaya leaf curl betasatellite (PaLCuB; accession. no. LN878112), respectively. The evolutionary relationships, average evolutionary divergence and the recombination events were also inferred. The MeYVMV exhibited 9.5% average evolutionary divergence and its CP and Rep had 9.3% and 12.2%, concomitantly; the alphasatellite and the betasatellite had 8.3% and 5.2%, respectively. The nt substitution rates (site^{-1} year^{-1}) were found to be 6.983×10^{-04} and 5.702×10^{-05} in the CP and Rep of MeYVMV, respectively. The dN/dS ratio and the Tajima D value of MeYVMV CP demonstrated its possible role in host switching. The absolute quantification of the begomovirus demonstrated that mild symptoms might have a correlation with low virus titer. This is the first identification of MeYVMV and associated DNA-satellites from ornamental sunflower in Pakistan. The role of sequence divergence, recombination and importance of MeYVMV along with DNA-satellites in extending its host range is discussed.

1. Introduction

Arthropod-transmitted begomoviruses with circular single-stranded DNA (cssDNA) genomes in the *Geminiviridae* family have been recently classified into nine genera (Zerbini et al., 2017). Out of these, the members in the genus *Begomovirus* represent the major sub-group of the *Geminiviridae* family encompassing ~409 virus species and cause global economic losses to the cultivated crops in the warm to temperate regions. Begomoviruses exclusively infect dicotyledonous plants through a non-propagative and circulative mode of transmission with whitefly *Bemisia tabaci* (Brown et al., 2015). These are further categorized into bipartite begomoviruses (two components with genomic size ca. 2600 nucleotides (nt) each) and monopartite begomoviruses (a genome homolog of DNA-A). The genome of begomoviruses is a very peculiar type and five-to-six open reading frames (ORFs) are encoded on DNA-A. All the ORFs are encoded in both virion-sense and complementary-sense orientation and are commonly transcribed by a common bi-directional promoter (Briddon et al., 2012). However, just two ORFs, nuclear shuttle protein (NSP) and movement protein (MP), are encoded by DNA-B components in opposite orientations (Noueiry et al., 1994, Sattar et al., 2013). The monopartite begomoviruses associated with sub-genomic cssDNA-satellites; alphasatellite, betasatellite and/or deltasatellite have been dominantly found in the Old World (OW). The genome of alphasatellites is half (ca 1350 nts) of the helper begomovirus encoding a single ORF, replication-associated protein (Rep), in the virion-sense

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orientation. Alphasatellites have been recently classified as a subfamily *Geminialphasatellitinae* of the family *Alphasatellitidae* (Briddon et al., 2018). Interestingly, a dominant majority of monopartite begomoviruses are often reported to be associated with alphasatellites and betasatellites within the same host plant. However, in the New World (NW), monopartite begomovirus infections are sporadic and alphasatellites are found associated with only a few bipartite begomoviruses (Paprotka et al., 2010, Romay et al., 2010). The precise role of gemini-alphasatellites is still unclear; however, they may exacerbate virus symptoms and/or interfere with begomovirus or betasatellite accumulation in the infected plants (Idris et al., 2011, Kon et al., 2009, Mar et al., 2017). Moreover, the Rep protein of some alphasatellites has a role in suppressing the post-transcriptional gene silencing (PTGS) of the host plants (Abbas et al., 2019, Nawaz-ul-Rehman et al., 2010). The members of the genus *Betasatellite* (family *Tolecusatellitidae*) are diverse cssDNA molecules (ca 1350 nts) mostly found in the OW (Adams et al., 2017). Their association with the helper begomovirus is a pre-requisite for their replication, encapsidation and insect transmission. These molecules encode a single ORF βC1 and assist their helper virus for successful infection, pathogenicity and overcome host plant defense (Briddon et al., 2003). The third type of cssDNA-satellites is ca. 700 nts called deltasatellites (genus *Deltasatellite*, family *Tolecusatellitidae*), which do not encode any functional ORF in their genome (Lozano et al., 2016).

Different biotic stresses, including viruses, pose a significant threat to sunflower cultivation in the Indo-Pak sub-continent (Ma et al., 2018). The wild and cultivated sunflower (*Helianthus* spp.) plants host many bipartite and monopartite begomoviruses including ageratum enation virus (AEV), croton yellow vein mosaic virus (CrYVMV), tomato leaf curl Karnataka virus (ToLCKV), tomato leaf curl New Delhi virus (ToLCNDV) and tomato leaf curl Gujrat virus (ToLCGV) (Govindappa et al., 2011, Kumar et al., 2016a,b, Sharma et al., 2018, Vanitha et al., 2013, Vindyashree et al., 2015). Begomoviruses infecting agro-economical important crops have been extensively investigated; however, less information is available about the begomoviruses infecting wild and/or ornamental host plant species. During the last few decades, the host range of begomoviruses has been extended to weeds, ornamental and wild plants due to many biotic factors including mutation, recombination, pseudo-recombination, emergence and spread of whitefly vectors and certain environmental factors (Qurashi et al., 2017, Sattar et al., 2018). Therefore, it is necessary to keep tracking the virome architect of these non-cultivated host plants. To our knowledge, no begomoviruses have yet been reported to infect sunflower species (cultivated, ornamental or wild) in Pakistan. In this study, a complete monopartite begomovirus-complex infecting ornamental sunflower was isolated and characterized.

2. Material and methods

2.1. Leaf samples and DNA isolation

In the year 2016, ornamental sunflower plants exhibiting begomovirus like mild leaf curl symptoms were collected from an orchard near Lahore, Pakistan (31.431899 "N; 74.34087 "E) (Fig. 1). The collected leaf samples of four symptomatic plants (Od1- Od4) exhibiting mild upward leaf curling and two asymptomatic plants; Ad1 (Fig. 1E) and Ad2 were subjected to total genomic DNA isolation using the GeneAll®Exgene™plantSV mini 100P kit (GeneAll Biotech. South Korea) as suggested by the manufacturer.

