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RESEARCH ARTICLE

Virome profiling of pigeonpea using high-throughput sequencing and development of LAMP assay for PPSMV detection

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Abstract

Pigeonpea (*Cajanus cajan* (L.) Millsp) is a major pulse crop vulnerable to various biotic stresses including viral diseases. In addition to known viruses, novel and emerging viruses further threaten its production. In the current study, a survey was conducted in pigeonpea fields of Karnataka, India during 2023-24. The incidence of pigeonpea viral diseases in the surveyed areas ranged from 2 to 34 %. During the survey thirty virus infected pigeonpea samples were collected for virome analysis. Total RNA extracted from fifteen samples each from Northern and Southern Karnataka was pooled separately into two equimolar RNA samples and subjected to virome profiling. The rRNA-depleted RNA was used to prepare mRNA libraries, which were sequenced on the Illumina NovaSeq 6000 platform. Virome analyses revealed the presence of three different viruses: pigeonpea sterility mosaic virus 1 (PPSMV 1), pigeonpea sterility mosaic virus 2 (PPSMV 2) and horse gram yellow mosaic virus (HgYMV) from Northern Karnataka and only PPSMV 1 from Southern Karnataka. Sequencing was validated by reverse transcription polymerase chain reaction (RT-PCR) in individual samples, which revealed the prevalence of PPMSV 1, PPMSV 2 and HgYMV in Karnataka, India. Further, a reverse transcription loop-mediated isothermal amplification (RT-LAMP) diagnostic assay was developed to detect PPMSV, which can be used for routine detection. Among the identified viruses in pigeonpea, HgYMV is reported for the first time from Karnataka, India.

Keywords: horse gram yellow mosaic virus; pigeonpea; pigeonpea sterility mosaic virus 1; pigeonpea sterility mosaic virus 2; reverse transcription loop-mediated isothermal amplification; virome profiling

Introduction

Pigeonpea (*Cajanus cajan* L. Millsp) is an important pulse crop in India. It is also known as arhar, tur, or red gram belongs to the Fabaceae family, plays a critical role in nutritional security and sustainable agriculture due to its high protein content (20–30 %) and soil fertility benefits (1). India is the largest producer of pulses and pigeonpea is the second most widely cultivated pulse after chickpea, covering an area of 4.06 million hectares, yielding 3.31 million tonnes and productivity of 814 kg/ha (2). Despite the large area under pigeonpea cultivation in India, productivity is very low due to many biotic (fusarium wilt, sterility mosaic, phytophthora blight and pod borer complex) and abiotic stresses (drought, salinity and water-logging) (3). Among these, viral diseases severely affect pigeonpea productivity. Nearly 15 viruses have been documented to naturally infect pigeonpea (4), among which sterility mosaic disease (SMD), often referred as the “green plague” of pigeonpea is of major concern. It was recognized as the most destructive and economically significant viral disease in India (5), resulting in substantial yield losses.

Pigeonpea plants affected by SMD exhibit symptoms such as yellow mosaic, bushy pale green foliage with excessive vegetative growth, reduced leaf size, stunting, leaf distortion and partial or complete cessation of reproductive structures. This disease is caused by the pigeonpea sterility mosaic virus 1 (PPSMV 1) and pigeonpea sterility mosaic virus 2 (PPSMV 2) (6) and is transmitted by the eriophyid mite, *Aceria cajani* Channabasavanna in a semi-persistent manner (7). Pigeonpea sterility mosaic virus belongs to the genus *Emaravirus* and family, *Fimoviridae*. PPSMV 1, has four to five RNA segments (RNA 1 to RNA 5) and PPSMV 2, consists of six RNA segments (RNA 1 to RNA 6). The largest

segment, RNA 1 (7022 bp) encodes an RNA-dependent RNA polymerase; RNA 2 (2223 bp) encodes a glycoprotein precursor; RNA 3 (1442 bp) encodes the nucleocapsid protein; RNA 4 (1563 bp) encodes a movement protein; while RNA 5 (1689 bp) encodes the p5 protein and a sequence of 1094 nucleotide base pairs encodes the p6 protein. The functions of the p5 and p6 proteins remain unknown (8).

Pigeonpea production is increasingly threatened by novel and emerging viral diseases. In addition to SMD, yellow mosaic disease (YMD) of pigeonpea has been reported in recent years. Yellow mosaic disease in pigeonpea was first documented in Sri Lanka (9). In India, although the disease was initially described based on symptomatology (10), mungbean yellow mosaic India virus (MYMIV) and mungbean yellow mosaic virus (MYMV) were subsequently identified as the causal agents (11). The increasing incidence of viral infections underscores an urgent need to develop reliable and sensitive detection methods to facilitate early diagnosis, effective management and resistance breeding (12).

Polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) are pivotal molecular techniques that offer high levels of sensitivity and specificity (13). Polymerase chain reaction amplifies DNA sequences, making it ideal for detecting DNA viruses, while RT-PCR targets RNA viruses by converting viral RNA into complementary DNA (cDNA) using reverse transcriptase enzyme. These methods have successfully detected viruses such as alfalfa mosaic virus (AMV) and cucumber mosaic virus (CMV) in Australian chickpeas and lentils, even at low titers (14). However, their utilization is largely restricted to laboratories with advanced infrastructure due to the dependence on sophisticated and expensive thermocyclers. Loop-mediated isothermal amplification (LAMP) is a rapid, cost-effective technique operating at a constant temperature (60–65 °C), enabling amplification within 30-60 min (15). Its simplicity and visual detection using different dyes make it suitable for on-site diagnosis of viruses (16).

$$\text{Percent disease incidence} = \frac{\text{Number of diseased plants}}{\text{Total number of plants examined}} \times 100 \quad \text{(Eqn. 1)}$$

High-throughput sequencing (HTS), enables an unbiased and comprehensive virome analysis in infected plants, facilitating the detection of both known and novel viruses, mixed infections and genetic variants, which are critical for understanding viral evolution and informing resistance breeding strategies (17). High-throughput sequencing has been effectively employed for virome profiling in several pulse crops, including black gram (18), green gram (19) and chickpea (20). However, HTS-based studies focusing on the virome profiling in pigeonpea remain scarce in India and still need to be explored. To bridge this knowledge gap, the present study was conducted to document viral disease incidence and characterise the virome associated with pigeonpea in Karnataka, India using HTS, with the aim of determining the status of the viral population in major pigeonpea growing areas of the state.

Materials and Methods

Survey for viral disease incidence

A roving survey was conducted to record the prevalence of viral diseases in pigeonpea crop across 12 districts of Karnataka (Fig. 1), India during December 2023- 24. The incidence of viral diseases was quantified by the percent disease incidence (PDI) method. The PDI was calculated by using equation given previously (21). The zigzag pattern was followed to collect required data in which randomly selected plants were evaluated at each location (22).

Diseased sample collection

The leaf samples from the pigeonpea plants showing typical symptoms of viral infection like mosaic, leaf curling and stunting (Fig. 2) was collected from surveyed fields across the different locations (Table 1). Samples from asymptomatic plants (healthy plants) were also collected from each location and the collected samples were stored in the -80 °C freezer for further study.

