



Virome analysis deciphered the infection of American plum line pattern virus, little cherry virus 1 and plum bark necrosis stem pitting-associated virus in plum from India

Zainul A. Khan · Pooja Thapa · Damini Diksha · Yazing S. Mailem ·
Susheel K. Sharma · Sajad U. Nabi · Sumiah Wani · Mehraj D. Shah ·
Mahendra K. Verma · Nitika Gupta · Virendra K. Baranwal

Accepted: 19 June 2024
© Koninklijke Nederlandse Planteziektenkundige Vereniging 2024

Abstract As very limited information is available on viruses and viroids infecting plum in India, virome analysis was undertaken to determine the virus and viroid spectrum infecting plum. High throughput sequencing (HTS) analysis of five plum samples (PM1-PM5) revealed the presence of near complete genome sequences of prunus necrotic ringspot virus (PNRSV), American plum line pattern virus (APLPV), apple chlorotic leaf spot virus (ACLSV) and hop stunt viroid (HSVd) in PM1;

little cherry virus 1 (LChV-1), plum bark necrosis stem pitting-associated virus (PBNSPaV), ACLSV and HSVd in PM2. No virus or viroid sequence was obtained in PM3, PM4 and PM5. Near complete genome sequences of these viruses were obtained using different assemblers. APLPV, LChV-1, PBNSPaV and HSVd are being reported here for the first time from plum in India. The presence of these viruses was also confirmed by reverse transcription polymerase chain reaction and Sanger sequencing in HTS samples as well as in additional 36 plum samples (PM6-PM41) collected from the same orchard from where PM1 and PM2 samples were obtained. The validation test in an additional 36 samples indicated predominance of APLPV and PNRSV which were detected in all the samples, while other viruses were present only in some of the samples. Moreover, fragments per kilobase of transcript per million reads mapped and virus accumulation studies showed dominance of PNRSV RNA3 in PM1 and LChV-1 in PM2. Analysis of single nucleotide variants (SNVs) showed that PNRSV RNA3 had the highest SNVs (99) and PBNSPaV had the lowest SNVs (1). The phylogenetic analysis revealed that these viruses are phylogenetically related to viruses from different countries. These findings advance our knowledge of the viral pathogens of plum, and would help in virus indexing and certification of clean plant for commercial plum cultivars.

Zainul A. Khan, Pooja Thapa and Susheel Kumar Sharma these authors contributed equally.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10658-024-02912-2>.

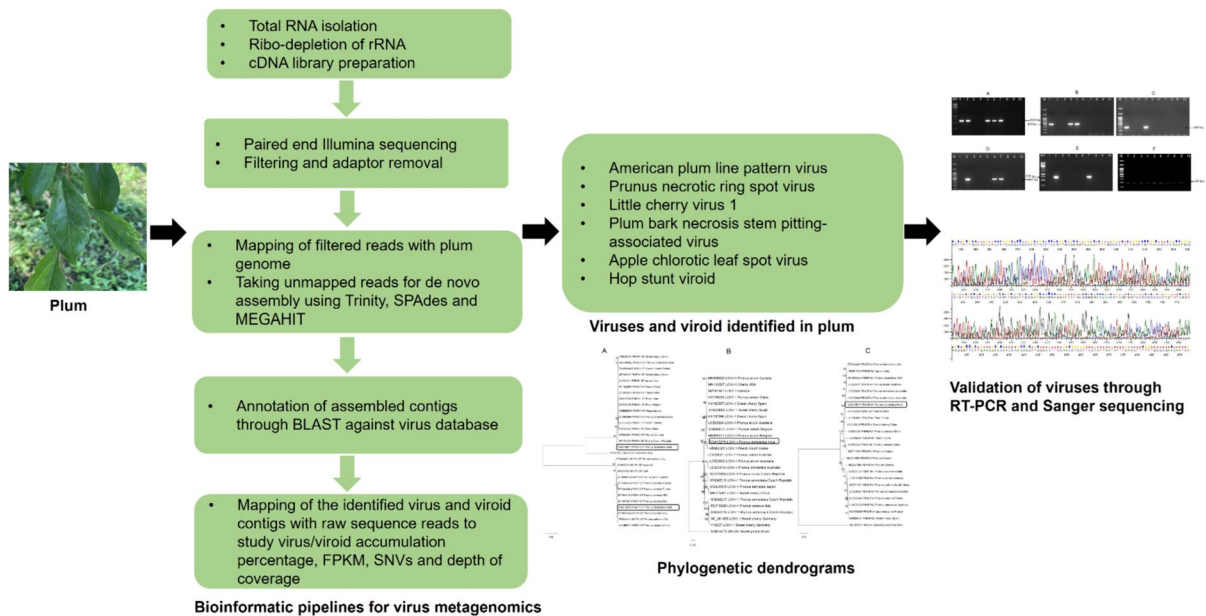
Z. A. Khan · P. Thapa · D. Diksha · Y. S. Mailem ·
S. K. Sharma · N. Gupta (✉) · V. K. Baranwal (✉)
Advanced Centre for Plant Virology, Division of Plant
Pathology, ICAR-Indian Agricultural Research Institute,
New Delhi, India
e-mail: nitika.iari@gmail.com

V. K. Baranwal
e-mail: vbaranwal2001@yahoo.com

S. U. Nabi · M. K. Verma
ICAR-Central Institute of Temperate Horticulture,
Srinagar, India

S. Wani · M. D. Shah
Division of Plant Pathology, Sher-e-Kashmir University
of Agricultural Sciences and Technology-Kashmir,
Shalimar, Srinagar, India

Graphical abstract



Keywords Plum virome · High throughput sequencing · Single nucleotide variants · FPKM · RT-PCR

Introduction

Plum (*Prunus domestica*) is a stone fruit cultivated in temperate regions such as Jammu & Kashmir, Himachal Pradesh, and Uttarakhand states of India. These regions contribute 77% of total plum production in India. During 2020–21, India produced 84,000 Metric Tonnes of plum from 24,000 Ha.

(https://agriwelfare.gov.in/Documents/Horticultural_Statistics_at_Glance_2021.pdf) Horticulture Statistics Division, Department of Agriculture & Farmers Welfare, Ministry of Agriculture & Farmers Welfare, Government of India).

Globally several viruses viz. American plum line pattern virus (APLPV) (Paulsen & Fulton, 1968), prunus necrotic ringspot virus (PNRSV) (Chandel et al., 2011), plum pox virus (PPV) (García et al., 2014), plum bark necrosis stem pitting-associated virus (PBNPaV) (Amenduni et al., 2005), apple chlorotic leaf spot virus (ACLSV) (Rana et al., 2010),

little cherry virus-1 (LChV-1), peach virus D (PeVD), peach leaf pitting-associated virus (PLPaV) (Jo et al., 2020) and a viroid namely hop stunt viroid (HSVd) (Jo et al., 2017, 2020) have been reported from plum. In India, only PNRSV and ACLSV have been previously reported (Chandel et al., 2013; Rana et al., 2010). PPV is the most damaging virus of plum and belongs to the genus *Potyvirus* and consists of a single stranded (ss) RNA (+) genome (García et al., 2014). PNRSV and APLPV are members of the genus *Ilarvirus* having three positive sense ss RNAs namely RNA1, RNA2 and RNA3 (Alayasa et al., 2003; Wang et al., 2018). PBNPaV and LChV-1 belongs to the genera *Ampelovirus* and *Velarivirus* of family *Closteroviridae*, respectively, consisting of positive sense ss RNA genomes (Katsiani et al., 2018; Qu et al., 2014). PLPaV is a member of the genus *Fabavirus* and has a bipartite positive sense ss RNA genome. PeVD and ACLSV belongs to genera *Marafivirus* and *Trichovirus*, respectively (Igori et al., 2017). HSVd belongs to the genus *Hostuviroid*, which has a circular ss RNA genome (Marquez-Molins et al., 2021).

The majority of the commercial plum varieties are clonally propagated through cuttings and grafting which can result in the transmission of viruses

and viroids from infected plants to the healthy plants (Jo et al., 2020). Early detection and eradication of viruses in orchards and nurseries can help to prevent virus spread and result in virus-free plant material. Targeted detection using enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR/reverse transcription-PCR) including different variants and gene-editing (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 12a: CRISPR/Cas12a) has been developed for the identification of viruses in perennial fruit crops (Jeong et al., 2014; Jiao et al., 2021; Kim et al., 2018; Komorowska et al., 2010; Menzel et al., 2002). However, these methods have limitations and require either antibodies or prior knowledge of the sequence of the pathogen. To circumvent these constraints, high-throughput sequencing (HTS) technology can be used to detect novel or unknown viruses in unbiased manner and has been used to unravel the virome in perennial fruit crops including apple, grapevine, pear, peach and plum (Jo et al., 2018, 2020; Kim et al., 2021; Nabi et al., 2022; Noda et al., 2017; Sidharthan et al., 2020; Wang et al., 2022).