2.2. Amplification, cloning, and sequencing

Total genomic DNA extracts from all sunflower plants were used to amplify putative viral circular molecules using Phi-29

Fig. 1. Ornamental sunflower (*Helianthus* spp.) plants showing mild leaf curling symptoms **(A)** Od1, **(B)** Od2, **(C)** Od3 and **(D)** Od4, and the healthy sunflower plant **(E)** Ad1.

DNA polymerase (Thermo Fisher Scientific) as described by Qurashi et al. (2017). The obtained rolling circle amplification (RCA) products were further diluted (5x) and used as a template in the subsequence PCR assays. The universal degenerate primers BegomoF/R (Shahid et al., 2007), DNA101/DNA102 (Bull et al., 2003), and β01/β02 (Briddon et al., 2002) were employed in independent PCR reactions to acquire the complete begomovirus, alphasatellite and betasatellite genomes, respectively. Restriction fragment length polymorphism (RFLP) of the resultant PCR amplicons was examined to decipher the presence of multiple begomoviruses using different restriction enzymes (including *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Sal*I, PstI). Each enzyme yielded a unanimous digestion pattern (data not shown), affirming the presence of a single begomovirus genome. After RFLP investigations, one PCR amplicon of each genome component (begomovirus, betasatellite and alphasatellite) from Od-1 plant was gel purified, cloned into the pTZ57R/T cloning vector (Thermo Fisher Scientific), and completely sequenced (FirstBASE Laboratories Sdn + Bhd, Malaysia).

2.3. Sequence comparisons and phylogenetic analysis

The full-length genome of the identified begomovirus and associated DNA-satellites genomes were compared to the closely matched sequences in a BLASTn search (http:www.ncbi.nlm.nih.gov). Based upon their highest nt sequence identities, the highly matched sequences were selectively retrieved from the database and values for pairwise nt sequence identities were calculated in

the species demarcation tool (SDT v1.2) for each component (Muhire et al., 2014).

Phylogenetic trees were constructed in MEGA7 software with Maximum-Likelihood (ML) algorithm and the predicted best-fit Kimura-2 parameter model with 1000 iteration values selected (Kumar et al., 2016a,b). The ORFs were predicted in ORF finder tool available at NCBI GenBank database.

2.4. Assessment of genetic variability, divergence and substitution rate

Genetic variability in the sequences of the isolated begomovirus components compared to their respective sequences retrieved from GenBank database was estimated in DnaSP v. 6.12 (Rozas et al., 2017). Various parameters viz. insertions and deletions (InDels), total number of mutations (Eta), total number of polymorphic (segregating) sites (S), total number of singleton mutations (Eta S), average number of nt difference between sequences (k), nt diversity (π), Watterson's estimate of the population mutation rate based on the total number of segregating sites (Θ-W) and total number of mutations (-Eta) were calculated (Lima et al., 2017). The extent of nt diversity (π) among MeYVMV sequences was assessed in DnaSP (ver 6.12) program (Rozas et al., 2017) with a window length of 100 and a step size of 25. The nt substitution rate per site and mutation at various codon positions of the begomovirus-encoded coat protein (CP) and Rep, alphasatellite-encoded Rep, and betasatellite-encoded βC1 were also assessed in BEAST (v.1.8.5) using Bayesian Markov Chain Carlo method (Drummond and Rambaut, 2007). Both strict and relaxed molecular clock (uncorrelated exponential and uncorrelated lognormal) were used to analyse each dataset. Tracer (v 1.5) was used to infer coalescent constant demographic and best-fit clock models. Effective sample sizes (ESS) >200 were observed for all the tested parameters.

MEGAX was used to infer the best-fit nt substitution model using Bayesian information criterion (BIC) and Akaike IC (AIC) scores, Tajima's neutrality test, and average evolutionary distances (Kumar et al., 2018). Additionally, the Pamilo–Bianchi–Li (PBL) method was executed, in MEGA X, to infer pairwise genetic differences at non-synonymous (dN), synonymous (dS) nt positions and their ratio.

2.5. Recombination analysis

An online tool, genetic algorithm for recombination detection (GARD; http://datamonkey.org/) and the recombination detection program (RDP5) were used to investigate the presence of any putative recombination events in begomovirus and DNA-satellites (Martin et al., 2015). The recombination analysis was carried out with default settings in RDP5 and the recombination events supported by at least five different algorithms, with an acceptable cut-off *P*-value at $1x10^{-5}$ were considered credible.

2.6. Quantification of the virus components

An optimized real-time quantitative PCR was conducted to accomplish the absolute quantification of begomovirus and associated DNA-satellite components (Shafiq et al., 2017). Absolute quantification was achieved by preparing tenfold serial dilutions of the standards as 20 ng, 2 ng, 0.2 ng, 0.02 ng, and 0.002 ng, respectively. The recombinant plasmids bearing the viral components were used to prepare the standards, and an equal amount of plant genomic DNA was spiked in the standards to eliminate the background bias. Pre-quantified genomic extracts of each symptomatic (Od1-to-Od4) plant and a non-symptomatic (Ad1) plant were used in three independent replicates to estimate the virus and DNA-satellite titers. Finally, the viral titers and copy numbers were calculated in 1 μg of the genomic DNA using online resource (http://cels.uri.edu/gsc/cndna.html).

2.7. Standard curve calculations and data analysis

For absolute quantification of each virus or DNA-satellites components, a standard curve was drawn by linear regression analysis after plotting the Ct value against the total amount of DNA. Standard error was calculated for three technical repeats of each value. SigmaPlot 10.0 (http://sigmaplot.software.informer.com/10.0/) was used to calculate the statistically significant differences based on Student's *t*-test.