High-throughput sequencing (HTS)

Total RNA was extracted from the pigeonpea leaf samples using the modified phenol-chloroform and lithium-chloride (LiCl) method (23, 24). The total RNA from the individual leaf samples was pooled into two equimolar RNA samples for NGS analysis by measuring the concentration (ng/µL) of each sample using a spectrophotometer

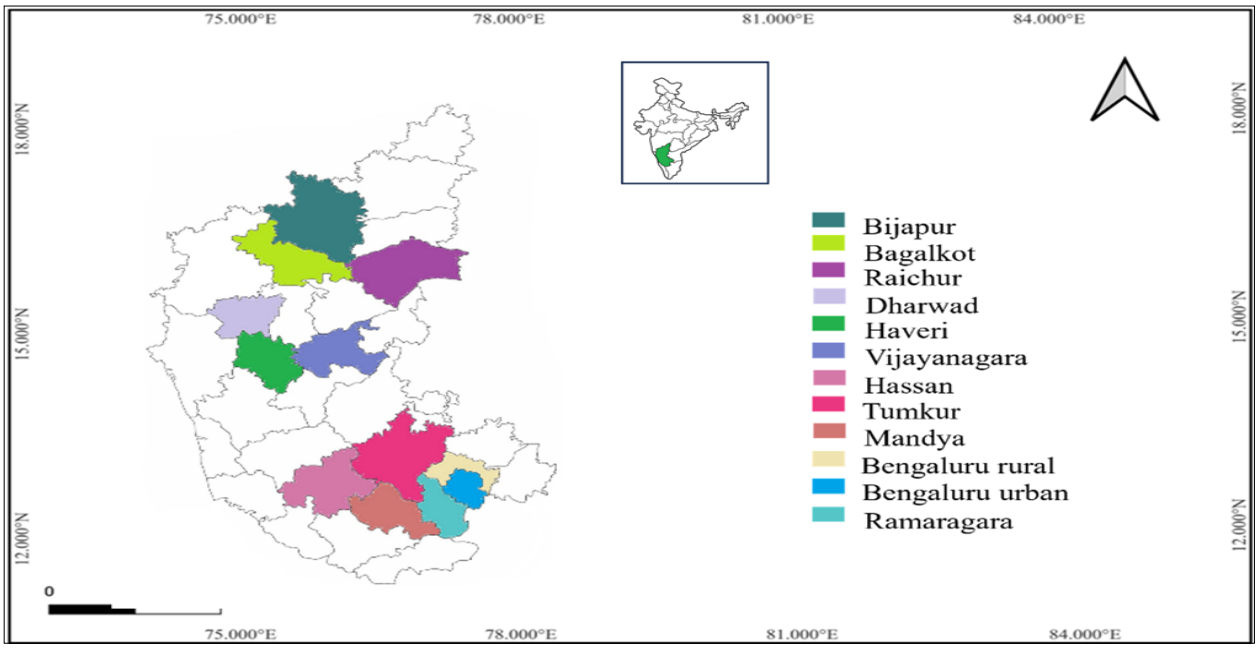

Fig. 1. Karnataka map representing the surveyed locations to record the incidence of viral disease in pigeonpea.

Table 1. Survey for the prevalence of viral diseases in pigeonpea in Karnataka during 2023 and 2024

District	Taluk	Village with GPS coordinates	Area (acre)	% Incidence	Average PDI	Symptoms
Haveri	Haveri	Varadi (14°79'N; 75°29'E)	1.25	16.00	12.83	Mosaic and partial sterility
		Byadagi (14°64'N; 75°52'E)	1.50	12.00		Sterility and mosaic
	Byadagi	Gummanahalli (14°66'N;75°46'E)	1.25	10.00		Mild mosaic
		Angaragatti (14°66'N;75°45'E)	1.75	13.33		Mosaic and leaf curling
	Bilagi	Yadahalli (16°19'N; 75°60'E)	0.50	21.42		Mild mosaic
Bagalkot	Hungund	Hirebadawadgi (16°05'N; 76°03'E)	20	22.50	18.32	Initial chlorotic spots, sterility
		Chittawadgi (16°02'N; 76°02'E)	1.75	16.32		Sterility and mosaic
	Badami	Adagal (15°96'N;75°67'E)	1.00	13.04		Complete sterility and mosaic
Raichur	Raichur	Gouhal (16°21'N;77°23'E)	0.75	18.36	13.51	Mild mosaic
		Venktapur (16°22'N;77°22'E)	0.50	13.33		Mosaic
		Murhanpur (16°23'N;77°17'E)	0.75	10.20		Mosaic
	Lingsur	Mudgal (16°23'N; 76°58'E)	2.00	14.58		Sterility and mosaic
		Abbasali doddi (16°26'N; 76°62'E)	2.25	11.11		Mild mosaic
Vijayapura	Vijayapura	Arakeri (16°93'N; 75°67'E)	0.50	14.58	18.60	Stunting and leaf curling
		Siddapura (16°95'N; 75°67'E)	0.75	15.55		Mosaic
	Basavana Bagewadi	Hunashyal (16°55'N; 76°04'E)	2.00	18.60		Mild mosaic
		Hulabenchi (16°56'N; 76°13'E)	1.00	20.40		Sterility
	Muddebihal	Bidarkundi (16°39'N; 76°12'E)	2.00	24.00		Sterility and mosaic
Vijayanagara	Kudlugi	Kudlugi (14°93'N; 76°36'E)	0.50	34.00	31.55	Severe mosaic
		Kudlugi (14°95'N; 76°35'E)	0.75	33.33		Mosaic and complete sterility
	Hagaribommanahalli	Hagaribommanahalli (14°97'N; 76°33'E)	1.00	28.88		Mosaic
	Hadagalli	Hadagalli (14°92'N; 76°06'E)	0.50	30.00		Mosaic
Dharwad	Dharwad	Dandikoppa (15°48'N; 75°19'E)	1.00	18.36	21.48	Sterility and mosaic
		Nayakanahulikatti (15°36'N; 75° 00'E)	0.25	26.66		Sterility and mosaic
	Hubli	Byahatti (15°44'N; 75°19'E)	1.25	13.04		Initial chlorotic spots
		Timmasagara (15°31'N; 75°11'E)	0.50	18.75		Mosaic and complete sterility
	Navalgund	Kumaragoppa (15°54'N; 75°33'E)	1.25	30.61		Severe mosaic
Hassan	Hassan	Attihalli (13°04'N; 76°07'E)	0.10	3.33	3.33	Mosaic
		Arekalahosahalli (13°02'N; 76°07'E)	0.25	4.16		Sterility and mosaic
		Uddarakoppalu (13°02'N; 76°07'E)	0.25	2.50		Mild mosaic
Bangalore rural	Doddaballapura	Gantiganahalli (13°36'N; 77°60'E)	0.25	10.00	6.24	Complete sterility and mosaic
		Kolur (13°33'N; 77°49'E)	0.10	8.33		Mosaic
		Hadonalli (13°37'N; 77°54'E)	0.50	6.52		Severe mosaic
	Hoskote	Upparahalli (13°08'N; 77°78'E)	0.25	4.16		Initial chlorotic spots
		Lakkondahalli (13°11'N; 77°77'E)	0.25	2.22		Mild mosaic
Mandya	Mandya	Ragimuddanahalli (12°49'N; 76° 79'E)	0.25	2.08	3.24	Sterility and mosaic
		Uramarkasalagere (12°47'N; 76° 80'E)	0.25	2.22		Mosaic
	Pandavapura	Hiremarali (12°51'N; 76°69'E)	0.10	5.26		Mosaic
		Chikkade (12°49'N; 76°69'E)	0.25	2.38		Sterility and mosaic
	Nagamangala	Hosahalli (12°84'N; 76°77'E)	0.50	4.16		Mosaic
		Chinnegowdana Kopalu (12°85'N; 76°77'E)	0.50	2.32		Mild mosaic
Bangalore urban	K. R. Pete	Kenchenahalli (13°23'N; 75°74'E)	1.00	4.34	9.84	Mosaic
	Yelahanka	GKVK (13°08'N; 77°57'E)	2.50	10.00		Complete sterility and mosaic
		IIHR (13°13;77°.49)	1.75	9.52		Severe mosaic
		ZARS (13°08'N; 77°57'E)	0.50	10.00		Partial sterility and mosaic
Kolar	Srinivasapura	Kasettipalli (13°44'N; 78°22'E)	0.50	10.20	7.89	Complete sterility and mosaic
		Kurigepalli (13°43'N; 78°22'E)	0.50	8.00		Mosaic
		Srinivasapura (13°32'N; 78°21'E)	1.00	8.88		Mosaic and leaf curling
Ramanagara	Malur	Kondasandra (13°27'N; 78°23'E)	0.50	6.12	2.83	Mild mosaic
		Sonnahalli (12°99'N; 77°98'E)	0.75	6.25		Mosaic
	Channapatna	Thaguchagere (12°62'N; 77°22'E)	0.25	2.12		Mild mosaic
		Sanabanahalli (12°60'N; 77°23'E)	0.50	4.34		Mosaic
	Magdi	Magdi (12°97'N; 77°23'E)	0.25	2.04		Initial chlorotic spots
Tumakuru	Tiptur	Rangapura (13°20'N; 76°47'E)	1.00	2.04	2.91	Mosaic
		Ballekatte (13°19'N; 76°46'E)	0.50	4.00		Complete sterility and mosaic
		Harisamudra (13°32'N; 76°46'E)	0.50	2.08		Mild mosaic
	Sira	Kusukunte kaval (13°75'N; 76°92'E)	0.25	2.38		Initial chlorotic spots
		Padmapura (13°70'N; 76°93'E)	0.75	2.04		Mild mosaic