As very limited information on viruses affecting plum in India was available, HTS was used for the identification of viruses and viroids in asymptomatic/symptomatic plum plants collected from Jammu & Kashmir and Himachal Pradesh states of India and the results of the same has been analysed and presented here.

Materials and methods

Sample collection, RNA isolation, library preparation and RNA sequencing

P. domestica cv. Stanley and *P. domestica* cv. Santa Rosa plant leaves showing mosaic and necrosis symptoms (Fig. S1) were collected from experimental fields of the ICAR-Central Institute of Temperate Horticulture (ICAR-CITH; 33.98427° N, 74.80209° E) and Sher-e-Kashmir University of Agricultural Sciences and Technology (SKUAST, 34.14941° N, 74.88407° E), Srinagar, Jammu and Kashmir, India, during May 2022. Similarly, asymptomatic plum leaves (*P. salicina*) were collected in May 2022 from the ICAR-National Bureau of Plant Genetic

Resources (NBPGR), Regional Station (31.09768° N, 77.16031° E), Phagli, Shimla, Himachal Pradesh, India. Two symptomatic samples (100 mg of leaves) from ICAR-CITH and SKUAST and asymptomatic sample from ICAR-NBPGR designated as PM1, PM2 and PM3, respectively, were used for RNA extraction. During September and November 2022, asymptomatic plum samples were collected from Srinagar and Shimla named as PM4 and PM5, respectively. Total RNA from five samples was extracted using the Spectrum™ Plant Total RNA Kit (Sigma, USA) according to the manufacturer's instructions. The RNA was tested for quality and integrity check using a NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, USA), an Agilent 4150 TapeStation (Agilent, USA) and a Qubit 4 Fluorometer (Invitrogen, USA). Ribozero treatment was given to deplete rRNA using the Pan-Plant riboPOOL kit (siTOOLS BIOTECH, Germany) according to the manufacturer's protocol. The NebNext Ultra II RNA Library Prep Kit for Illumina (NEB, USA) was used to prepare RNA-seq libraries following the manufacturer's instructions. The quality and quantity of the libraries were evaluated using the Agilent 4150 TapeStation (Agilent, USA) and the Qubit 4 Fluorometer (Invitrogen, USA). On the NovaSeq 6000 V1.5 (Illumine, USA), high quality total RNA-Seq libraries were paired end sequenced (2 × 150 bp).

De novo assembly and bioinformatics analysis

Each sample's paired end raw data (FastQ files) were quality checked and pre-processed with the fastp (0.20.1) (Chen et al., 2018). To align clean filtered sequencing reads to the *P. domestica* genome (https://www.rosaceae.org/species/prunus_domestica/genome_v1.0.a1), the Bowtie2 (2.4.5) was used (Langdon, 2015). Trinity v.2.8.5, SPAdes v.1.3.3 and MEGAHIT v.1.2.9 were used to *de novo* assemble the host unaligned sequence reads (Bankevich et al., 2012; Grabherr et al., 2011; Li et al., 2015). The non-human virus database was downloaded from NCBI (<https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/>) on October 29, 2022, and used to annotate the assembled contigs. The virus genome's ORFs were identified using the ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). The assembled contigs representing virus and viroid associated genome sequences viz. PNRSV,

APLPV, LChV-1, PBNSPaV, ACLSV and HSVd were submitted to GenBank.

Single-nucleotide variants (SNVs), genome mapping, depth of coverage and fragments per kilobase of transcript per million reads mapped (FPKM) determination

The genomic variants such as SNVs, insertions and deletions (InDels) of each virus was identified using HaplotypeCaller tool (Ren et al., 2018). To map sequence data with the reference virus and viroid genomes, BWA-MEM algorithm was used (Li, 2013). Host unmapped filtered reads from individual samples were aligned with the reference virus and viroid genome sequences. From sequence alignment map (SAM) files, the virus and viroid specific reads were extracted, and these reads were further used for the computation of virus/viroid accumulation, depth of coverage, and fragments per kilobase of transcript per million reads mapped (FPKM) as described by Khan et al. (2023).

The percentage of reads per virus/viroid was calculated after retrieving the number of reads associated with each virus/viroid from the SAM file. The percentage was calculated by dividing each virus/viroid's reads by the total number of reads from all viruses and viroids and multiplying by 100. FPKM was determined by multiplying individual virus/viroid reads with 10^9 and divide by total accumulated virus and viroid reads multiplied by virus/viroid length (Khan et al., 2023).

Phylogenetic analyses and taxonomic classification

For phylogenetic analysis, complete sequences of viruses including LChV-1 and PBNSPaV, and coat protein (CP) of APLPV and PNRSV which shared highest nucleotide sequence identity and query coverage with the HTS identified virus contigs, were retrieved from GenBank and subjected to multiple sequence alignment using ClustalW. Phylogenetic trees were prepared using Neighbor-Joining method in Molecular Evolutionary Genetic Analysis (MEGA) version 11 (Tamura et al., 2021). Further, the viruses identified in PM1 and PM2 HTS data were validated with Kraken 2 which is a taxonomic classification system based on exact *k*-mer matches (Wood et al., 2019). Kraken 2 results were visualised with Krona

that displays hierarchical data in multilayered pie charts (Ondov et al., 2011).

Validation of viruses/viroids through RT-PCR and sanger sequencing

Total RNA was isolated from symptomatic and asymptomatic plum samples using IRIS method (Ghawana et al., 2011). The quality and quantity of the RNA were measured using the NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, USA). First strand cDNA synthesis was done using the FIRE Script® RT cDNA synthesis kit (Solis BioDyne, Estonia) as per manufacturer's instructions. The PCR was performed using 10 µM virus/viroid specific primers (Table 1), 50 ng of cDNA, and 1X Dream Taq PCR Master Mix (Thermo Scientific, USA). The PCR cycle conditions included an initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50–60 °C for 45 s (depending on the specific primer pair) and extension at 72 °C for 30–45 s (depending on the specific primer pair) and final extension at 72 °C for 10 min (Table 1). PCR amplicon was stained with EtBr and visualised using the Gel documentation system (BIO-RAD, USA). The amplified DNA fragments were cloned into pGEM®-T Easy Vector (Promega, USA) and sequenced using Sanger technology. Leaf samples were collected from 36 plum trees namely PM6 to PM25 in June 2023 and PM26 to PM41 in May 2024 from the same field of Srinagar, Jammu & Kashmir, from where the original PM1 and PM2 HTS samples were collected to validate the viruses and viroid in plum trees.

Data availability

The SRR23529962, SRR23530428, SRR23531015, SRR25501240 and SRR25500943 SRA accession numbers in the NCBI correspond to the raw sequence reads of PM1, PM2, PM3, PM4 and PM5, respectively. The genome sequences of viruses and viroid were submitted in NCBI with the accession numbers OQ513265 (PNRSV RNA1), OQ513266 (PNRSV RNA2), OQ513267 (PNRSV RNA3), OQ513268 (APLPV RNA1), OQ513269 (APLPV RNA2), OQ513270 (APLPV RNA3), OQ513271 (ACLSV), OQ513272 (HSVd), OQ513273 (ACLSV),

Table 1 List of primers used for the detection of viruses in plum

Virus and viroid ^a	Primer name	Sequence of primers (5' to 3')	Target gene ^b	PCR amplicon (bp)	Annealing temperature (°C)	Extension time (S)	References
PNRSV	PNRSV CP For	ACTCAGGGC TCAACAAAG GG	CP	450	60	45	This study
	PNRSV CP Rev	GTACGCGCA AAAGTGTCG AA					
APLPV	APLPV CP For	CACCACTCA GTTCGCTCA AC	CP	309	60	45	This study
	APLPV CP Rev	GATGCGCAG AGTACGGTC AT					
LChV-1	LCV1 RdRp For	GTCTGGTGT CTCCGAATA TTCAC	RdRp	728	58	45	This study
	LCV1 RdRp Rev	CACGGAGTA AAGTGGGAT GC					
PBNSPaV	PBNSPaV CP For	AAAACGCGT TGTCCTTCA GC	CP	457	60	45	This study
	PBNSPaV CP Rev	CCACCGGAC TGATTACCA CC					
ACLSV	ACLSV CP For	CAGACCCCT TCATGGAAA GACAG	CP	645	50	45	(Kumar et al. 2014)
	ACLSV CP Rev	TGACTCTTT ATACTCTTT CATGGGTTC					
HSVd	HSVd_FP	ACCTGAGAA AGGAGCCCC GGG	Plant Mitochondrial Nad5 gene	178	62	30	This study
	HSVd_RP	GGCATCACC TCTCGGTTC GT					
	HSVd-78 FP	AACCCGGGG CAACTCTTC TC		307	55	30	(Zhang et al., 2009)
	HSVd-98 RP	AACCCGGGG CTCCTTCT CA					
	Nad5-s FP	GATGCTTCT TGGGGCTTC TTGTT		181	58	30	(Menzel et al., 2002)
	Nad5-as RP	CTCCAGTCA CCAACATTG GCATAA					

^aPNRSV, prunus necrotic ringspot virus, APLPV, American plum line pattern virus, ACLSV, apple chlorotic leaf spot virus, LChV-1, little cherry virus 1, PBNSPaV, plum bark necrosis stem pitting-associated virus, HSVd, Hop stunt viroid

^bCP, coat protein, RdRp, RNA dependent RNA polymerase

OQ513274 (PBNPaV), OQ513275 (LChV-1) and OQ513276 (HSVd).