3. Results

3.1. Sequence comparisons and phylogenetic analysis

Successful amplification of the predictable begomovirus and DNA-satellites was achieved from the symptomatic sunflower plants (Od1-to-Od4), whereas the non-symptomatic plants could not produce any amplification. One full-length clone, each of a begomovirus (Od1-A), alphasatellite (Od1-a) and betasatellite (Od1-b), were sequenced entirely from Od-1 plant and deposited at NCBI GenBank database with accession numbers MH628534, MH628535, and MH628536, respectively. The complete sequence of begomovirus clone Od1-A was 2749 nt in length and had the typical genome organization of Eastern hemisphere begomoviruses with six predicted ORFs (Table 1). The SDT analysis showed a maximum nt sequence identity of Od1-A at 96.8% with an isolate of mesta yellow vein mosaic virus (MeYVMV) reported from *Alcea rosea* in Pakistan (unpublished data, accession number FR772081). Following the current criteria for demarcation of new begomovirus species by the International Committee for the Taxonomy of Viruses (ICTV) set at > 91% (Brown et al., 2015), Od1-A is representing a new isolate of MeYVMV species infecting ornamental sunflower in Pakistan. The phylogenetic dendrogram also supported this conclusion by grouping Od1-A isolate with MeYVMV isolates reported previously in the same clade supported by 100% bootstrap iterations (Fig. 2A).

The clone Od1-a was 1367 nts in length and resembled alphasatellites genomes with a single predicted ORF, alpha-Rep, on the virion-sense strand. The homology analysis of the clone Od1-a showed maximum nt sequence identity at 97.4% with ageratum enation alphasatellite (AEA) (accession number FR772085) infecting *A. rosea* in Pakistan (unpublished data). Thus, species demarcation criteria for alphasatellites set at < 88% (Briddon et al., 2018) showed Od1-a as a new isolate of AEA in the genus *Colecusatellite* (family, *alphasatellitidae*). The isolate Od1-a was segregated well within AEA clade in the phylogenetic dendrogram with 100% bootstrap value (Fig. 2B). The complete betasatellite clone (Od1-b) was 1387 nts in length and the highest percentage identity of nt sequences was noted at 98.2% with papaya leaf curl betasatellite (PaLCuB; accession number LN878112) identified from tomato in Pakistan. In the phylogenetic dendrogram, the clone Od1-b also grouped well (100% bootstrap value) with other PaLCuB isolates (Fig. 2C). Thus, the species demarcation criteria suggested at > 79% (Briddon et al., 2008) showed that Od1-b is a new isolate of PaLCuB from sunflower in Pakistan.

3.2. Estimation of genetic variation and substitution rate of begomovirus and DNA-satellites

Haplotype sequence polymorphisms, diversity and INDELs were computed for Od1-A, Od1-a and Od1-b based on their respective complete genome sequences retrieved from the database (Table 2).

Table 1
Percent nucleotide (nt) sequence identities of the full-length genome of MeYVMV and its individual open reading frames (ORFs) with the selected begomovirus species using MUSCLE alignment in the species demarcation tool (SDT).

Accession	Virus species	Host species	Country	% nt sequence identity						
				Full-length Od1-A	C1 (Rep)	C2 (TrAP)	C3 (REn)	C4	V1 (CP)	V2 (Pre-CP)
HQ257375	RaLCuV	Okra	India	79.8	84.9	74.5	77.0	91.9	77.6	77.8
AY705380	CLCuBaV	*Gossypium barbadense*	India	85.5	90.0	89.1	90.9	92.2	78.2	89.9
GU112003	CLCuBaV	Okra	India	84.9	87.5	88.9	89.8	86.7	78.0	90.2
HM448898	MaYVV	Tomato	China	82.7	84.5	88.1	92.1	78.1	77.6	86.8
FN806779	MaYVV	*Sidaacuta*	China	84.6	87.6	88.4	92.7	87.4	78.7	88.5
JQ897969	ToLCNDV2	Tomato	India	84.8	89.1	87.3	90.3	91.3	78.9	89.9
KF999982	MaYMV	Malvastrum	China	82.8	85.3	89.6	91.8	86.4	77.6	86.5
FN552749	MaYMV	Malvastrum	China	86.3	88.9	88.9	92.1	89.6	84.7	85.6
FJ159264	MeYVMV	*Hibiscus cannabinus*	India	90.9	91.7	92.1	94.0	89.0	93.5	91.7
FJ159265	MeYVMV	*H. cannabinus*	India	90.5	91.7	92.1	94.0	89.0	92.2	91.7
HG937524	PaLCuV	Cotton	Pakistan	77.7	73.7	73.1	74.2	73.5	92.5	80.8
JN817516	PaLCuV	*Croton bonplandianus*	India	78.0	74.7	72.6	74.4	76.4	92.2	80.2
KT253643	PaLCuV	cluster bean	India	78.0	74.2	72.1	73.7	73.1	91.8	78.7
KT253646	PaLCuV	cluster bean	India	77.9	74.7	73.1	75.1	72.2	91.4	79.0
HE578897	MeYVMV		Pakistan	95.6	95.0	**98.3**	**97.8**	94.2	**98.3**	**97.1**
FR772081	MeYVMV	Hollyhock	Pakistan	**96.8**	**98.4**	97.3	97.8	**98.7**	95.6	95.7
FR772082	HoLCV	Hollyhock	Pakistan	87.9	93.9	87.1	89.2	97.4	78.1	91.4
LT716980	HoLCV	*Malva parviflora*	Pakistan	85.7	89.8	88.9	89.2	91.6	78.2	89.4
GQ478343	HoLCV	*Ecliptaprostrata*	Pakistan	86.4	88.6	90.6	91.8	87.1	78.6	95.1

Regarding haplotypes distributions in MeYVMV and AEA sequences including Od1-A and Od1-a, all the tested sequences (23) were found haplotypes, while 56 haplotypes (out of 57) were detected in PaLCuB sequences. All the sub-populations showed high haplotype diversity (hd > 0.95). The highest number of segregating sites (S) were found in Od1-A (869), followed by 619 and 373 in Od1-b and Od1-a, respectively. The average nt difference (K) of Od1-A , Od1-a and Od1-b was found as 251, 106 and 63, respectively. A high degree of nucleotide diversity (Θ-Eta) was observed in Od1-b (0.15) with an average of 785 nt differences followed by Od1-A (0.11) and Od1-a (0.10), respectively. The highest numbers of Indels observed in Od1-b were 160 followed by Od1-A (91) and Od1-a (63), respectively (Table 2). The overall distribution of nucleotide diversity (π) was observed at all nt positions and in each ORF of MeYVMV genome. However, the highest peak of nt diversity was found at the N-terminus and middle region of Rep (C1) protein. The other region with highest nt diversity was the middle region of CP and the least nt diversity was found in pre-CP (V2), transcription associated protein (TrAP/C2), replication enhancer protein (REn/C3 and C4 coding regions (Fig. 3).