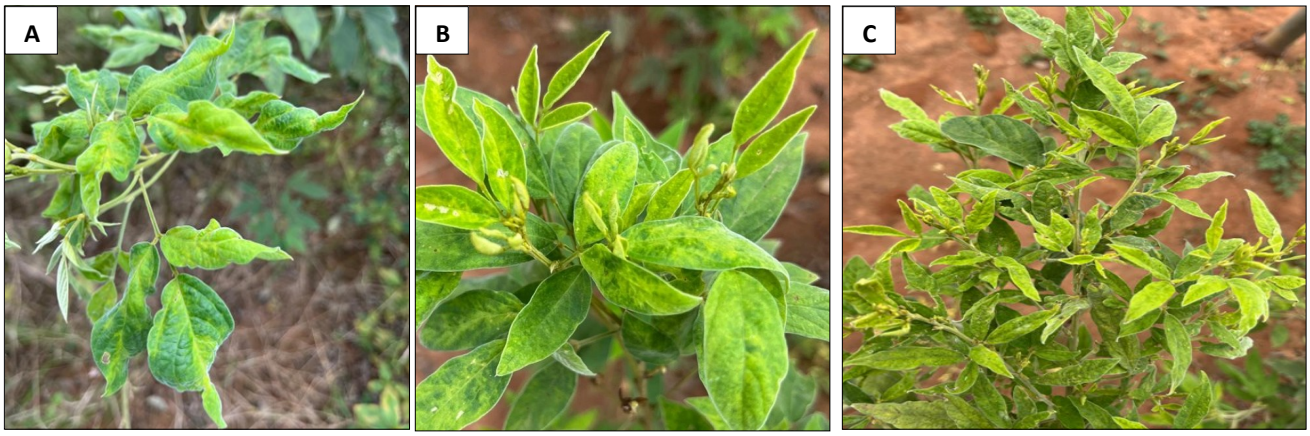

Fig. 2. Pigeonpea plants showing different viral symptoms, from which samples were collected for virome profiling. The symptoms observed in each sample are as follows: (A) leaf curling; (B) mosaic; (C) stunting and sterility.

(Thermo scientific Nanodrop 8000) and pooled to ensure equal concentration of RNA (in nanogram) across all samples. The first pooled sample comprised the pooling of fifteen pigeonpea RNA samples collected from Northern Karnataka (PN) and second sample comprised pooled RNA of fifteen pigeonpea samples collected from Southern Karnataka (PS). From these pooled samples ribosomal RNA (rRNA) was removed using the Ribo-Zero rRNA removal kit (Illumina, San Diego, CA, USA). Messenger RNA (mRNA) libraries were then prepared using the Illumina TrueSeq stranded mRNA library preparation kit (Illumina, San Diego, CA, USA). Library quan-tification was performed using a Qubit 4.0 fluorometer (Thermo Fisher Scientific, USA) with a DNA HS assay kit (Thermo Fisher Scientific, USA). Finally, the libraries were sequenced on an Illumina NovaSeq 6000 platform.

De novo assembly and virus identification

The quality of raw mRNAome data quality was first assessed by using a most popular bioinformatic tool FastQC version 0.11.9 (25) which provide quality score of data include, basic statistics, per base sequence quality, sequence length distribution, per sequence quality scores, per sequence GC content, per base sequence content, per base N content, over represented sequences (sequencing adapters) and sequence duplication levels. Sequence adapters and low-quality reads were trimmed using Trim Galore version 0.6.5, applying a Phred quality score of q= 30 (26) and the quality of the reads was once again confirmed with FastQC. Good quality mRNA reads were then selected for *de novo* assembly using Trinity version 2.13.2 (27).

The contigs generated from the individual *de novo* assemblies were subjected to homology search using standalone BLASTn version 2.12.0 (28) to identify closely related sequences (MEGABLAST) with an e-value threshold of 1e-5 and a query coverage of ≥ 90 % in the virus database, which includes complete viral reference sequences from the National Center for Biotechnology Information (NCBI) GenBank.

Reconstruction of viral genomes

Contigs corresponding to identified viruses were assembled and compared against the virus reference database available in NCBI. The sequences showing higher similarity were selected as reference genomes. Multiple sequence alignment was then carried out using Clustal X version 2.0 software (29) using the reference sequences and contigs of varying lengths to identify overlapping regions and extend fragmented assemblies. The reconstructed virome with maximum genome coverage was refined using BioEdit version 7.2 software (30).

Phylogenetic and recombination profiling of viral genomes

The genomes of the identified viruses, along with their corresponding reference sequences retrieved from the NCBI GenBank database were utilized for sequence comparison, phylogenetic analysis and recombination detection. Pairwise sequence identity was estimated by aligning the assembled viral genomes with their respective reference sequences using ClustalW implemented in the Sequence Demarcation Tool (SDT) version 1.2 (31). Phylogenetic trees were generated in MEGA version 11 platform (32) using the Neighbor-Joining (NJ) method based on the Kimura 2-Parameter (K2P) model with 1000 bootstrap replicates to assess branch support. Recombination detection was conducted in RDP5 (33), which is integrated with six algorithms: RDP, GENECONV, MaxChi, Chimaera, SiScan and 3Seq. Recombination events supported by at least three of these algorithms were considered significant.