Results

Identification of viruses and viroid from plum HTS data

Raw reads, high quality filtered reads and host unmapped reads are presented in Table 2. Host unmapped reads of PM1, PM2, PM3, PM4 and PM5 were 16,369,070, 16,320,851, 21,038,690, 10,601,100 and 11,906,241, respectively (Table 2). The host unmapped reads were assembled with Trinity into 25,384, 22,294,19,317, 265,326 and 352,179 contigs of >200 bp for PM1, PM2, PM3, PM4 and PM5, respectively (Table S1). The contigs assembled with SPAdes for PM1, PM2, PM3, PM4 and PM5 were 27,942, 20,222, 17,796, 183,744 and 256,724, respectively (Table S2). MEGAHIT assembled 73,658, 93,907, 70,149, 841,520 and 42,900 contigs for PM1, PM2, PM3, PM4 and PM5, respectively (Table S3). N50 values ranged between 306 and 1200 (Table S1-S3).

BLAST annotation results showed the presence of PNRSV, APLPV, ACLSV, HSVd in PM1 and LChV-1, PBNPaV, ACLSV, HSVd in PM2. While no virus or viroid was detected in PM3, PM4 and PM5 (Table 3). ACLSV and HSVd were identified in both PM1 and PM2 samples. Near complete genome of PNRSV (RNA1, RNA2 and RNA3), APLPV (RNA1, RNA2 and RNA3), LChV-1 and HSVd were recovered using Trinity. Partial genomic sequences of PBNPaV and ACLSV were also identified through Trinity and SPAdes. Near complete genome of ACLSV and PBNPaV were recovered when MEGAHIT assembler was used (Table S4).

PNRSV RNA1, PNRSV RNA2, PNRSV RNA3, APLPV RNA1, APLPV RNA2, APLPV RNA3, ACLSV and HSVd of PM1 showed 99.4%, 99.3%, 97.2%, 99.1%, 98%, 98.4% and 82.4% nucleotide sequence identity with PNRSV RNA1 (MF069044), PNRSV RNA2 (OL800570), PNRSV RNA3 (OP357944), APLPV RNA1 (KY883315), APLPV RNA2 (NC_003452), APLPV RNA3 (NC_003453), ACLSV (OR640150) and HSVd (KF534763) sequences available at GenBank, respectively. Viruses and viroid sequences including PBNPaV, LChV-1, ACLSV and HSVd identified

Table 2 Read statistics of plum high throughput sequencing data

Plum samples	Statistics term											
	BioProject	BioSample	SRA accession	Total raw reads	Total filtered reads (HQ)	HQ reads (%)	Host aligned reads	Host unaligned reads	Aligned reads (%)	Q20 (%)	Q30 (%)	GC content (%)
PM1	PRJNA936634	SAMN33359145	SRR23529962	45,935,272	44,990,784	97.94	28,621,714	16,369,070	63.62	98.17	93.99	43.24
PM2	PRJNA936636	SAMN33359146	SRR23530428	41,749,628	40,469,018	96.93	24,148,167	16,320,851	59.67	98.10	93.86	42.50
PM3	PRJNA936637	SAMN33359161	SRR23531015	51,582,534	49,439,614	95.84	28,400,924	21,038,690	57.45	98.29	94.22	42.73
PM4	PRJNA1001639	SAMN36814151	SRR25501240	37,180,812	34,210,860	92.01	23,609,760	10,601,100	69.01	97.38	92.51	49.56
PM5	PRJNA1001611	SAMN36813020	SRR25500943	37,342,408	36,081,656	96.62	24,175,415	11,906,241	67	98.67	95.65	51.6

Table 3 Viruses and viroid identified through high throughput sequencing and their validation in plum leaves using RT-PCR










































Plum	Symptoms	Viruses and viroids identified through HTS ¹	Validation through RT-PCR	Plum	Symptoms	Viruses and viroids identified through HTS	Validation through RT-PCR
PM1		APLPV, PNRSV, ACLSV, HSVd	PNRSV, APLPV, ACLSV	PM22		NP	APLPV, PNRSV, LChV-1, ACLSV
PM2		LChV-1, PBNSPaV, ACLSV, HSVd	LChV-1, PBNSPaV, ACLSV	PM23		NP	APLPV, PNRSV, PBNSPaV
PM3		No viruses and viroids detected	No viruses and viroids detected	PM24		NP	APLPV, PNRSV, PBNSPaV
PM4		No viruses and viroids detected	No viruses and viroids detected	PM25		NP	APLPV, PNRSV, PBNSPaV
PM5		No viruses and viroids detected	No viruses and viroids detected	PM26		NP	APLPV, PNRSV, PBNSPaV
PM6		NP ²	APLPV, PNRSV, LChV-1, PBNSPaV	PM27		NP	PNRSV, LChV-1, PBNSPaV
PM7		NP	APLPV, PNRSV, LChV-1, ACLSV	PM28		NP	APLPV, PNRSV, PBNSPaV
PM8		NP	APLPV, PNRSV, LChV-1, PBNSPaV, ACLSV	PM29		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM9		NP	APLPV, PNRSV, LChV-1, PBNSPaV, ACLSV	PM30		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM10		NP	APLPV, PNRSV, PBNSPaV, ACLSV	PM31		NP	APLPV, PNRSV, LChV-1, PBNSPaV

Table 3 (continued)

PM11		NP	APLPV, PNRSV, LChV-1, PBNSPaV, ACLSV	PM32		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM12		NP	APLPV, PNRSV, PBNSPaV, ACLSV	PM33		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM13		NP	APLPV, PNRSV, PBNSPaV, ACLSV	PM34		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM14		NP	APLPV, PNRSV, PBNSPaV	PM35		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM15		NP	APLPV, PNRSV, LChV-1, PBNSPaV	PM36		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM16		NP	APLPV, PNRSV, LChV-1, PBNSPaV	PM37		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM17		NP	APLPV, PNRSV, LChV-1, PBNSPaV	PM38		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM18		NP	APLPV, PNRSV, LChV-1, PBNSPaV	PM39		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM19		NP	APLPV, PNRSV, LChV-1	PM40		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM20		NP	APLPV, PNRSV, LChV-1, PBNSPaV	PM41		NP	APLPV, PNRSV, LChV-1, PBNSPaV
PM21		NP	APLPV, PNRSV, LChV-1, PBNSPaV				

^aAPLPV, American plum line pattern virus, PNRSV, prunus necrotic ringspot virus, LChV-1, little cherry virus 1, PBNSPaV, plum bark necrosis stem pitting-associated virus, ACLSV, apple chlorotic leaf spot virus, HSVd, hop stunt viroid

^bNP, not performed

in PM2 exhibited 97.6%, 94.5%, 82.3% and 99% nucleotide sequence identity with PBNSPaV (LC523034), LChV-1 (KR080325), ACLSV (KC847061) and HSVd (EF523825) sequences present in GenBank, respectively.