The mean nt substitution rate of Od1-a-encoded Rep was found to be highest (5.449×10^{-1}), followed by Od1-b-encoded βC1 (1.0301×10^{-3}), Od1-A-encoded CP ($6.983x10^{-4}$) and Rep ($5.702 x10^{-5}$), respectively (Table 3). These findings are suggestive of rapid evolution in Od1-a (AEA) than the other two components. Mutations are a key player in the selection process leading to genetic variation in the begomoviruses (Lima et al., 2017). Therefore, the mutation rate of all three codon positions in the selected ORFs was also calculated. Our results demonstrated that Od1-A-encoded CP and Od1-a-encoded Rep had a higher mutation rate in codon position 3. Whereas Od1-A-encoded Rep had the high mutation rate at codon position 1 (Table 3).

The comparison of Od1-A isolate with the nt sequences of other MeYVMV isolates exhibited an average evolutionary divergence of 9.5% (Table 4). While, the average evolutionary divergence of Od1-a and Od1-b isolates with other AEA and PaLCuB isolates was 8.3 and 5.2%, respectively. Subsequently, average evolutionary divergence of the coding ORFs, CP and Rep, of Od1-A was 9.3% and 12.2%, respectively. Whereas, the average evolutionary divergence of Od1-a encoded Rep was 7.9% and Od1-b-encoded βC1 was 3.6% (Table 4). The dN/dS ratio is a measure of the selective constraint pattern in evolutionary relationships (Nei and Gojobori, 1986). The dN/dS ratio of Od1-b was higher than the Od1-A and Od1-a. The Od1-A encoded Rep has a higher dN/dS ratio (2.237) as compared to the CP (0.100), Od1-b encoded βC1 has a higher dN/dS ratio (0.269) than Od1-a encoded Rep (0.114) (Table 4).

3.3. Recombination analysis

The predicted six ORFs of the isolate Od1-A showed variable nt sequence identities with respective ORFs of different begomoviruses, suggesting a recombinant origin (Table 1). Thus, a recombination analysis was carried out to further validate this speculation. The GARD examined 7653 models and predicted four recombination breakpoints in Od1-A. In case of Od1-b, GARD examined 17,595 models and predicted 6 breakpoints while, up to 8 breakpoints were predicted in Od1-a after examining 22,710 models (Fig. 4). To further validate, RDP analysis was carried out using RDP5, which predicted two recombination events in MeYVMV isolate Od1-A at the nt coordinates 433–1082 and 2242–2630 with the lowest P-values calculated as $4.761x10^{-52}$-$1.861x10^{-19}$ and $5.232x10^{-16}$-$2.569x10^{-05}$, respectively (Fig. 4). Likewise, one major recombination event in the AEA isolate Od1-a at the nt coordinates 307–450 with the lowest acceptable P-values $1.173x10^{-12}$ -$3.427x10^{-05}$. Whereas, in the PaLCuB isolate Od1-b a recombination event was predicted at the nt coordinates 295–1082 with the lowest acceptable P-values $8.549x10^{-24}$-$1.208x10^{-05}$ (Table 5).

3.4. Quantification of begomovirus and DNA-satellites

For the absolute quantification of the begomovirus and DNA-satellite components, all the obtained Ct values were within the dynamic range of the respective standards employed in this experiment. All the tested plants (Od1-to-Od4), showed a single melting curve indicative of a single product. The highest MeYVMV copies (per μg of genomic DNA) were found in the Od-3 plant ($1.44x10^9$) followed by Od-2 ($1.30x10^9$), Od-4 ($1.11x10^9$) and Od-1 plant ($8.35x10^8$), respectively (Fig. 5). The accumulation of the PaLCuB (per μg of genomic DNA) was highest in the Od-1 plant ($2.96x10^9$), followed by Od-4 ($2.72x10^9$), Od-2 ($2.48x10^9$) and Od-3 plant ($2.01x10^9$), respectively. The highest copies of the AEA genome (per μg of genomic DNA) was found in the Od-1 plant ($2.82x10^9$), followed by Od-3 ($1.73x10^9$), Od-2 ($1.27x10^9$) and Od-4 plant ($1.18x10^9$), respectively (Fig. 5).