Development of RT-PCR and RT-LAMP assays for the detection of identified viruses

The presence of the identified viruses was validated through RT-PCR assay developed by designing specific primers targeting the viral coat protein regions using Primer3 software. These primers were designed considering parameters like primer length (18–24 nucleotides), melting temperature (Tm) between 55–60 °C, GC content of 40–60 %. Along with these, the avoidance of secondary structures (hairpins, self-dimers and cross-dimers) were also ensured. A 1000 ng of each pooled RNA sample was used to synthesise cDNA of each viral sequences identified through HTS using virus specific reverse primers. For cDNA synthesis, 20 µL reaction volume containing 1000 ng/ µL of RNA sample, 1 µL reverse primer (10 pM), 4 µL of RT buffer, 2.5 µL of dNTPs mixture, 0.5 µL of reverse transcriptase enzyme and reaction mixture was made upto 20 µL using RNAase free water. It was incubated for 90 min on ProFlex PCR system (Applied Biosystems, Thermo Fisher Scientific, USA) followed by RT-PCR with reaction volume of 20 µL containing 2 µL of cDNA (100 ng/ µL) template, 1.5 µL of forward primer (10 pM), 1.5 µL of reverse primer (10pM), 10 µL of 2X PCR Master mix, 5 µL of sterilized milipore water with reaction condition of ini-tial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec horsegram yellow mosaic virus (HgYMV) and 57 °C for 30 sec (PPSMV 1 and PPSMV 2) and extension at 72 °C for 45 sec, with a final exten-sion step at 72 °C for 10 min on ProFlex PCR system (Applied Biosystems, Thermo Fisher Scientific, USA) and amplified products were analysed by

electrophoresis on 1 % agarose gel (70 V for 50 min) stained with ethidium bromide (10 mg/mL) visualised on Gel Doc XR imaging system (BioRad, USA).

The RT-LAMP assay was developed for detection of PPSMV 1, by designing three primers sets targeting coat protein region using PrimerExplorer software. The reaction mixture of 25 µL containing 2 µL of cDNA (100 ng/µL) template, 1 µL (10 µM) of F3, B3, 2 µL (10 µM) of each FIP, BIP, LF and LB primers, 1.5 µL of 10 mM dNTPs, 1.0 µL of 5 M betaine, 6.5 µL of sterile double-distilled water, 2.5 µL of 1X ThermoPol Reaction buffer (20 mM Tris– HCl, 10 mM (NH_4) SO_4, 10 mM KCl, 2 mM $MgSO_4$, 0.1 % Triton X-100, pH 8.8 at 25 °C), 0.5 µL of 100 mM $MgSO_4$ and 1.0 µL of 8U Bst DNA Polymerase (New England Biologicals, USA). The reaction mixture was incubated in a ProFlex PCR system (Applied Biosystems, Thermo Fisher Scientific, USA) at 60 °C for 1 hr followed by 80 °C for 10 min to terminate the reaction. Loop-mediated isothermal amplification reaction products were analysed by electrophoresis on 2 % agarose gels, which were run at 65 V for 3 hr. The gels were stained with ethidium bromide (10 mg/mL) and visualised using Gel Doc XR Imaging System (BioRad, USA).

Results

Viral disease incidence and symptomatology

The incidence of pigeonpea viral diseases in the surveyed areas ranged from 2 to 34 % Among the 12 districts surveyed (Fig. 3, less than 10 % average disease incidence was recorded in Tumakuru (2.91 %), Ramanagara (2.83 %), Hassan (3.33 %), Mandya (3.24 %), Bengaluru Rural (6.24 %), Kolar (7.89 %) and Bengaluru Urban (9.84 %) districts (Fig. 2). In contrast, more than 10 % average disease incidence was reported from Dharwad (21.48 %), Vijayanagara (18.60 %), Bagalkot (18.32 %), Raichur (13.51 %) and Haveri (12.83 %) (Table 1). Further, fifteen pigeonpea samples each representing Northern and Southern Karnataka regions displaying typical viral disease symptoms, such as mosaic pattern, leaf curling, sterility and stunting were collected for further analysis. The samples from Northern Karnataka were designated as PN 1–15 and Southern Karnataka as PS 1–15 (Tables 2 and 3).

High-throughput sequencing (HTS)

A total of 30 pigeonpea samples were processed for HTS and two equimolar RNA pools were generated representing Northern Karnataka (PN) and Southern Karnataka (PS). Sequencing on the Illumina platform produced 13.8 million raw reads for PN and 8.2 million (5.70 GB) for PS (Supplementary Table 1). Although mRNA-based library preparation enriches for polyadenylated transcripts and may reduce recovery of non-polyadenylated viral RNAs, both datasets yielded sufficient viral reads for virome analysis. *De novo* assembly using Trinity produced 38262 contigs for PN and 8,773 contigs for PS. MEGABLAST analysis of assembled contigs against complete viral reference genomes in NCBI GenBank revealed the presence of both RNA and DNA viruses. In the PN dataset, contigs corresponding to PPSMV 1, PPSMV 2 and the DNA virus, HgYMV were detected, whereas only PPSMV 1–associated contigs were identified in the PS dataset (Table 4). Virus-associated contigs were extracted and mapped to their respective reference genomes, showing partial genome recovery (< 50 %) but high nucleotide identity (> 90 %), thereby confirming the presence of these viruses despite possible under-representation of non-polyadenylated viral genomes.

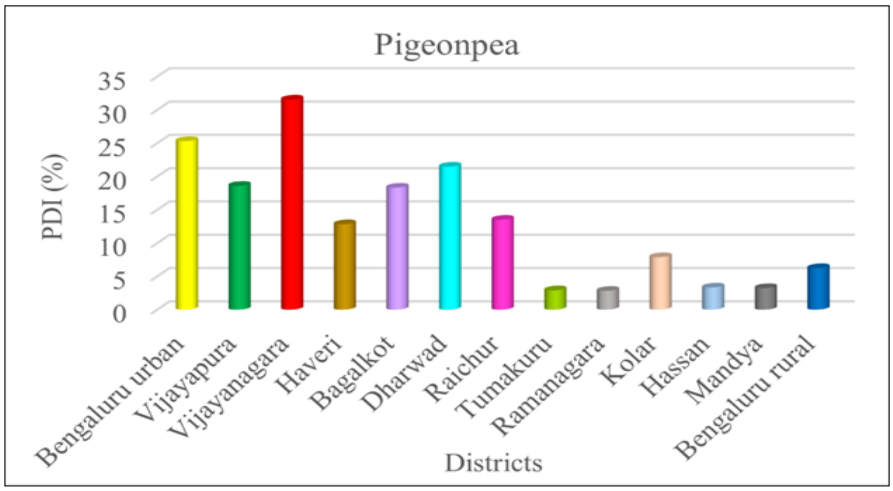

Fig. 3. Graphical representation of district wise average PDI of viral diseases.

Table 2. Details of pigeonpea samples selected for virome profiling collected from different locations of Northern Karnataka, India

District	Taluk	Village with GPS coordinates	Sample code
Haveri	Haveri	Varadi (14.79°N; 75.29°E)	PN1
	Byadagi	Gummanahalli (14.66°N; 75.46°E)	PN2
		Angaragatti (14.6624°N;75.4514°E)	PN3
Bagalkot	Hungund	Hirebadawadgi (16.0562°N; 76.0343°E)	PN4
	Badami	Adagal (15.9621°N;75.6764°E)	PN5
Raichur	Raichur	Gouhal (16.2123°N;77.2346°E)	PN6
		Murhanpur (16.2304°N; 77.1712°E)	PN7
	Lingsur	Mudgal (16.2334°N; 76.5834°E)	PN8
Vijayapura	Vijayapura	Siddapura (16.9599°N; 75.6720°E)	PN9
	Basavana Bagewadi	Hunashyal (16.5594°N; 76.0472°E)	PN10
	Muddebihal	Bidarkundi (16.3931°N; 76.1230°E)	PN11
Vijayanagara	Kudlugi	Kudlugi (14.9357°N; 76.3652°E)	PN12
	Hagaribommanahalli	Hagaribommanahalli (14.9714°N; 76.3364°E)	PN13
	Hadagalli	Hadagalli (14.9251°N; 76.0691°E)	PN14
Dharwad	Dharwad	Nayakanahulikatti (15.3647°N; 75.0053°E)	PN15

Table 3. Details of pigeonpea samples selected for virome profiling from different locations of Southern Karnataka, India