Viral population and SNVs analysis

Each HTS data had a different number of sequenced reads associated with identified viruses and viroids. The number of ACLSV reads in PM1 and PM2 was 342 and 113, respectively (Table 4). The accumulation of individual virus in each HTS data was calculated by dividing individual virus reads with total virus-associated reads and multiplying them by 100. PNRSV RNA3 (34.5%) was the dominant genome segment, followed by APLPV RNA3 (31.4%), APLPV RNA1 (12.9%), PNRSV RNA1 (7.9%), PNRSV RNA2 (7%), APLPV RNA 2 (6.2%) and ACLSV (0.06%) in PM1 (Table 4, Fig. S2). The accumulation of HSVd in PM1 was extremely low. In

PM2, LChV-1 was predominant having 97% accumulation and ACLSV showed the lowest (0.5%) accumulation (Table 4, Fig. S2).

We used the FPKM method to determine the viral and viroid RNA in PM1 and PM2 HTS data. RNA3 of both PNRSV and APLPV shared highest FPKM 177581.16 and 152,848.22, respectively compared to RNA1 and RNA2 (Table 4). Among the viruses minimum FPKM was recorded for ACLSV. HSVd exhibited 72.98 and 116.67 FPKM for PM1 and PM2 respectively (Table 4). Additionally, to estimate the richness of RNA3 (5' UTR and movement protein: MP) and subgenomic RNA4 (CP and 3' UTR), we separately mapped these two sequences of PNRSV and APLPV RNA3. The results indicated that PNRSV CP had 1.2-fold higher accumulation than PNRSV MP, while APLPV CP had 0.1-fold lower accumulation than that of APLPV MP.

Furthermore, SNVs of PNRSV, APLPV, LChV-1, ACLSV and PBNSPaV were analysed in PM1 and PM2 samples. In PM1, PNRSV had the most SNVs,

Table 4 Virus and viroid reads, depth of coverage, FPKM and single nucleotide variants of individual virus and viroid identified in HTS data of plum samples

Plum Samples	Viruses and viroid ^a	Virus/viroid reads	Length (bp)	Virus/viroid accumulation (%)	Depth of coverage	FPKM ^b	SNVs ^c
PM1	PNRSV RNA1	40,155	3352	7.91	11,898.31	23,604.34	32
	PNRSV RNA2	35,780	2591	7.05	11,553.45	27,210.04	25
	PNRSV RNA3	175,111	1943	34.50	17,227.16	177,581.16	99
	APLPV RNA1	65,593	3303	12.92	12,271.42	39,129.58	3
	APLPV RNA2	31,262	2404	6.16	9991.21	25,623.51	9
	APLPV RNA3	159,255	2053	31.38	18,125.74	152,848.22	5
	ACLSV	342	7555	0.07	639.70	89.20	8
	HSVd	11	297	0.002	334.81	72.98	0
PM2	LChV-1	21,425	16,931	96.96	5159.67	189.81	14
	PBNSPaV	328	14,214	1.45	607.83	3.46	1
	ACLSV	113	7555	0.51	401.42	2.25	7
	HSVd	231	297	1.05	2984.86	116.67	12
PM3	NP ^d						
PM4	NP						
PM5	NP						

^aPNRSV, prunus necrotic ring spot virus, APLPV, American plum line pattern virus, ACLSV, apple chlorotic leaf spot virus, LChV-1, little cherry virus 1, PBNSPaV, plum bark necrosis stem pitting-associated virus, HSVd, hop stunt viroid

^bFPKM, fragments per kilobase of transcript per million reads mapped

^cSNVs, single nucleotide variants

^dNP, not present

followed by APLPV and ACLSV. The majority of the SNVs in PM2 were found in LChV-1, followed by ACLSV and PBNPaV (Table 4). In PNRSV RNA1, 26 SNVs were found in the replicase region, and 6 SNVs were located in the 3' untranslated region (UTR). In PNRSV RNA2, 25 SNVs were identified in the polymerase region. For PNRSV RNA3, 1, 47, 45, and 6 SNVs were found in the 5' UTR, movement protein (MP), CP, and 3' UTR, respectively. In the APLPV genome, RNA1 had 3 SNVs in the replicase region, while RNA2 contained 5 and 4 SNVs in the polymerase and 3' UTR, respectively. In APLPV RNA3, 1, 2, 1, and 1 SNVs were found in the 5' UTR, MP, CP, and 3' UTR, respectively. ACLSV's replicase region had 8 SNVs. In the LChV-1 genome, 5, 2, 1, 4, and 2 SNVs were identified in the polyprotein, viral HSP90, CP, CP minor, and hypothetical protein regions, respectively. ACLSV's replicase region contained 7 SNVs, while PBNPaV's polyprotein had 1 SNV.

Phylogenetic analyses of viruses identified in HTS data and Kraken taxonomic classification

We chose CP of PNRSV and APLPV for the construction of phylogenetic tree. Phylogenetic analysis revealed that the PNRSV and APLPV identified in PM1 HTS data closely related to PNRSV and APLPV reported from Czech Republic (AF170158) and Italy (EF494407 and EF503724), respectively (Fig. 1a). Both the viruses represent two separate clades in the tree. Further, LChV-1 identified in present study grouped together with South Korean isolate of LChV-1 (KR080325) in dendrogram (Fig. 1b). PBNPaV identified in this study clustered together with PBNPaV isolate (LC503034) infecting *P. domestica* in Australia (Fig. 1c). Kraken 2 was used to perform taxonomic classification on all reads of PM1, PM2, PM3, PM4 and PM5. The samples contained a varied range of viral, bacterial, and archaeal populations. A taxonomic classification

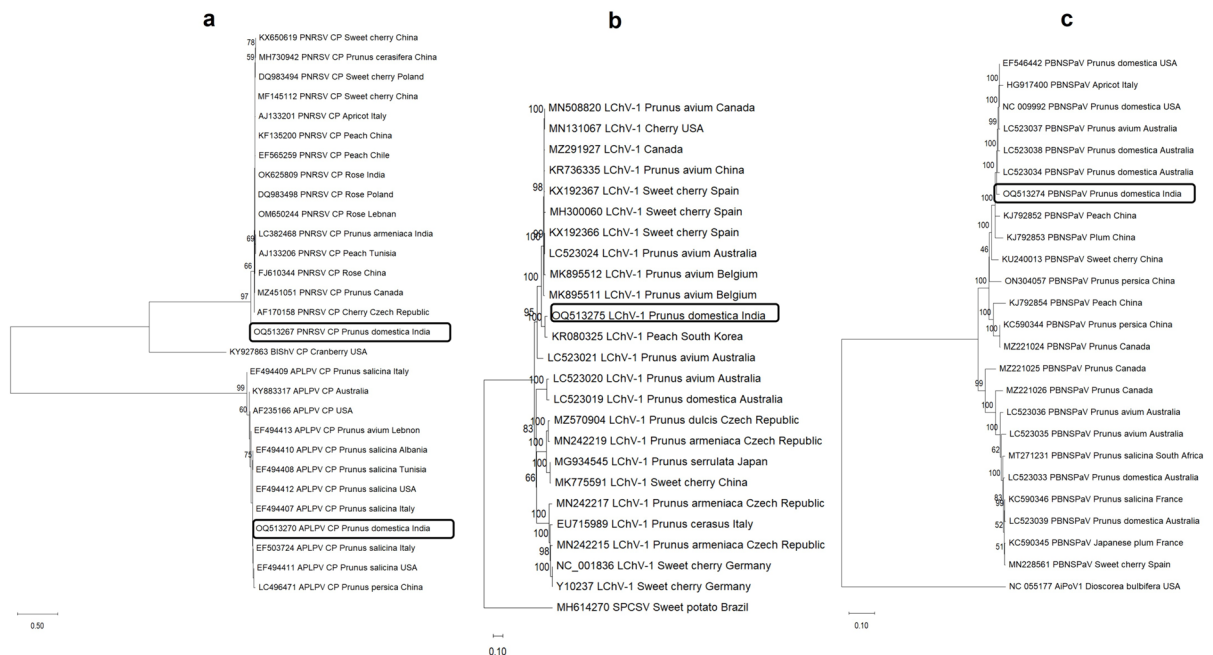


Fig. 1 Phylogenetic dendrograms showing the relationship of **a**) CP of APLPV (OQ513270) and PNRSV (OQ513267), and complete genome of **b**) LChV-1 (OQ513275) and **c**) PBNPaV (OQ513274) of this study (highlighted) with other isolates of APLPV, PNRSV, LChV-1 and PBNPaV retrieved from GenBank. The evolutionary distances were calculated using the Maximum Composite Likelihood method, and the evolutionary history was estimated using Neighbor-Joining method in

MEGA11. At each node, the percentage of bootstrap scores from 1000 replicates is indicated. APLPV, American plum line pattern virus; PNRSV, prunus necrotic ringspot virus; LChV-1, little cherry virus 1; PBNPaV, plum bark necrosis stem pitting-associated virus; BISHV, blueberry shock virus; SPCSV, sweet potato chlorotic stunt virus; AiPoV1, air potato virus 1; CP, coat protein

obtained from PM1 revealed a dominance of bacteria (80.76%), followed by viruses (11.46%) and archaea (0.73%). In PM2 bacteria predominated (94.01%) followed by archaea (1.36%) and viruses (0.15%). Similar patterns were observed in PM3, PM4 and PM5 as of PM2 while the virus percentage was very low (0.02 to 0.06%). Among plant viral communities, APLPV was found to be dominant over the PNRSV in the PM1 (Fig. S3). PBNSPaV dominated over LChV-1 and ACLSV in PM2 (Fig. S4). No plant viruses and viroids were detected in PM3, PM4 and PM5 (Fig. S5, S6, S7).