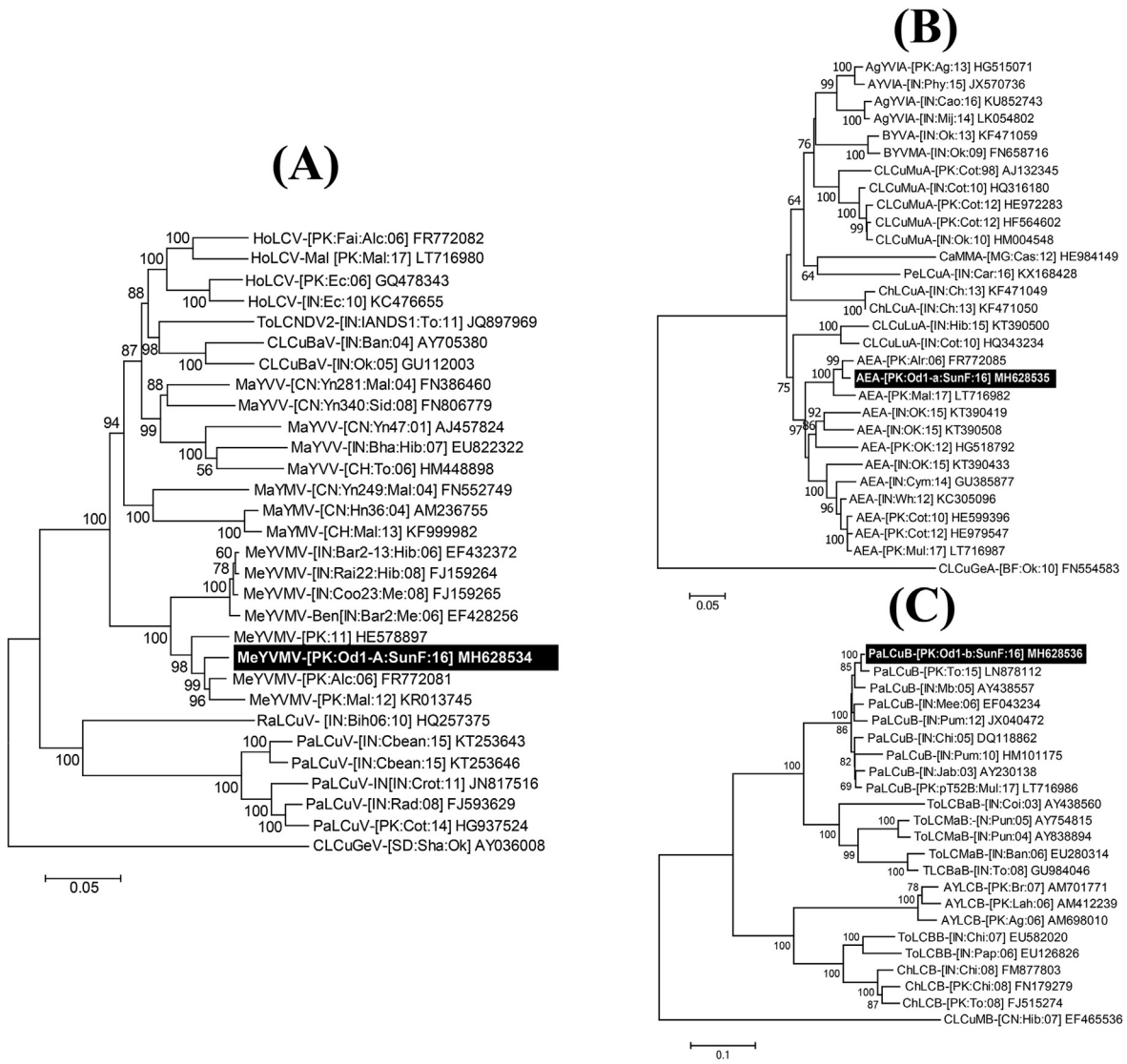

Fig. 2. Phylogenetic dendrograms showing evolutionary relationships of complete nucleotide (nt) sequences of begomovirus **(A)**, alphasatellite **(B)** and betasatellite **(C)** isoaltes identified in this study. The phylogetic dendrograms were constructed with Maximum-Likelihood (ML) algorithm in MEGA7 software using cotton leaf curl Gezira virus (CLCuGeV) as an outlier. All the respective isolates identified in this study are represented in bold white text on black rectangular background. The horizontal lines are showing nt substitutions per site whereas, the numbers on branch nodes are showing percent bootstrap values > 60 with 1000 replicates to authenticate the data. All isolates were retrieved from NCBI GenBank database and are represented in the phylogenetic dendrograms with their abbreviations and respective accession numbers. The full names of all begomovirus, alphasatellite and betasatellite species were abbreviated following Brown et al. (2015), Briddon et al. (2018) and (Briddon et al., 2012), respectively.

Table 2
Nucleotide diversity of MeYVMV, AEV and PaLCuB.

Virus Component	No. of seq	InDel sites	S	Eta (h)	Eta (S)	Hd	K	π	h	Θ-W	Θ-Eta
Od1-A (with MeYVMV only)	23	91	869	1066	279	1.0	251	0.09	23	0.09	0.11
Od1-a (with AEA only)	23	63	373	468	174	1.0	106	0.08	23	0.08	0.10
Od1-b (with PaLCuB only)	57	160	619	785	417	0.99	63	0.05	56	0.11	0.15

InDels = Insertions and deletions S = total number of polymorphic (segregating) sites, Eta = total number of mutations, Eta S = total number of singleton mutations, Hd = haplotype diversity, K = average number of nucleotide difference between sequences, π = nucleotide diversity, h = haplotypes.

4. Discussion

From the last couple of decades, several biological and environmental factors have contributed to the rapid emergence and adaptation of begomovirus species on new multiple plant hosts (Rojas et al., 2005, Seal et al., 2006). Thus, begomoviruses have increasingly been found infecting new crops, weeds, ornamental plants and/or trees, which were previously thought to be the non-host for these viruses (Sattar et al., 2018). The present study corroborated with previous reports and showed a change of host from weeds to an ornamental sunflower. The weed infecting MeYVMV has been reported from different weeds mesta, hibiscus, sonchus, malvastrum, hollyhock and kenaf in the Indian sub-continent either alone (Chatterjee and Ghosh, 2007a,b) or in association with different betasatellites (Acharyya et al., 2011, Chatterjee and Ghosh, 2007a,b, Das et al., 2008, Meena et al., 2019, Roy et al., 2009).