District	Taluk	Village with GPS coordinates	Sample code
Hassan	Hassan	Attihalli (13.0438°N; 76.0744°E)	PS1
		Arekalahosahalli (13.0237°N; 76.0717°E)	PS2
Bangalore rural	Doddaballapura	Gantiganahalli (13.3640°N; 77.6010°E)	PS3
	Hoskote	Lakkondahalli (13.1189°N; 77.7714°E)	PS4
Mandya	Mandya	Uramarkasalagere (12.4775°N; 76.8048°E)	PS5
	Pandavapura	Hiremarali (12.5192°N; 76.6908°E)	PS6
Bangalore urban	Nagamangala	Chinnegowdana Kopalu (12.8523°N; 76.7719°E)	PS7
	Yelahanka	GKVK (13.0853°N; 77.5795°E)	PS8
Kolar	Srinivasapura	Kurigepalli (13.4466°N; 78.2249°E)	PS9
	Malur	Sonnahalli (12.9949°N; 77.9878°E)	PS10
Ramanagara	Channapatna	Sanabanahalli (12.6063°N; 77.2378°E)	PS11
	Magdi	Magdi (12.9732°N; 77.2390°E)	PS12
Tumakuru	Tiptur	Rangapura (13.2027°N; 76.4703°E)	PS13
		Harisamudra (13.3227°N; 76.4652°E)	PS14
	Sira	Padmapura (13.7033°N; 76.9303°E)	PS15

Sequence comparison and phylogenetic analyses

The recovered viral genomes of PPSMV 1, PPSMV 2 and HgYMV from pigeonpea virome were used for further analysis. Sequence comparison and phylogenetic analysis were performed using these genomes and along with their respective reference sequences retrieved from NCBI GenBank. The nucleotide sequences of isolates obtained in the present study were named based on the sampling location and host. For example, the pigeonpea isolate of PPSMV 1 segment RNA 2 from Northern Karnataka were assigned the descriptor, pigeonpea North [India: Karnataka: Pigeonpea sterility mosaic virus 1 segment RNA 2: 2025] and designated as PN [IN:Kar:P1 RNA2:25]. Similar descriptor was assigned for all other isolates.

The phylogenetic analysis of RNA 2 segment of PPSMV 1 and PPSMV 2 obtained from the HTS of Northern and Southern Karnataka revealed that PN [IN:Kar:P1 RNA2:25] isolate shared the highest nucleotide identity (97.95 %) with the PPSMV 1 Bihar isolate (NC 029556), followed by the Andhra Pradesh isolate (NC 029555) (97.93 %). The PN [IN:Kar:P2 RNA2:25] isolate showed the highest nucleotide identity of 99.50 % with PPSMV 2 (LN651182) isolate from Chevella, followed by 99.20 % identity with PPSMV 2 isolate (PQ782228) from Hisar. The PS [IN:Kar:P1 RNA2:25] isolate showed a maximum nucleotide identity of 98.23 % with PPSMV 1 isolate (KX363907) from Pune. These 3 isolates grouped closely with PPSMV 1 (NC029556) isolate from Bihar, PPSMV 2 isolate Dharwad (MT683534) and Hisar (PQ782228) and PPSMV 1 (MH374921) from Badnapur, respectively. Similarly, nucleotide sequences of RNA 4 segment of PN [IN:Kar:P1 RNA4: 25] isolate obtained in the present study showed maximum nucleotide identity of 96.99 % with PPSMV 1 (KX363938) isolate from Pune. It was showing close clustering with isolates from Pune (KX363938) Andhra Pradesh (NC 029557), Hyderabad (LM652700) and Badnapur (MH374923). Likewise, nucleotide sequences of RNA 6 segment of PN [IN:Kar:P2 RNA6:25] isolate obtained in the present study showed maximum nucleotide identity of 98.74 % with PPSMV 1 isolate (MT376975) from Salem and has close clustering with isolate KX363944 (Coimbatore-1). For HgYMV isolate, PN [IN:Kar:HgYMV DNAB:25] revealed maximum nucleotide identity of 99.82 % with (MW791234) Bangalore isolate followed by 98.34 % with (MW814718) Raichur isolate showing close genetic relationship to the Bangalore isolate (MW791234), supported by 98 % bootstrap support (Fig. 4) (Supplementary Tables 2-5).

Detection of recombination among the identified viruses

Possible recombination events in genomic segments of PPSMV and HgYMV were identified using RDP 5 program. Localization of possible recombination breakpoint value and recombination events in sequences were identified by taking values of six methods: RDP, GENECONV, MaxChi, Chimaera, SiScan and 3 Seq.

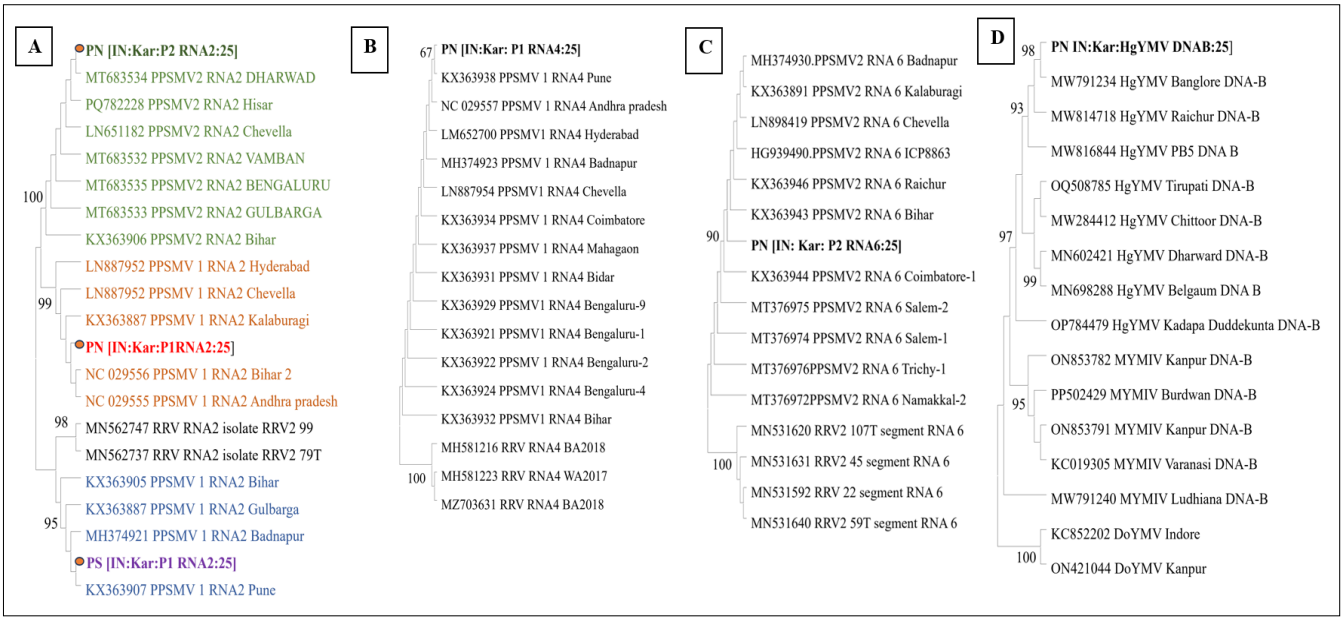

Fig. 4. Phylogenetic analyses of identified viruses. The Phylogenies were constructed using neighbourhood joining (NJ) method and Kimura 2-parameter (K2P) model with 1000 bootstrap replicates using 0.05 scale length in MEGA 11 programme: A) RNA 2, B) RNA 4, C) RNA6 and D) HgYMV DNA B segment of pigeonpea virus isolates.

