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AcademicPres

Rohini R *et al*. (2025)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 53, Issue 1, Article number 14329
DOI:10.15835/nbha53114329
Research Article

Notulae Botanicae Horti Agrobotanici Cluj-Napoca

Molecular insights of pigeonpea sterility mosaic virus mixed infections and its variability in pigeonpea

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Abstract

Pigeonpea sterility mosaic virus-1 (PPSMV-1) and Pigeonpea sterility mosaic virus-2 (PPSMV-2) are the two distinct Emaraviruses causing Sterility Mosaic Disease (SMD), which is an emerging threat to the pigeonpea (*Cajanus cajan* L. Millspaugh) production in India. The dissemination of virus is progressing at an accelerated rate and existence of PPSMV-1 (*Emaravirus cajani*) and PPSMV-2 (*Emaravirus toordali*) exhibiting diversity across the ecosystems. A total of 34 symptomatic and healthy samples were collected from six districts covering Western, North-Eastern, Cauvery Delta Zone and North-Western agro-climatic zones of Tamil Nadu. The disease incidence ranged from 12.8% to 71.6% and the highest mean incidence of 52.67% was recorded in the North-Eastern agroclimatic zone of Tamil Nadu. The RT-PCR and multiplex RT-PCR analysis differentiated the presence of both PPSMV-1, PPSMV-2 and mixed infections. Sequence analysis of RNA1 and RNA3 segments of the virus genomes demonstrated significant genetic heterogeneity among isolates, while phylogenetic relatedness of PPSMV-1 and PPSMV-2 were grouped into distinct clusters. Intra-specific recombination events were observed in the RNA3 segments of both PPSMV-1 and PPSMV-2. There were no recombination events in RNA1 segments of the virus genomes. These results emphasise the widespread distribution and genetic diversity of PPSMV-1 and PPSMV-2 in Tamil Nadu and provide valuable insights for the development of rapid diagnostic tools for effective field management.

Keywords: mixed infections; multiplex RT-PCR; pigeonpea; PPSMV-1; PPSMV-2; Sterility Mosaic Disease; Tamil Nadu

Received: 23 Dec 2024. Received in revised form: 22 Jan 2025. Accepted: 07 Mar 2025. Published online: 17 Mar 2025.

From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Introduction

Pigeonpea (*Cajanus cajan* L. Millspaugh) (Family: *Fabaceae*) is the fourth most important pulse crop cultivated in India and other South Asian countries (Jones *et al*., 2004). In India, it is commonly referred to as redgram, *tur*, or *arhar* and the production was 4.34 million tonnes from an area of 5.05 million ha with a productivity of 859 kg/ha (MoAF&W, 2022). In Tamil Nadu, pigeonpea is being cultivated both as monocrop and intercrop along with groundnut, cotton and millets.

SMD was initially reported in 1931 from the state of Bihar (Mitra, 1931) and is caused by two distinct emaraviruses: *Emaravirus cajani* (PPSMV-1) and *Emaravirus toordali* (PPSMV-2), a putative RNA virus of negative polarity (Elbeaino *et al*., 2014, 2015; Kumar *et al*., 2017; Patil *et al*., 2017; Sayiprathap *et al*., 2020). This disease is transmitted by eriophyid mite *Aceria cajani* Channabasavanna (Acari: *Arthropoda*) in a semi-persistent manner (Kulkarni *et al*., 2002). Symptoms associated with SMD are mosaic mottling or chlorotic ringspots, reduction of leaf size, stunting, excessive vegetative growth, and partial to complete cessation of flowering and sterility (Jones *et al*., 2004; Pande *et al*., 2012; Patil and Kumar, 2015).

Pigeonpea is susceptible to many diseases under field conditions (Saxena *et al*., 2004), however only a few of them are economically important and common over large areas. In India, the most widespread and significant diseases of pigeonpea are sterility mosaic disease (SMD), *Fusarium* wilt, *Phytophthora* blight and *Alternaria* blight (Reddy *et al*., 1998). Among them, SMD cause significant constrain in pigeonpea production in the Indian subcontinent (Mitra, 1931). Kannaiyan *et al*., (1984) estimated that over 0.2 million tonnes of reduction in the production of pigeonpea is due to SMD alone in India. Singh *et al*. (2013) reported that the varied distribution of incidence of SMD in main and ratooned crops in the Central Zone (Maharashtra), Southern Zone (Karnataka and Tamil Nadu) and North Eastern Plain Zone (Bihar and Uttar Pradesh). Siril *et al*. (2022) reported a significant variation in SMD incidence ranging from 6.27-25.58 per cent in northern parts of Karnataka