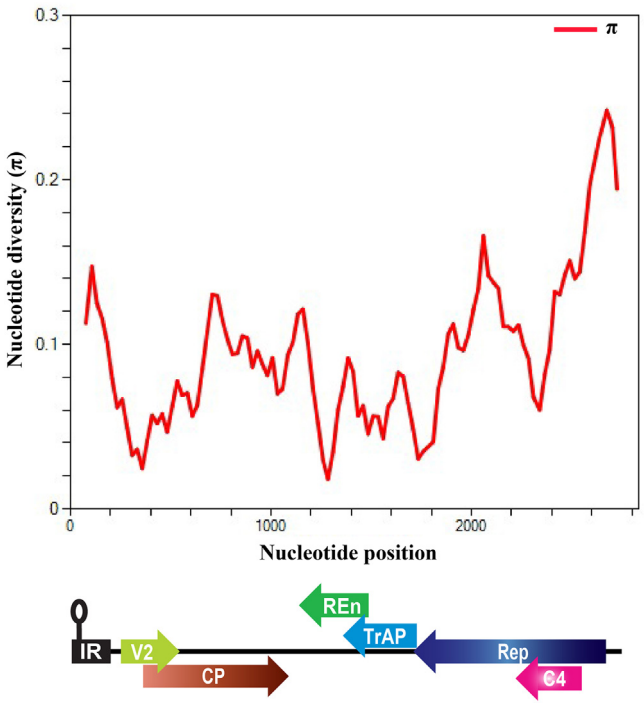

Fig. 3. Distribution of nucleotide diversity (π) in the genome of MeYVMV population. The linear genome organization of MeYVMV is used to indicate the nt variation rate at each nt position and each ORF region. The window length was set 100 nt wide with a 25 nt step size.

Diversification and emergence of new begomoviruses mainly rely on recombination, mutation and satellite capture; however, the evolution of begomoviruses is predominantly driven by mutations (Juárez et al., 2019, Lima et al., 2017). Our nt and mutation rate data of codon position revealed a high mutation rate in the Rep of AEA, βC1 of PaLCuB, and CP and Rep of MeYVMV, corroborating the previous findings (Mishra et al., 2020, Saleem et al., 2016). In concurrence with earlier reports, our study's findings accentuate that mutation might be a major force driving the emergence and selection of MeYVMV and associated DNA satellites (Ho et al., 2014, Mishra et al., 2020, Silva et al., 2012).

A higher sequence divergence (12.2%) and dN/dS ratio (2.237) was observed in the Rep of MeYVMV, strengthening the earlier findings (Lima et al., 2017). The High genetic variability in the specific regions of Rep and CP of begomoviruses is associated with purifying selection and a few sporadic cases (Lima et al., 2017). The highest nt diversity (π) was noted in the full-length genomes of MeYVMV (the data was collected from GenBank in November 2020). However, all these mutations were unevenly distributed throughout the genomes and regions of highest nt diversity were existed in the CP and Rep proteins. The unevenly distributed genetic variation in the CP of MeYVMV might provide critical insight into the adaptation to a new host. The negative Tajima's D value also pinpointed that CP might help the MeYVMV to switch to sunflower plants. The CP of begomoviruses is conserved and helps in intra- or intercellular movement, insect-mediated transmission from plant to plant (Rojas et al., 2005), interact with host factors, encode nuclear localization signals (NLS), nuclear export signals (NES) (Unseld et al., 2001), binds to ss- and dsDNA (Liu et al., 1997), and successful virus infection (Iqbal et al., 2012, Iqbal et al., 2017). The CP is very conserved at nt level as deciphered by its structural and functional roles. However, the 3′ region of CP is more flexible to variations at the nt level than the other two (5′ and central) regions.

All identified virus components showed recombination events. Although different recombination breakpoints were observed with RDP5 and GARD analysis, but these readily explain the specificity and utility of different algorithms. Non-redundant and non-random recombination breakpoints are the characteristic feature of ssDNA viruses, which occurred during rolling-circle-replication (Lefeuvre et al., 2007, Martin et al., 2011a,b). In the case of MeYVMV, recombination events were detected, both by RDP and GARD programs in the CP and Rep regions, the known region of recombination in begomoviruses genome (Lima et al., 2017, Martin et al., 2011a,b). Together both these factors, recombination and high genetic variability, might be responsible for the host switching. However, it is far from a conclusion and needs further empirical studies to prove this notion.

Association of betasatellites may help the begomoviruses to overcome host defense response, broaden host range, and may indirectly affect the *in planta* virus accumulation (Briddon et al., 2001). Betasatellites genome has no specific sequence homology with the begomoviruses except nona-nucleotide sequences (TAATATT/AC), which resembles geminivirus-like nona-nucleotide sequences (Hanley-Bowdoin et al., 2000). Betasatellites are very flexible in their *trans*-replication by a multiple ranges of begomoviruses or even other geminiviruses (Kharazmi et al., 2012, Sattar et al., 2019). The Rep protein of most of the helper begomoviruses helps in the replication of multiple betasatellites even though these are the non-cognate to a begomovirus (lacking the associated begomovirus iteron sequences). It is thus speculated that begomovirus-betasatellite interactions are not very specific in terms of *trans*-replication, *in planta* long-distance movement and transmission (Saunders et al., 2008). The specific sequences in betasatellites impersonate the iterons for rep binding, which shows its promiscuous replicative nature (Nawaz-ul-Rehman et al., 2009). Probably because of the same reason, MeYVMV can successfully *trans*-replicate diverse betasatellites. Besides the satellite capture, the other main driving force for geminivurs evolution is their recombination ability (Seal et al., 2006, Silva et al., 2014). The present data showed that MeYVMV and its associated

Table 3
Mean substitution and codon position mutation rate of the ORFs encoded by the begomovirus and associated DNA-satellites.

	Od1-A (CP)		Od1-A (Rep)		Od1-a (Rep)		Od1-b (βC1)	
	Strict clock (ESS value)	Relaxed clock (ESS value)	Strict clock (ESS value)	Relaxed clock (ESS value)	Strict clock (ESS value)	Relaxed clock (ESS value)	Strict clock (ESS value)	Relaxed clock (ESS value)
Mean nt substitution rate (site^{-1} year^{-1})	6.983E-04 (261)	3.305E-06 (228)	5.702E-05 (233)	7.449E-05 (228)	5.449E-01 (201)	8.454E-04 (250)	1.0301E-03 (219)	9.432E-04 (264)
At 95% HPD interval	5.0113E-14, 8.0189E-06	2.005E-06, 2.591E-04	2.557E-17, 2.704E-04	2.773E-06, 2.526E-04	5.249E-11, 9.304E-04	8.863E-05, 1.679E-03	5.671E-04, 1.513E-03	2.163E-07, 2.641E-03
CP1 mu	0.337 (7419)	0.334 (7050)	1.807 (7483)	1.812 (5965)	0.545 (7846)	0.548 (7144)	0.733 (6303)	0.738 (4477)
CP2 mu	0.312 (7431)	0.310 (5739)	0.653 (6738)	0.654 (5599)	0.201 (6745)	0.203 (7101)	0.626 (6318)	0.633 (3628)
CP3 mu	2.351 (6440)	2.356 (4861)	0.538 (7404)	0.532 (6945)	2.254 (6834)	2.249 (6180)	1.640 (5722)	1.629 (3563)

Od1-A = MeYVMV, Od1-a = AEA, Od1-b = PaLCuB, HPD = highest posterior density, CP = codon position

Table 4
Sequence variability analysis of MeYVMV, AEA, PaLCuB, and of their selected ORFs.