Table 4. Genome coverage of identified viruses in pigeonpea samples from Karnataka from RNAome data with reference sequences available at NCBI, GenBank

Pooled RNA sample	Identified virus	Region	Reference genome	Reference virus	Genome recovered (nt)	Reference genome length (nt)	% Genome recovery	% Nucleotide identity with reference genome
PN	PPSMV 1	RNA2	PPSMV 1 RNA2	NC_029556	340	1544	22.02	97.55
		RNA 4	PPSMV 1 RNA4	NC_029557	284	2223	12.77	98.29
	PPSMV 2	RNA 2	PPSMV 2 RNA2	NC_030662	252	2229	11.30	99.55
		RNA 6	PPSMV 2 RNA6	NC_030659	658	1764	37.30	96.5
	HgYMV	DNA B	HgYMV DNA B	NC_005636	243	2542	9.55	93.8
PS	PPSMV1	RNA2	PPSMV 1 RNA2	NC_029556	252	2223	11.33	96.82

No recombination events were detected in the genomic segments of PPSMV 1 RNA2, PPSMV 1 RNA 4, PPSMV 2 RNA 2, PPSMV 2 RNA 6 and HgYMV viruses recovered from the pigeonpea samples collected from Karnataka, India.

Reverse transcription-PCR based validation of viruses detected in the pigeonpea virome from Northern Karnataka

All fifteen samples pooled for virome analysis were individually validated for the presence of PPSMV 1 by RT-PCR using specific primers designed (Table 5) to target the coat protein (CP) region of PPSMV 1. Amplified products were confirmed through gel electrophoresis on 1 % agarose gel. The results revealed clear and distinct amplicon of the expected size (~209 bp) (Fig. 5). Amplification confirmed PPSMV 1 in all fifteen samples collected from the northern districts of Karnataka. No amplification was observed in the healthy control or water controls, indicating the absence of false positives. Similar validation for PPSMV 2 with designed primers for CP specific primers produced an amplicon of the expected size (~154 bp) in 10 samples out of 15 (PN3, PN4, PN6, PN8, PN9, PN10, PN12, PN13, PN14 and PN15) (Fig. 5) collected from Northern districts of Karnataka. With respect to HgYMV using specific primers yielded an amplicon of the expected size (~1 kb), in 3 samples of pigeonpea (PN6, PN7 and PN13) collected from the Northern districts of Karnataka (Fig. 5). Notably, co-infection of three viruses viz., PPSMV 1, PPSMV 2 and HgYMV were detected in sample PN6 from Gouhal village, Raichur district and sample PN13 from Hagaribommanahalli, Vijayanagara district (Table 6).

Table 5. Primers designed from coat protein genes of viruses identified in virome analysis for use in RT-PCR assay

Virus	Primer	Sequence (5'-3')
PPSMV I	PPSMV I F	TCATCAATCGGTTAGCTGGTG
	PPSMV I R	CGCCTACACTAGAAGACATAGC
PPSMV II	PPSMV II F	TGACACTGTTGCTGCACATC
	PPSMV II R	TGGCCAGCTAGACGATTGAT
HgYMV	HgYMV F	CAGAAGTCGCCGTCATARTGG
	HgYMV R	GGAGAACCTCATCATCTCCATG

Reverse transcription-PCR based validation of virus detected in the pigeonpea virome from Southern Karnataka

In the pooled sample from Southern Karnataka, only PPSMV 1 was identified. Accordingly, validation was performed on the individual samples from Southern Karnataka using CP region specific primers. This revealed that all the samples tested positive for the presence of PPSMV 1. The clear and distinct bands were observed at expected amplicon length of ~209 bp. This confirmed the prevalence of PPSMV 1 in Southern districts of Karnataka (Fig. 6).

Development of RT-LAMP

Since the sensitivity level of conventional RT-PCR is low, in the current study, RT-LAMP was developed for the detection of PPSMV 1 in the plant sample. Reverse transcription-LAMP assay was carried out to validate the presence of PPSMV 1 in pooled RNA sample by using designed three sets of primers (Table 7). The results revealed ladder-like bands on 2 % agarose gel confirming the presence of PPSMV 1 in the pooled RNA sample and no bands in healthy control indicate the specificity of the developed RT-LAMP assay for the detection of PPMSV 1 (Fig. 7).

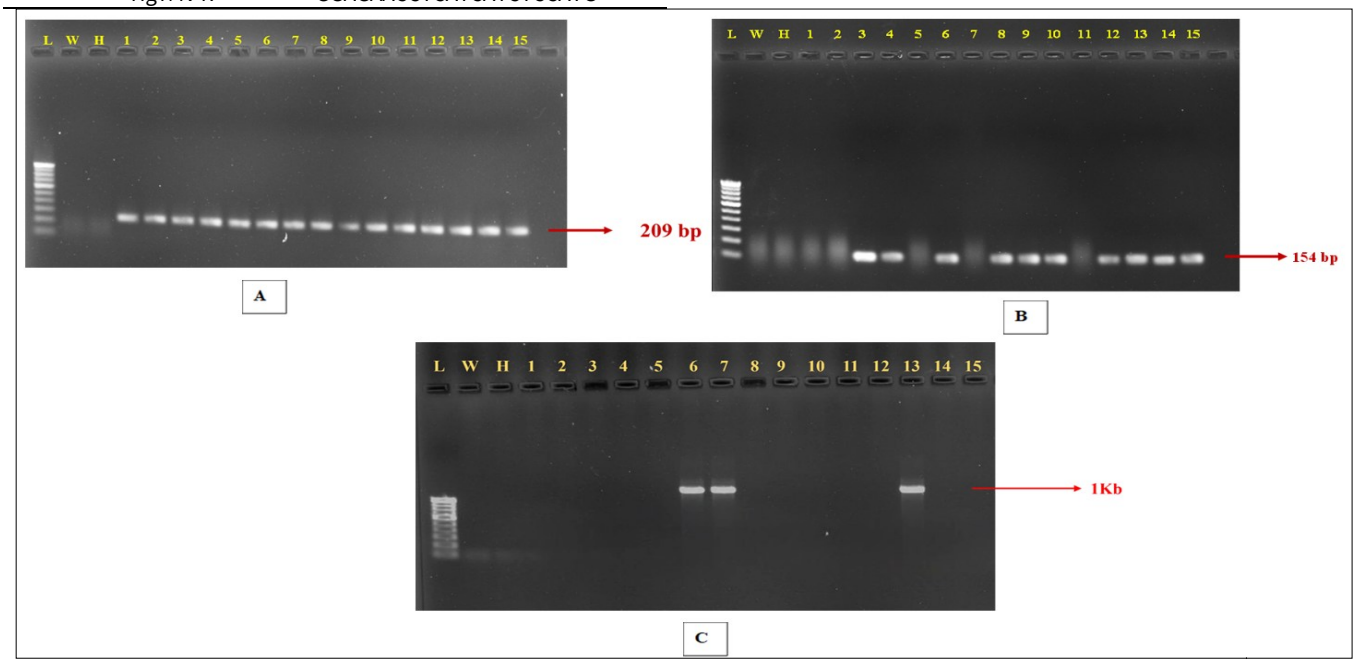

Fig. 5. Validation of identified viruses: A) PPSMV 1, B) PPSMV 2 and C) HgYMV from Northern Karnataka samples using RT-PCR assay. Lane L: 100 bp ladder, Lane W: Water control, Lane H: Healthy control and Lane 1-15: Pigeonpea samples (PN1- PN15).