Confirmation of viruses by RT-PCR and Sanger sequencing

RT-PCR and Sanger sequencing of cloned viral fragments was done to validate the presence of virus and viroid contigs identified through HTS. ACLSV was detected in PM1, PM2 and three other plum samples of the same orchard from where PM1 and PM2 HTS data were obtained (Fig. 2a, Table 3). PNRSV was identified in all three PM1 samples while APLPV was detected only two PM1 plum sample (Fig. 2b, c, Table 3). In PM2, PBNSPaV was confirmed in all

three plum samples while LChV-1 was identified in two plum sample (Fig. 2d, e, Table 3). RT-PCR of a housekeeping gene using Nad5-s and Nad5-as primers showed equal expression of *Nad5* gene in all plum samples (Fig. 2f). Upon several attempts we could not validate the presence of HSVd in both PM1 and PM2 using RT-PCR. In addition, the infection of PNRSV, APLPV, LChV-1, PBNSPaV and ACLSV was confirmed by Sanger sequencing results. The cloned viral fragments of PNRSV, APLPV, LChV-1, PBNSPaV and ACLSV showed maximum 87.53%, 85.11%, 82.85%, 85.56% and 92.30% sequence identity with PNRSV (MK392166; Bulgaria; sweet cherry), APLPV (EF494413; Lebanon; *Prunus avium*), LChV-1 (KR080325; South Korea; peach), PBNSPaV (LC523037; Australia; *Prunus avium*) and ACLSV (KX579123; Germany; apple), respectively.

The validation work was carried out using additional samples, namely PM6 to PM25, collected in June 2023 and PM26 to PM41 collected in May 2024 in the same field of Srinagar, Jammu & Kashmir from where the original PM1 and PM2 HTS samples were collected (Table 3). RT-PCR was performed for APLPV, PNRSV, LChV-1, PBNSPaV, ACLSV and HSVd. RT-PCR results

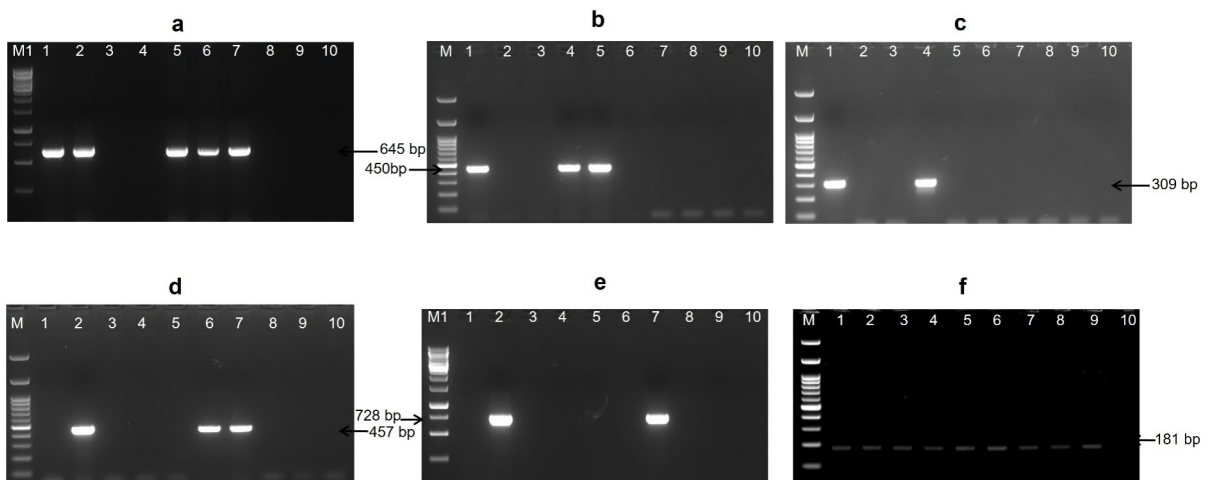


Fig. 2 Results of RT-PCR showing amplification of partial fragments of a) CP of ACLSV, b) CP of PNRSV, c) CP of APLPV, d) CP of PBNSPaV, e) RdRp of LChV-1-CP and f) *nad5* as internal control, from plum samples using specific primer pairs mentioned in Table 1. Lane M, 100 bp DNAladder, lane M1, 1 kb DNA ladder; lane 1, PM1; lane 2, PM2; lane 3, PM3; lane 4 and 5, other samples from same orchard

of PM1; lane 6 and 7, other samples from same orchard of PM2; lane 8, PM4; lane 9, PM5 and lane 10, negative control. APLPV, American plum line pattern virus; PNRSV, prunus necrotic ringspot virus; LChV-1, little cherry virus 1; PBNSPaV, plum bark necrosis stem pitting-associated virus; ACLSV, apple chlorotic leaf spot virus; CP, coat protein; RdRp, RNA dependent RNA polymerase

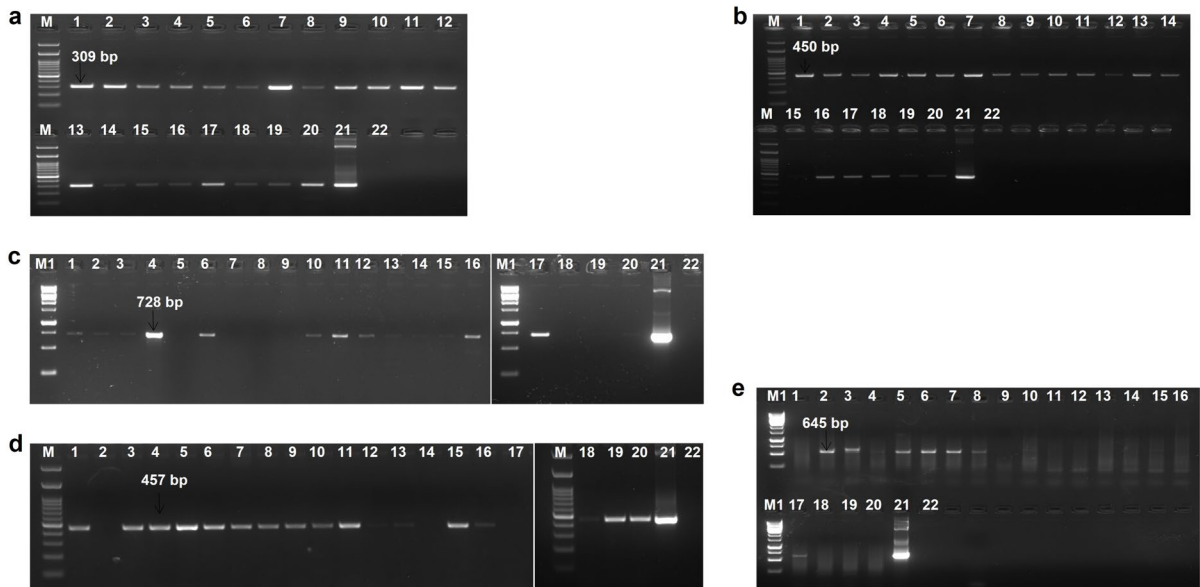


Fig. 3 Results of RT-PCR showing amplification of partial fragments of **a**) CP of APLPV, **b**) CP of PNRSV, **c**) RdRp of LChV-1CP, **d**) CP of PBNSPaV, **e**) CP of ACLSV in 20 plum samples, namely PM6 to PM25 (Lane 1–20), collected in June 2023 from Srinagar, Jammu & Kashmir. Lane M, 100 bp DNA ladder; Lane M1, 1 kbp DNA ladder; Lane 21, positive con-

trol; Lane 22, negative control. CP, coat protein; RdRp, RNA-dependent RNA polymerase; APLPV, American plum line pattern virus; PNRSV, prunus necrotic ringspot virus; LChV-1, little cherry virus 1; PBNSPaV, plum bark necrosis stem pitting-associated virus; ACLSV, apple chlorotic leaf spot virus

revealed that APLPV, PNRSV, LChV-1, PBNSPaV and ACLSV were present in varying frequencies in 36 plum samples (Fig. 3a-e, Table 3). Out of 36 plum samples, 35 were positive for APLPV and all