Virus Component	Best Model	Mean distance (d)	dN	dS	dN/dS	Tajima D
Od1-A	HKY + G	0.095 ± 0.003	0.082 ± 0.004	0.125 ± 0.009	0.656	0.181
Od1-A (CP)	T92 + G	0.093 ± 0.014	0.029 ± 0.004	0.289 ± 0.029	0.100	−0.232
Od1-A (Rep)	HKY + G	0.122 ± 0.018	0.132 ± 0.009	0.059 ± 0.009	2.237	0.306
Od1-a	T92 + G	0.083 ± 0.004	0.058 ± 0.006	0.184 ± 0.015	0.315	0.045
Od1-a (Rep)	T92 + G	0.079 ± 0.012	0.028 ± 0.005	0.245 ± 0.025	0.114	0.291
Od1-b	T92 + G	0.052 ± 0.009	0.056 ± 0.004	0.043 ± 0.005	1.302	−1.889
Od1-b (βC1)	T92 + G	0.036 ± 0.005	0.021 ± 0.004	0.078 ± 0.016	0.269	−2.107

Od1-A = MeYVMV, Od1-a = AEA, Od1-b = PaLCuB, dN = non-synonymous, dS = synonymous

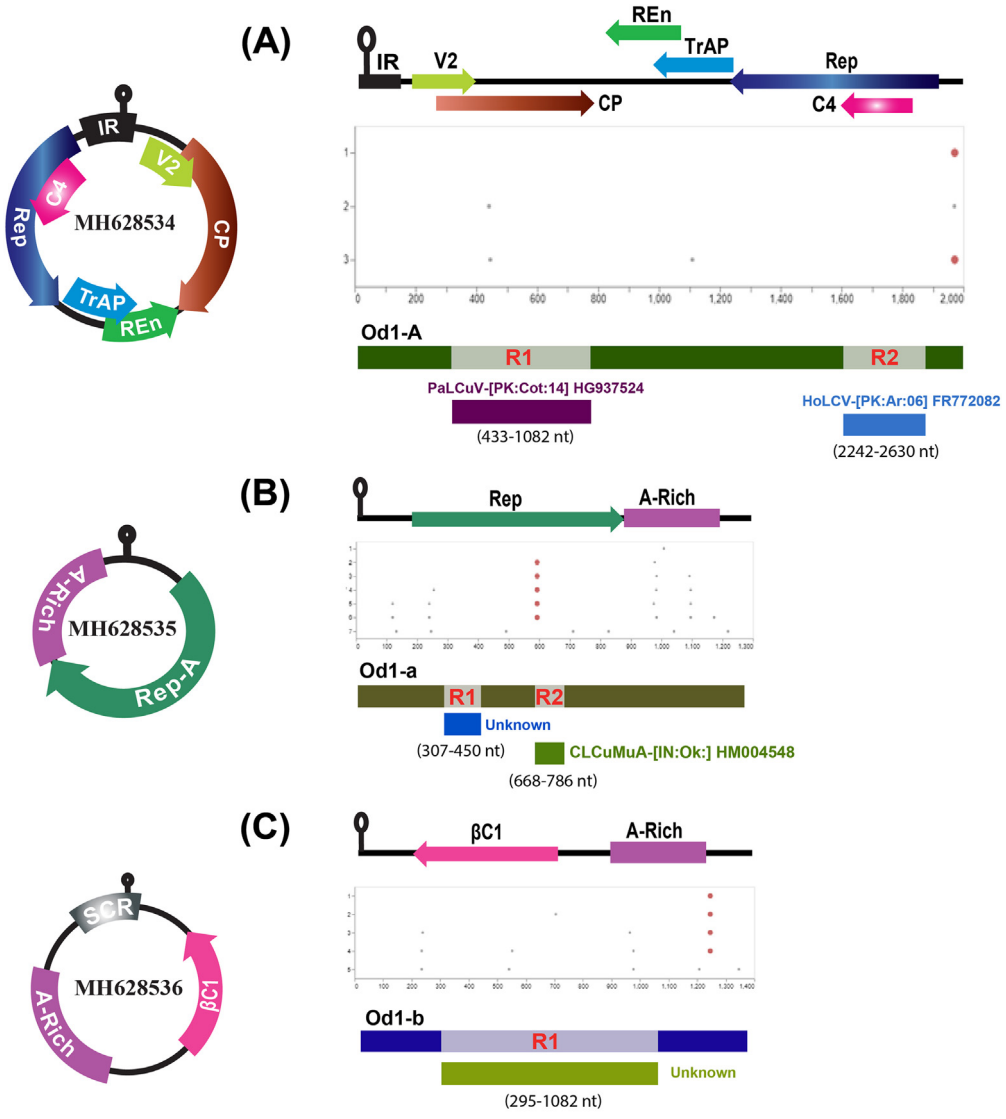

Fig. 4. Recombination analysis of the isolated begomovirus and DNA-satellite clones. Linear and circular genome maps of begomovirus **(A)**, alphasatellite **(B)** and betasatelite **(C)** are plotted. The colored arrows are showing position and orientation of genes. The recombination events detected with GARD and RDP are shown in colored boxes under each genome, respectively.