Table 6. Detection of PPSMV 1, PPSMV 2 and HgYMV in pigeonpea samples collected from Northern districts of Karnataka

Location	Sample code	Identified viruses		
		PPSMV 1	PPSMV 2	HgYMV
Haveri, Varadi	PN1	+	-	-
Haveri, Gummanahalli	PN2	+	-	-
Haveri, Angaragatti	PN3	+	+	-
Bagalkot, Hirebadawadgi	PN4	+	+	-
Bagalkot, Adagal	PN5	+	-	-
Raichur, Gouhal	PN6	+	+	+
Raichur, Murhanpur	PN7	+	-	+
Raichur, Mudgal	PN8	+	+	-
Vijayapura, Siddapura	PN9	+	+	-
Vijayapura, Hunashyal	PN10	+	+	-
Vijayapura, Bidarkundi	PN11	+	-	-
Vijayanagara, Kudlugi	PN12	+	+	-
Vijayanagara, Hagaribommanahalli	PN13	+	+	+
Vijayanagara. Hadagalli	PN14	+	+	-
Dharwad, Nayakanahulikatti	PN15	+	+	-

Note: +: amplified; -: not amplified

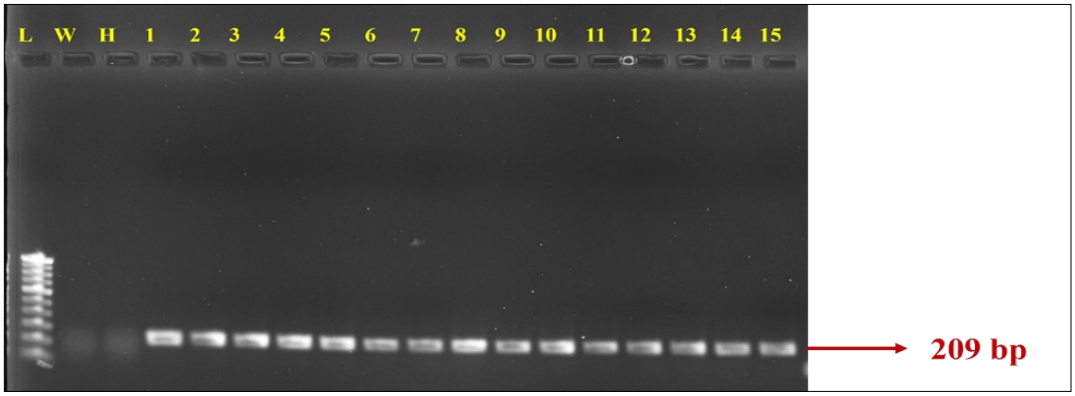

Fig. 6. Validation of PPSMV 1 from Southern Karnataka samples using RT-PCR assay. Lane L: 100 bp ladder, Lane W: Water control, Lane H: Healthy control and Lane 1-15: Pigeonpea samples (PS1-PS15).

Table 7. Primers designed from coat protein gene of PPSMV 1 identified in virome analysis for use in RT- LAMP assay

Virus	Primer	Sequence (5'-3')
PPSMV I	F3 (Forward outer)	CAATAATGGCTGCTGGAATC
	PPSMV I B3 (Backward outer)	GGGTAAAGTTCATAAAGAAACTCA
	PPSMV I FIP (Forward inner)	CTTAGTCTTGTTGTCAGTCTTGA-CACTCAAGCATACATTTGATGAGG
	PPSMV I BIP (Backward inner)	GTTCCCAATCCTGCAATTATCAA-TCACAATCATCCAATAATATGGGTTAC
	PPSMV I LB (Loop Backward)	TCTAGCTGGCCAGATGGGTT

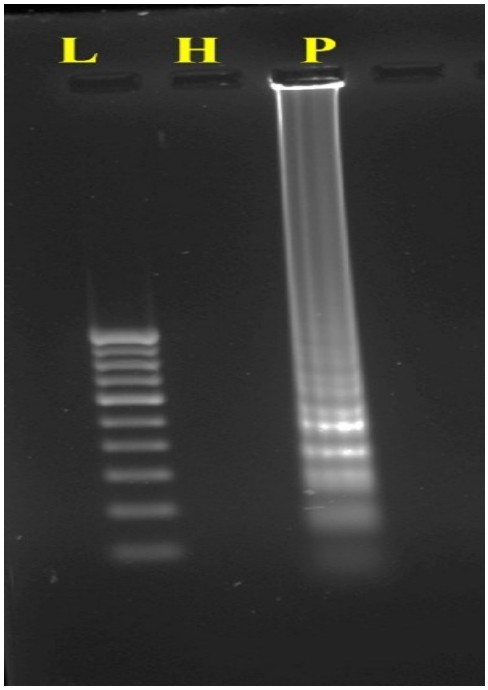

Fig. 7. Validation of PPSMV 1 with RT-LAMP assay. Lane L: Ladder (100 bp), Lane H: Healthy control, Lane P: Pooled RNA sample used for pigeonpea virome analyses.

Discussion

Viral diseases have emerged as major constraints to pigeonpea productivity, possibly due to the increased activity of vectors under changing climatic conditions (7). However, research on viral diseases affecting pigeonpea crop in India remains in its early stages, with significant gaps in understanding the population structure of plant viruses. Our goal in this study was to assess the virome of pigeonpea plants using HTS (Fig. 8) to identify both known and previously unreported viruses, if any in addition to develop diagnostic tools.

The survey shows the incidence of viral diseases in pigeonpea growing districts of Karnataka varied from 2 to 34 %. However, we noticed less than 10 % average disease incidence in Southern districts of Karnataka, while more than 10 % average disease incidence was reported from Northern districts of Karnataka. The higher viral incidence in Northern Karnataka (3) could be due to the cultivation of susceptible cultivars such as Gulyal local, TS-3R, ICP 8863 and other local varieties in Karnataka (34). Addition to this, practice of ratoon cropping from leftover infected pigeonpea plants after harvesting might serving as possible sources of virus infection, thus providing an opportunity for repeated cycles of infection.