36 samples tested positive for PNRSV respectively (Fig. 3a and Fig. 4a, Fig. 3b and Fig. 4b, Table 3). LChV-1 and PBNSPaV were found in 27 and 33 plum samples, respectively (Fig. 3c and Fig. 4c,

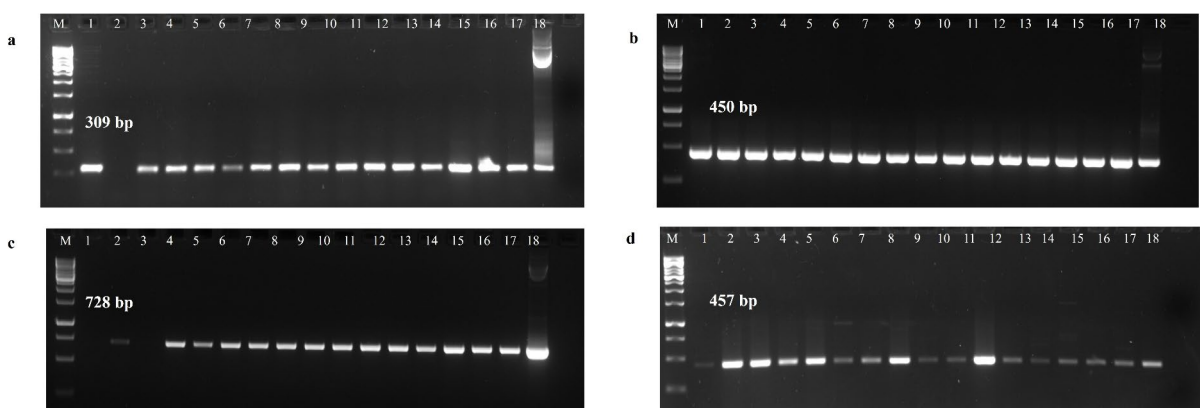


Fig. 4 Results of RT-PCR showing amplification of partial fragments of **a**) CP of APLPV, **b**) CP of PNRSV, **c**) RdRp of LChV-1CP, **d**) CP of PBNSPaV, in 16 plum samples, namely PM26 to PM41 (Lane 1–16), collected in May 2024 from Srinagar, Jammu & Kashmir. Lane M: 1Kb DNA ladder; Lane 17,

positive control; Lane 18, negative control. CP, coat protein; RdRp, RNA-dependent RNA polymerase; APLPV, American plum line pattern virus; PNRSV, prunus necrotic ringspot virus; LChV-1, little cherry virus 1; PBNSPaV, plum bark necrosis stem pitting-associated virus

Fig. 3d and Fig. 4d, Table 3), while ACLSV was detected only in 8 plum samples (Fig. 3e, Table 3). Despite several attempts HSVd could not be amplified in RT-PCR.

Discussion

Since plum trees are propagated vegetatively, cuttings and grafting may lead to the spread and accumulation of viruses. Several viruses have been reported from plum (Amenduni et al., 2005; Chandel et al., 2011; García et al., 2014; Jo et al., 2017, 2020; Rana et al., 2010). The overall process of producing clean plant material must include virus detection and cleaning of viral pathogen. HTS is a better tool to determine the virus spectrum in the plant and once the whole virus spectrum is known, targeted virus detection by methods such as RT-PCR, ELISA, RPA and CRISPR/Cas12a can be employed. Until now, the presence of ACLSV and PNRSV have been reported in plum from India (Chandel et al., 2013; Rana et al., 2010). However, HTS-based technology could identify near complete sequence of five viruses (PNRSV, APLPV, LChV-1, PBNSPaV and ACLSV) and one viroid (HSVd) and the presence of APLPV, LChV-1, PBNSPaV and HSVd is being reported for the first time in plum from India. Recently HTS identified six viruses namely ACLSV, LChV-1, PeVD, PLPaV, PNRSV, PBNSPaV and one viroid i.e. HSVd in plum cultivars from Korea (Jo et al., 2020). RT-PCR assessment of 36 plum samples showed the presence of APLPV, PNRSV, LChV-1, PBNSPaV and ACLSV in varying frequencies.

RNA isolation and Ribo-zero treatment for the removal of abundance of rRNA before library preparation is crucial for efficient virome analysis in plants. Ribo-zero treatment facilitates the detection of low abundance transcripts (Petrova et al., 2017). Our study clearly indicated the detection of near complete sequences of APLPV, PNRSV, LChV-1, PBNSPaV and ACLSV and a viroid namely HSVd in Ribo-zero treated libraries. Despite obtaining near-complete genomes of PBNSPaV and HSVd, the coverage was very low. The virome (viral metagenomics) primarily relies on *de novo* assembly of short sequencing reads to recover compositional and functional information (Sutton et al., 2019). To identify near complete

sequences of known or unknown viruses from diseased fruit crops, *de novo* assemblers are used now a days (Di Gaspero et al., 2022; Jo et al., 2015, 2018; Sidharthan et al., 2020). Trinity, SPAdes and MEGAHIT assemblers identified a similar number of viruses, but MEGAHIT had more virus sequence coverage than Trinity and SPAdes in our study. PBNSPaV and ACLSV were recovered partially using Trinity and SPAdes while these viruses were nearly completely identified when MEGAHIT assembler was used. Using multiple assemblers has advantages because viruses that evade detection by one assembler may be detected by the other (Massart et al., 2019).

APLPV was first reported in the United States (Paulsen & Fulton, 1968), and it was further recorded in Canada and several Mediterranean countries, including Italy, Tunisia, Albania, Lebanon and Palestine (Alayasa et al., 2003; Myrta et al., 2002). Recently APLPV has been reported from flowering cherry (*P. serrulata*) in Japan (Candresse et al., 2017). In our study, APLPV was confirmed in plum sample collected from ICAR-CITH, Srinagar and phylogenetic analysis showed its close resemblance with other APLPV reported from Italy in *P. salicina*. Similarly, LChV-1 and PBNSPaV were detected in plum samples of SKUAST, Srinagar. LChV-1 was first identified from Germany in sweet and sour cherry (Keim-Konrad & Jelkmann, 1996) and later it was detected in apricot, almond, plum and peach from other countries such as Italy, Czech Republic, Iran, Morocco and Chile, however, it was assumed that LChV-1 infection was latent (Fiore et al., 2018; Matic et al., 2007; Pourrahim & Farzadfar, 2018; Šafářová et al., 2020, 2022; Tahzima et al., 2019). The evolutionary study of LChV-1 and PBNSPaV from India revealed close resemblance with LChV-1 and PBNSPaV reported from Peach in South Korea and *P. domestica* in Australia, respectively. APLPV, LChV-1 and PBNSPaV are known to be transmitted only by infected propagating materials (Marini et al., 2002; Šafářová et al., 2022). It may be speculated that APLPV, LChV-1 and PBNSPaV came to India through imported plant materials. Further, the taxonomic classification by Kraken2 has shown the validation of the virus and viroid reads present in plum HTS data. Despite having limitations in terms of accuracy and sensitivity when counting the amount of plant viral

reads present, it was nevertheless able to recognise the plant viral species that were present in the HTS data. It has previously been determined that Kraken2's performance is enough for identifying microorganisms (Bush et al., 2020; Hadzega et al., 2021).

The mutation rate in RNA viruses is very high, up to a million times more frequently than their hosts, that enhances their virulence and make them capable of evolving into new species (Duffy, 2018). As a result of the mutation, quasispecies of variants are produced (Schneider & Roossinck, 2001). The characteristics of the viral population are influenced by the viral quasispecies, which are a collection of diverse variants (Lauring & Andino, 2010). Viruses adapt to their environments by acquiring novel SNVs as well as recombination events such as insertions, deletions, and duplications (Wang et al., 2022). SNV is a single nucleotide substitution for another, which can be common in one population but very rare in another population. SNV analyses revealed the mutation frequency for each virus contigs. The SNVs were more frequently found in PM1 and the highest SNVs were reported for RNA3 of PNRSV. Additionally, MP had more SNVs compared to CP. The lowest SNV i.e., 1 was recorded for PBNSPaV in PM2. The existence of more variations in the PM1 sample may be the cause of the larger number of SNVs found in PNRSV RNA3. The broad host range and diverse cellular environments provide opportunities for plant viruses to adapt and accumulate SNVs, leading to the presence of multiple genetic variants of the same virus.