DNA-satellites have a potential recombinant origin (Fig. 4, Table 5). It is therefore assumed that after successful recombination and/or DNA satellite capture, MeYVMV can extend its host range. The previous studies and the outcome of the present study, the association of MeYVMV with AEA and PaLCuB in ornamental sunflower, further supports this speculation. However, experimental evidence is a pre-requisite to infer a concrete conclusion.

Several methods are used to study the characterization and epidemiology of the begomoviruses, conventional PCR is a well-opted method but it cannot determine the quantity of the begomovirus in a host. The use of qPCR in virus epidemiology and disease etiology has offered accuracy, sensitivity and speed over conventional PCR and hybridization techniques. Furthermore, qPCR results can define the relationship between symptoms and virus titer. In our results,

Table 5
Summary of recombination breakpoints in the isolated begomovirus and DNA-satellite clones calculated by different algorithms in recombination detection program (RDP5).

Recombination event	Recombination breakpoint (nt position)	Major parent	Minor parent	RDP methods	Average P Values
Od1-A (MH628534)					
R1	433–1082	HoLCV (GQ478343)	PaLCuV (HG937524)	RDP	$4.761x10^{-52}$
				GENECONV	$5.204\ x10^{-49}$
				BootScan	$7.760\ x10^{-54}$
				MaxChi	$8.198\ x10^{-20}$
				Chimaera	$3.220\ x10^{-20}$
				SiScan	$1.861\ x10^{-19}$
				3Seq	$2.299\ x10^{-51}$
R2	2242–2630	MeYVV (EF428256)	HoLCV (FR772082)	RDP	5.527×10^{-06}
				GENECONV	4.284×10^{-11}
				BootScan	1.208×10^{-06}
				MaxChi	5.778×10^{-07}
				Chimaera	1.013×10^{-07}
				SiScan	5.232×10^{-16}
				3Seq	2.569×10^{-05}
Od1-a (MH628535)					
R1	307–450	AEA (KT390419)	Unknown	RDP	2.857×10^{-10}
				GENECONV	1.263×10^{-12}
				BootScan	1.173×10^{-12}
				MaxChi	6.767×10^{-07}
				Chimaera	3.427×10^{-05}
				SiScan	1.727×10^{-08}
				3Seq	8.977×10^{-06}
R2*	668–786	AEA (FR772085)	CLCuMuA (HM004548)	GENECONV	7.798×10^{-06}
				BootScan	2.143×10^{-06}
				3Seq	5.142×10^{-03}
Od1-b (MH628536)					
R1	295–1082	ToLCBB (EU126826)	Unknown	RDP	2.655×10^{-02}
				BootScan	2.770×10^{-05}
				MaxChi	2.454×10^{-05}
				Chimera	3.104×10^{-06}
				SiScan	$8.549 \times 10–24$
				3Seq	1.208×10^{-05}

* The recombination events, which were not supported by at least four different algorithms in RDP5 were not considered credible.

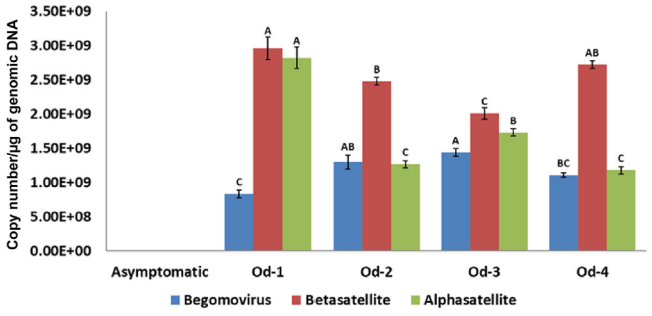

Fig. 5. Absolute quantification of MeYVMV and associated DNA-satellites from the collected symptomatic (Od1-Od4) and asymptomatic (Ad1) ornamental sunflower plants. Results shown were mean of three technical repeats and error bar represents standard error. The values in similar letters are non-significant while, the values in different letters are either significant or highly significant with $P < 0.05$, respectively.

the copy number of PaLCuB was found to be higher in all the four tested plants than the other two components (Fig. 3). The higher PaLCuB titer may be helping the MeYVMV to establish into a new host, as betasatellites help their cognate viruses in extending their host-range and enhancing *in planta* accumulation (Briddon et al., 2001, Iqbal et al., 2017, Saunders et al., 2000). Furthermore, the sole ORF in betasatellite (βC1) is a pathogenicity determinant and can also play role in TGS and PTGS suppression (Zhou, 2013). The copy numbers of AEA was found to be slightly higher than MeYVMV; this best explained the self-replicating nature of alphasatellites (Saunders and Stanley, 1999). Moreover, a higher AEA titer may provide a selective advantage to the infection because of Rep encoded by alphasatellites may act as a suppressor of PTGS activity (Abbas et al., 2019, Nawaz-ul-Rehman et al., 2010). Surprisingly, the copy number of MeYVMV was the lowest as compared to PaLCuB and AEA in all the tested plants. As the collected plants were showing very mild symptoms, this may likely be due to either host resistance (but not immunity) or low MeYVMV titer. These qPCR findings showed that symptom severity in plants could serve as a general guide to the virus and DNA satellites *in planta* accumulation.

This is the first description of MeYVMV associated with the DNA-satellite complex from ornamental sunflower in Pakistan. The host-switching of a weed infecting begomovirus may indicate an imminent threat to sunflower production in the Indian subcontinent. However, this is far from conclusive and requires a comprehensive investigation to explore the virus epidemiology and host range. In addition, evaluation of resistant/tolerant sunflower germplasm is a pre-requisite to curtail potential epidemic effect on sunflower production in the region.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Research involving human participants and/or animals

No animals/humans were involved in this experiment.

Authors contribution

MNS and MK conceived the idea. SNA performed the experiments and MS quantified the viral titre. MNS performed the phylogenetic and RDP analysis. ZI calculated genetic diversity, population structure, nucleotide substation rate and performed GARD analysis. MNS and ZI drafted the first manuscript. Proof reading was done by IA and MK. Final manuscript was read and approved by all authors.

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