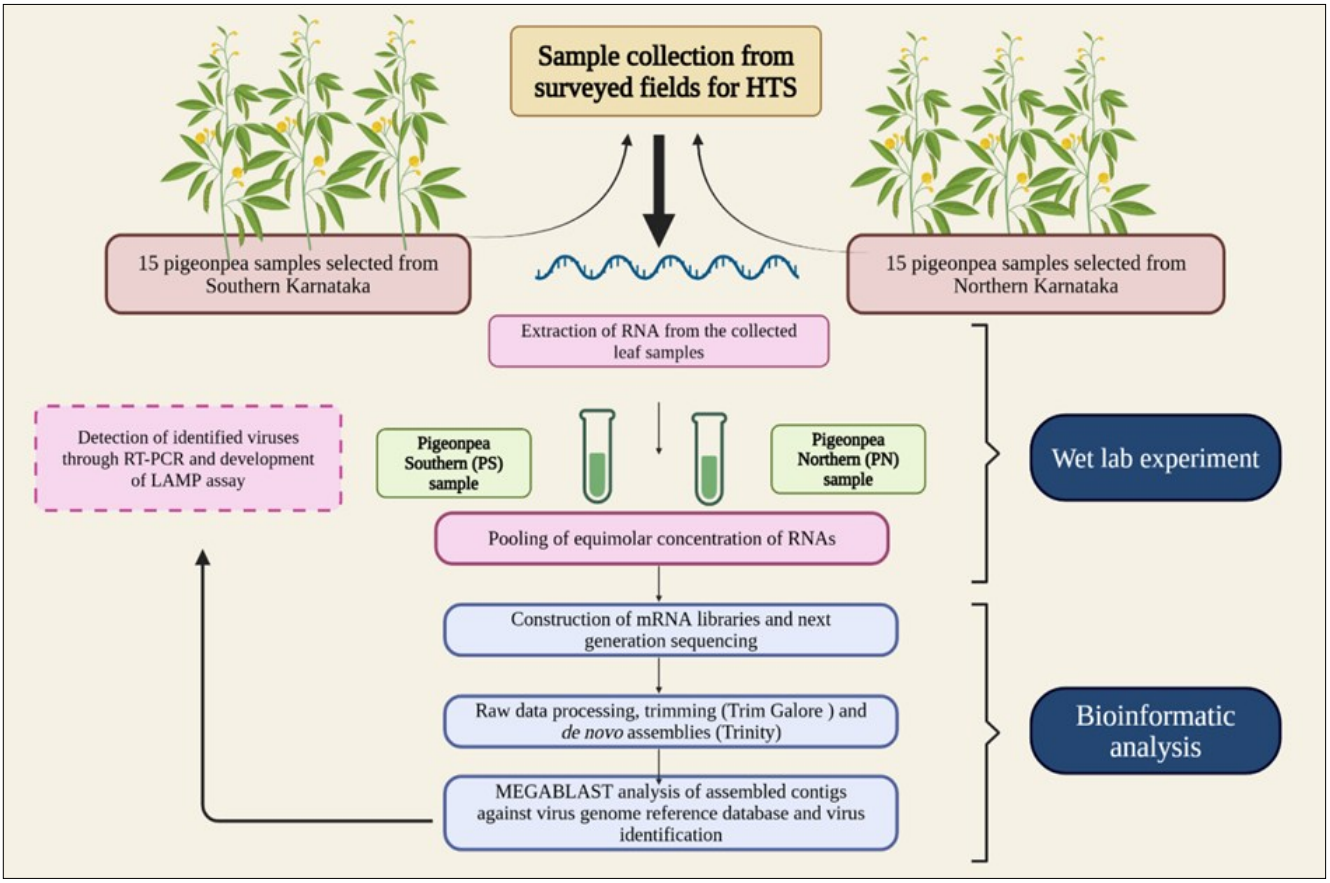

Fig. 8. Virome analysis workflow in pigeonpea.

Thirty virus infected samples from pigeonpea were collected for virome analysis and extracted RNA by modified phenol chloroform LiCl methods (24, 35). This method offers several advantages such as selectivity for RNA over DNA and proteins and the ability to denature RNases that could degrade the RNA (36). Virome analysis was performed using rRNA depleted total RNA, a method shown to effectively detect both RNA and DNA viruses (37). Paired-end raw reads were generated and subjected to *de novo* assembly using Trinity version 2.13.2 to profile the pigeonpea virome, a tool that has also been widely used in previous studies for virome profiling (35, 38, 39).

High-throughput sequencing and subsequent sequence search of the assembled reads revealed the presence of three viruses: two RNA viruses (PPSMV 1 and PPSMV 2) and one DNA virus (HgYMV) in pooled PN sample and only PPSMV 1 in PS sample. In a similar study, (38) used HTS to assess viral diversity from pulse crop, common bean and identified three viruses bean common mosaic virus (BCMV), bean common mosaic necrosis virus (BCMNV) and clover yellow vein virus (ClYVV). Similarly, 35 and 39 used HTS for identification of viruses associated with vegetable crops. The length of viral contigs is a key factor influencing the accuracy of virus identification in HTS datasets. We recovered only partial genomes (<50 %) of the identified viruses in this study, which is consistent with previous reports documenting partial viral genome recovery (38, 40). This could be attributed to low viral titre in the samples or the presence of highly divergent viral sequences that assemble poorly or fail to align with existing reference genomes, resulting in incomplete genome reconstruction (41). High-throughput sequencing has greatly expanded the detection of plant viruses, enabling the identification of both known and unknown pathogens, irrespective of their genome type or structure (17, 35, 39).

Based on the sequence identity and phylogenetic analysis, the PPSMV 1, PPSMV 2 and HgYMV isolate from current study were identified as a variant of an already reported species. Recombination is the formation of a new genome by covalent linkage of genetic material from two or more different parental genomes. No recombination events were detected in the genomic segments of PPSMV and HgYMV viruses recovered from the pigeonpea samples collected from Karnataka. This observation is not unexpected, as recombination is known to occur more frequently in retroviruses, pararetroviruses (e.g., cauliflower mosaic virus) (42) and many positive-sense RNA viruses (43), In contrast, double stranded and negative-stranded RNA viruses recombine with low frequency and the mechanism involved in (viral elements responsible for recombination and mutation rate) these events are still poorly understood (44). The absence of recombination in the current isolates suggests that they primarily evolve through purifying selection acting on existing genetic variation rather than through genetic exchange (45) with other viral strains, shows its conserved evolutionary pattern across different regions.

The presence of PPSMV 1, PPSMV 2 and HgYMV in pooled samples was validated through RT-PCR assays. Molecular confirmation of these viruses reinforces the HTS-based virome findings, providing additional validation for the detected viral pathogens. We success-fully amplified the target genes for identified viruses. Mixed infection of plants with different viruses occurs commonly in nature as the consequence of successive vector inoculations (12). We observe the association of PPSMV and HgYMV infection in our study, indicates a complex viral ecosystem, potentially influenced by vector, cropping patterns and climatic factors. Although HgYMV primarily infects horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.),

common bean (*Phaseolus vulgaris* L.) and soyabean (*Glycine max* (L.) Merr.) Its presence in pigeonpea suggests alternate host adaptation within legume systems (46). A recent study from Andhra Pradesh also reported pigeonpea as a host for HgYMV based on molecular evidence (47). Our findings provide the first report of HgYMV infecting pigeonpea from Karnataka, India using HTS. Such interspecies transmission of begomoviruses has been documented in other leguminous crops, reflecting vector promiscuity and overlapping host ranges (48). Pigeonpea also serves as a winter host for YMD causing viruses, facilitating the spread of the disease by whiteflies into the next growing season (49). This pattern may further be supported by ectothermic physiology of whiteflies (50), where they are sensitive to climatic changes and exhibit behavioural changes in response to seasonal variations. Together, these factors favour the increasing incidence of YMD in pigeonpea in India.

In this study, we also developed a LAMP assay for detection of PPSMV 1, representing the first diagnostic tool of its kind for future screening efforts. The choice of LAMP over PCR was due to its superior specificity and sensitivity. This method is a one-step, single-tube, reverse transcription and amplification reaction that detects a target RNA sequence with high sensitivity and specificity under a constant temperature of 60–65 °C within 60 min (15).

Conclusion

This study represents the first example of virome profil-ing in pigeonpea in India, highlighting the superior capabilities of HTS compared to traditional virus detection methods, especially during sudden outbreaks of emerging viruses. This investigation provides comprehensive data on the diversity and population structure of viruses present in the pigeonpea crop ecosystem. The results of this study show that YMD is increasingly becoming a significant concern in pigeonpea cultivation systems. These findings underscore the importance of early detection, development of sensitive diagnostic tools, continuous vector monitoring and adoption of management practices. Moreover, integrating resistant sources through targeted breeding strategies is crucial to develop durable, virus-resistant pulse varieties and ensure sustainable pulse productivity.

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Authors' contributions

KSA generated the samples for sequencing and done the bioinformatic analyses and validation work. RK and MB carried out bioinformatic analyses. MM and SMK carried out review, editing and visualization. TMN, GKS, VV and KSS carried out editing and supervision, CRJB and CNLR conceptualized and obtained funds and provided the overall direction. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process: Generative AI tool, Quillbot was used solely for rephrasing sentences and correcting grammatical errors, without contributing to the scientific content or analysis. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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