RNA3 segment of both the ilarviruses showed highest accumulation compared to other RNA fragments such as RNA1 and RNA2 in PM1. RNA4 is a subgenomic RNA of RNA3 that encodes CP. The study showed that the higher accumulation of RNA3 in PNRSV is due to the increased expression of CP from subgenomic RNA4. In contrast, for APLPV, the higher accumulation of RNA3 is due to the increased expression of MP transcribed by RNA3. Therefore, we cannot conclude that the higher accumulation of RNA3 in PNRSV and APLPV RNA3 in PM1 is solely due to the higher expression of CP from subgenomic RNA4. The virus CP's primary function is to form the capsid, which protects the virus genome from degradation. This may be one

of the reasons why CP is expressed more than other viral genes in infected plant cells. Earlier studies through small RNA sequencing revealed that CP transcription unit is not a hotspot for RNA silencing which can result in a very high accumulation of CP in infected cells (Akbergenov et al., 2006; Aregger et al., 2012).

Despite of several attempts we could not validate the presence of HSVd in all the samples (PM1 - PM41) using RT-PCR. This may be due to presence of low concentration of viroid in the samples. As the HTS on Illumina platform was performed at high depth of coverage, the viroids of low concentration were recovered, which might not be detected in RT-PCR.

Finally, the study provides a comprehensive overview of viral diversity in plum in India and describes HTS-based virus detection in plum plants. APLPV, LChV-1 and PBNSPaV were reported for the first time from India in plum. Moreover, FPKM, SNVs and phylogenetic analysis contribute valuable knowledge on the virus richness, mutations and genetic diversity of plum viruses. The findings contribute to our understanding of plum viral pathogens and will aid in virus indexing and certification for commercial plum cultivars. More research is needed to understand ecological virology and host-virus interactions to determine the virus pathogenicity in the host as well as other related fruit crops. Further studies are required to check whether these viruses are causing a decline in the quality and quantity of fruit production in these areas.

Acknowledgements We are thankful to the Director, ICAR-Central Institute of Temperate Horticulture, Srinagar, Jammu & Kashmir and Head, ICAR-National Bureau of Plant Genetic Resources, Regional Station, Phagli, Shimla, Himachal Pradesh for providing plum leaf samples.

Author contribution Z.A.K., V.K.B., N.G. and S.K.S. designed the study. Z.A.K., S.U.N., S.W., M.D.S. and V.K.B. performed the field work and collected the samples. Z.A.K., P.T., D.D. and Y.S.M. performed the lab experiments. Z.A.K., D.D., P.T., S.K.S., S.U.N., N.G. and V.K.B. analysed the data. Z.A.K. wrote the manuscript. N.G., S.K.S., M.K.V. and V.K.B. reviewed and edited the manuscript. All authors have read and approved the manuscript.

Funding This work was supported by the Indian Council of Agricultural Research under the National Professor Scheme (Ag.Edn.F.No. /27/01/NP/2022-HRD) to Professor V.K. Baranwal.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical statement The study in the article did not involve human or animals subjects.

References

- Akbergenov, R., Si-Ammour, A., Blevins, T., Amin, I., Kutter, C., Vanderschuren, H., et al. (2006). Molecular characterization of geminivirus-derived small RNAs in different plant species. *Nucleic Acids Research*, *34*(2), 462–471.
- Alayasa, N., Al Rwahnih, M., Myrta, A., Herranz, M. C., Minafra, A., Boscia, D., et al. (2003). Identification and characterization of an American plum line pattern virus isolate from Palestine. *Journal of Plant Pathology*, *85*, 3–7.
- Amenduni, T., Hobeika, C., Minafra, A., Boscia, D., Castellano, M. A., & Savino, V. (2005). Plum bark necrosis stem pitting-associated virus in different stone fruit species in Italy. *Journal of Plant Pathology*, *87*, 131–134.
- Aregger, M., Borah, B. K., Seguin, J., Rajeswaran, R., Gubaeva, E. G., Zvereva, A. S., et al. (2012). Primary and secondary siRNAs in geminivirus-induced gene silencing. *PLoS Pathogens*, *8*(9), e1002941. <https://doi.org/10.1371/journal.ppat.1002941>
- Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*, *19*(5), 455–477. <https://doi.org/10.1089/cmb.2012.0021>
- Bush, S. J., Connor, T. R., Peto, T. E. A., Crook, D. W., & Walker, A. S. (2020). Evaluation of methods for detecting human reads in microbial sequencing datasets. *Microbial Genomics*, *6*(7), mgen000393.
- Candresse, T., Faure, C., Theil, S., & Marais, A. (2017). First report of American plum line pattern virus infecting flowering cherry (*Prunus serrulata*) in Japan. *Plant Disease*, *101*(8), 1561.
- Chandel, V., Rana, T., & Hallan, V. (2013). Prunus necrotic ringspot virus: Incidence on stone and pome fruits and diversity analysis. *Archives of Phytopathology and Plant Protection*, *46*(19), 2376–2386.
- Chandel, V., Rana, T., Hallan, V., & Zaidi, A. A. (2011). Detection of Prunus necrotic ring spot virus in plum, cherry and almond by serological and molecular techniques from India. *Archives of Phytopathology and Plant Protection*, *44*(18), 1779–1784.
- Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). Fastp: An ultrafast all-in-one FASTQ preprocessor. *Bioinformatics*, *34*(17), i884–i890.
- Di Gaspero, G., Radovic, S., De Luca, E., Spadotto, A., Magris, G., Falginella, L., et al. (2022). Evaluation of sensitivity and specificity in RNA-Seq-based detection of grapevine viral pathogens. *Journal of Virological Methods*, *300*, 114383.
- Duffy, S. (2018). Why are RNA virus mutation rates so damn high? *PLoS Biology*, *16*(8), e3000003.
- Fiore, N., Fernandez, C., Quiroga, N., Pino, A. M., Rivera, L., Sagredo, K., & Zamorano, A. (2018). First report of little cherry virus 1 in Chile. *Plant Disease*, *102*(3), 689.
- García, J. A., Glasa, M., Cambra, M., & Candresse, T. (2014). Plum pox virus and sharka: A model potyvirus and a major disease. *Molecular Plant Pathology*, *15*(3), 226–241.
- Ghawana, S., Paul, A., Kumar, H., Kumar, A., Singh, H., Bhardwaj, P. K., et al. (2011). An RNA isolation system for plant tissues rich in secondary metabolites. *BMC Research Notes*, *4*(1), 1–5.
- Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, *29*(7), 644–652. <https://doi.org/10.1038/nbt.1883>
- Hadzega, D., Minarik, G., Karaba, M., Kalavska, K., Benca, J., Ciernikova, S., et al. (2021). Uncovering microbial composition in human breast cancer primary tumour tissue using transcriptomic RNA-seq. *International Journal of Molecular Sciences*, *22*(16), 9058.
- Igori, D., Lim, S., Baek, D., Kim, S. Y., Seo, E., Cho, I.-S., et al. (2017). Complete nucleotide sequence and genome organization of peach virus D, a putative new member of the genus Marafivirus. *Archives of Virology*, *162*, 1769–1772.
- Jeong, J., Ju, H., & Noh, J. (2014). A review of detection methods for the plant viruses. *Research in Plant Disease*, *20*(3), 173–181.
- Jiao, J., Kong, K., Han, J., Song, S., Bai, T., Song, C., et al. (2021). Field detection of multiple RNA viruses/viroids in apple using a CRISPR/Cas12a-based visual assay. *Plant Biotechnology Journal*, *19*(2), 394–405.
- Jo, Y., Choi, H., Kyong Cho, J., Yoon, J.-Y., Choi, S.-K., & Kyong Cho, W. (2015). In silico approach to reveal viral populations in grapevine cultivar Tannat using transcriptome data. *Scientific Reports*, *5*(1), 15841.
- Jo, Y., Choi, H., Lian, S., Cho, J. K., Chu, H., & Cho, W. K. (2020). Identification of viruses infecting six plum cultivars in Korea by RNA-sequencing. *PeerJ*, *8*, e9588.
- Jo, Y., Chu, H., Kim, H., Cho, J. K., Lian, S., Choi, H., et al. (2017). Comprehensive analysis of genomic variation of hop stunt viroid. *European Journal of Plant Pathology*, *148*, 119–127.
- Jo, Y., Lian, S., Chu, H., Cho, J. K., Yoo, S.-H., Choi, H., et al. (2018). Peach RNA viromes in six different peach cultivars. *Scientific Reports*, *8*(1), 1844.
- Katsiani, A., Maliogka, V. I., Katis, N., Svanella-Dumas, L., Olmos, A., Ruiz-García, A. B., et al. (2018). High-throughput sequencing reveals further diversity of little cherry virus 1 with implications for diagnostics. *Viruses*, *10*(7), 385.
- Keim-Konrad, R., & Jelkmann, W. (1996). Genome analysis of the 3'-terminal part of the little cherry disease associated dsRNA reveals a monopartite clostero-like virus. *Archives of Virology*, *141*, 1437–1451.
- Khan, Z. A., Diksha, D., Thapa, P., Mailem, Y. S., Sharma, S. K., Gupta, N., et al. (2023). Genome analysis of viruses of Phenuiviridae, Betaflexiviridae and Bromoviridae,

- and apple scar skin viroid in pear by high-throughput sequencing revealing host expansion of a rubodvirus and an ilarvirus. *Physiological and Molecular Plant Pathology*, 129, 102196.
- Kim, N.-Y., Lee, H.-J., Kim, H.-S., Lee, S.-H., Moon, J.-S., & Jeong, R.-D. (2021). Identification of plant viruses infecting pear using RNA sequencing. *The Plant Pathology Journal*, 37(3), 258.
- Kim, N.-Y., Oh, J., Lee, S.-H., Kim, H., Moon, J. S., & Jeong, R.-D. (2018). Rapid and specific detection of apple stem grooving virus by reverse transcription-recombinase polymerase amplification. *The Plant Pathology Journal*, 34(6), 575.
- Komorowska, B., Malinowski, T., & Michalczyk, L. (2010). Evaluation of several RT-PCR primer pairs for the detection of apple stem pitting virus. *Journal of Virological Methods*, 168(1–2), 242–247.
- Kumar, S., Singh, L., Ram, R., Zaidi, A. A., & Hallan, V. (2014). Simultaneous detection of major pome fruit viruses and a viroid. *Indian Journal of Microbiology*, 54(2), 203–210.
- Langdon, W. B. (2015). Performance of genetic programming optimised Bowtie2 on genome comparison and analytic testing (GCAT) benchmarks. *Biodata Mining*, 8(1), 1–7.
- Lauring, A. S., & Andino, R. (2010). Quasispecies theory and the behavior of RNA viruses. *PLoS Pathogens*, 6(7), e1001005.
- Li, D., Liu, C.-M., Luo, R., Sadakane, K., & Lam, T.-W. (2015). MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics*, 31(10), 1674–1676.
- Li, H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv preprint arXiv:1303.3997*. Oxford University Press.
- Marini, D., Rowhani, A., & Uyemoto, J. (2002). Graft-transmissible agent causes bark necrosis and stem pitting in plum trees. *California Agriculture*, 56(3), 108–111.
- Marquez-Molins, J., Gomez, G., & Pallas, V. (2021). Hop stunt viroid: A polyphagous pathogenic RNA that has shed light on viroid–host interactions. *Molecular Plant Pathology*, 22(2), 153–162.
- Massart, S., Chiumenti, M., De Jonghe, K., Glover, R., Haegeman, A., Koloniuk, I., et al. (2019). Virus detection by high-throughput sequencing of small RNAs: Large-scale performance testing of sequence analysis strategies. *Phytopathology*, 109(3), 488–497.
- Matic, S., Myrta, A., & Minafra, A. (2007). First report of little cherry virus 1 in cherry, plum, almond and peach in Italy. *Journal of Plant Pathology*, 89, S75.
- Menzel, W., Jelkmann, W., & Maiss, E. (2002). Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. *Journal of Virological Methods*, 99(1–2), 81–92.
- Myrta, A., Abbadi, H., Herranz, M. C., Al Rwahnih, M., Di Terlizzi, B., Minafra, A., & Pallas, V. (2002). First report of American plum line pattern virus (APLPV) in Albania, Italy and Tunisia. *Journal of Plant Pathology*, 84, 188.
- Nabi, S. U., Baranwal, V. K., Rao, G. P., Mansoor, S., Vladulescu, C., Raja, W. H., et al. (2022). High-throughput RNA sequencing of mosaic infected and non-infected apple (*Malus domestica* Borkh.) cultivars: From detection to the reconstruction of whole genome of viruses and viroid. *Plants*, 11(5), 675.
- Noda, H., Yamagishi, N., Yaegashi, H., Xing, F., Xie, J., Li, S., et al. (2017). Apple necrotic mosaic virus, a novel ilarvirus from mosaic-diseased apple trees in Japan and China. *Journal of General Plant Pathology*, 83, 83–90.
- Ondov, B. D., Bergman, N. H., & Phillippy, A. M. (2011). Interactive metagenomic visualization in a web browser. *BMC Bioinformatics*, 12(1), 1–10.
- Paulsen, A. Q., & Fulton, R. W. (1968). Hosts and properties of a plum line pattern virus. *Phytopathology*, 58, 766–772.
- Petrova, O. E., Garcia-Alcalde, F., Zampaloni, C., & Sauer, K. (2017). Comparative evaluation of rRNA depletion procedures for the improved analysis of bacterial biofilm and mixed pathogen culture transcriptomes. *Scientific Reports*, 7(1), 1–15.
- Pourrahim, R., & Farzadfar, S. (2018). Molecular detection of little cherry virus 1 (LChV-1) from Iran. *Iranian Journal of Virology*, 12(1), 1–5.
- Qu, L., Cui, H., Wu, G., Zhou, J., Su, J., Wang, G., & Hong, N. (2014). Genetic diversity and molecular evolution of plum bark necrosis stem pitting-associated virus from China. *PLoS One*, 9(8), e105443.
- Rana, T., Chandel, V., Kumar, Y., Ram, R., Hallan, V., & Zaidi, A. A. (2010). Molecular variability analyses of apple chlorotic leaf spot virus capsid protein. *Journal of Biosciences*, 35, 605–615.
- Ren, S., Bertels, K., & Al-Ars, Z. (2018). Efficient acceleration of the pair-hmms forward algorithm for gatk haplotype-caller on graphics processing units. *Evolutionary Bioinformatics*, 14, 1176934318760543.
- Šafářová, D., Navrátil, M., & Lemberk, J. (2022). Characterisation of the little cherry virus 1 isolate infecting almonds in the Czech Republic. *Plant Protection Science*, 58(4), 292–297.
- Šafářová, D., Ševčíková, V., Neumanová, K., Suchá, J., Nečas, T., & Navrátil, M. (2020). Molecular characterisation of little cherry virus 1 infecting apricots in the Czech Republic. *European Journal of Plant Pathology*, 158, 83–97.
- Schneider, W. L., & Roossinck, M. J. (2001). Genetic diversity in RNA virus quasispecies is controlled by host-virus interactions. *Journal of Virology*, 75(14), 6566–6571.
- Sidharthan, V. K., Sevanti, A. M., Jaiswal, S., & Baranwal, V. K. (2020). Robust virome profiling and whole genome reconstruction of viruses and viroids enabled by use of available mRNA and sRNA-Seq datasets in grapevine (*Vitis vinifera* L.). *Frontiers in Microbiology*, 11, 1232.
- Sutton, T. D. S., Clooney, A. G., Ryan, F. J., Ross, R. P., & Hill, C. (2019). Choice of assembly software has a critical impact on virome characterisation. *Microbiome*, 7(1), 1–15.
- Tahzima, R., Qessaoui, R., Foucart, Y., Massart, S., & De Jonghe, K. (2019). First report of little cherry virus 1 infecting apricot (*Prunus armeniaca*) in Morocco. *Plant Disease*, 103(11), 2975.
- Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*, 38(7), 3022–3027.

- Wang, J., Zhai, Y., Zhu, D., Liu, W., Pappu, H. R., & Liu, Q. (2018). Whole-genome characterization of *Prunus necrotic ringspot virus* infecting sweet cherry in China. *Genome Announcements*, *6*(9), e00060–e00018.
- Wang, Y., Wang, Y., Wang, G., Li, Q., Zhang, Z., Li, L., et al. (2022). Molecular characteristics and incidence of apple rubbery wood virus 2 and citrus virus a infecting pear trees in China. *Viruses*, *14*(3), 576.
- Wood, D. E., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with kraken 2. *Genome Biology*, *20*, 1–13.
- Zhang, B., Liu, G., Liu, C., Wu, Z., Jiang, D., & Li, S. (2009). Characterisation of hop stunt viroid (HSVd) isolates from jujube trees (*Ziziphus jujuba*). *European Journal of Plant Pathology*, *125*, 665–669.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.