On the basis of their genome organization and morphological characteristics, both viruses were taxonomically classified as *Emaraviruses* under the new family *Fimoviridae* of order *Elliovirales*. Recent investigations revealed the complete genome sequences of PPSMV-1, which includes four to five RNA segments (RNA-1 to RNA-5), and PPSMV-2, which comprises six RNA segments (RNA-1 to RNA-6) (Elbeaino *et al*., 2015; Kumar *et al*., 2017; Patil *et al*., 2017). The largest segment, RNA-1 is of 7022 nucleotides in length encodes an RNA-dependent RNA polymerase. RNA-2 with a sequence length of 2223 nucleotides encodes a glycoprotein precursor; RNA-3 with 1442 nucleotides long encodes the nucleocapsid protein; RNA-4 with 1563 nucleotides in length encodes a movement protein; while RNA-5with a length of 1689 nucleotides encodes the p5 protein, and a sequence of 1094 nucleotides encodes the p6 protein. The functions of the p5 and p6 proteins remain unknown till now (Elbeaino *et al*., 2013,2014,2018). Furthermore, a significant factor contributing to the sequence variability is the recombination and reassortment between the segments of viral RNA genomes of plant viruses (Kallinen *et al*., 2009; Walia *et al*., 2014). Though earlier studies are available on genetic diversity of PPSMV-1 and PPSMV-2 associated with SMD by Patil (2017), the intra-specific relationship between *Emaravirus cajani* and *Emaravirus toordali* from major pigeonpea growing areas across Tamil Nadu has not been well established as on date. A comprehensive insight in the inherent genetic diversity of the *Emaraviruses* causing SMD in pigeonpea would be helpful for the development of reliable and effective diagnostic tools as well as for better mitigation approaches. In view of these facts mentioned, the investigations about the genetic variation and disease prevalence associated with SMD in different agro-climatic regions of Tamil Nadu, India were carried out during *kharif 2023-2024*.

Materials and Methods

Incidence and severity of SMD

A roving survey was conducted during *kharif* 2023-2024 to record the per cent disease incidence of pigeonpea sterility mosaic disease covering different agro-climatic zones of Tamil Nadu namely Western zone of Coimbatore district, North Western zone of Salem, Dharmapuri and Krishnagiri district, Cauvery Delta zone of Perambalur district and North Eastern zone of Vellore district. In each district, nine fields from major pigeonpea-growing areas were surveyed to determine the incidence of the disease. Samples were collected from symptomatic plants showing yellow mosaic, chlorotic ring spots, reduced leaf size, and severe stunting, along with healthy samples from farmers' fields. These samples were transported to the laboratory in cold packs, snap frozen in liquid nitrogen, and stored at -80 °C for further analysis. In addition to this, other observations such as the culture of the crop and varieties grown were also recorded. The disease incidence (%) was calculated using the formula provided by Wheeler (1969).

$$\%\text{SMD incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants observed}} \times 100$$

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Approximately, 100 mg of both symptomatic and healthy pigeonpea fresh leaf tissues were crushed with liquid nitrogen. Total RNA was extracted from the resulting leaf homogenate using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) as directed by the manufacturer's instructions. The NanoDrop 8000, Thermo-Fisher Scientific spectrophotometer was used to evaluate the purity and concentration of the RNA and was stored at -20 °C (Patil *et al*., 2017).

In RT-PCR, total RNA (3µg) was transcribed using RevertAid First Strand cDNA synthesis kits (Thermo Scientific, USA) according to the manufacturer's instructions. This cDNA synthesis cycle was performed in a Veritipro Thermal Cycler with one cycle of 42 °C for 60 mins and 72 °C for 5mins. Amplification of different segments of PPSMV-1 and PPSMV-2 isolates were performed with 1µL of diluted cDNA (1:10) using *Emaravirus*-species specific PCR primers listed in Table 1. The PCR mixture tube was incubated by one cycle of denaturation at 94 °C for 4 min, followed by 35 cycles at 92 °C for 30s, 45-65 °C for 30s, and 72 °C for 30s. The final extension was at 72 °C for 7 min in a Veritipro Thermal Cycler. The amplified products were resolved in 1% TAE (Tris-acetate-EDTA) agarose gels stained with ethidium bromide and documented by a gel–doc system (Major Science image analyzer).

Table 1. Details of primers used in this study

<table>
<tr><th>Primer ID</th><th>5'-3' sequence</th><th>Target region</th><th>Amplicon size</th><th>Diagnostic tool</th><th>Reference</th></tr>
<tr><td>PPSMV1 RNA 1 F</td><td>ATCTAGGTGGTGTGTTTGACA</td><td rowspan="4">RNA-dependent RNA polymerase</td><td rowspan="2">~332 bp</td><td rowspan="4">RT-PCR</td><td rowspan="4">Sayiprathap et al., 2022</td></tr>
<tr><td>PPSMV1 RNA 1 R</td><td>AACTTGCTCAAAATTCTCAAGC</td></tr>
<tr><td>PPSMV2 RNA1 F</td><td>ATCAATACTCCATAGTGCACCT</td><td rowspan="2">~332 bp</td></tr>
<tr><td>PPSMV2 RNA1 R</td><td>ACACCAACAGAAATATTCTTGGTG</td></tr>
<tr><td>PPSMV 1 RNA 3 F</td><td>ACCGCTCATACATACATCTAATCAGC</td><td rowspan="4">Nucleocapsid protein</td><td rowspan="2">~998 bp</td><td rowspan="4">Multiplex RT-PCR</td><td rowspan="2">Patil, 2017</td></tr>
<tr><td>PPSMV 1 RNA 3 R</td><td>AAGAAGCACAACTTAAAGGCAAACTN</td></tr>
<tr><td>PPSMV2 RNA3 F</td><td>GAGAGTAGTGAGTTGGAACCGAT</td><td rowspan="2">~284 bp</td><td rowspan="2">Sayiprathap et al., 2022</td></tr>
<tr><td>PPSMV2 RNA3 R</td><td>GAGTATCCCAGCAGCCATTATT</td></tr>
</table>

Diagnostic multiplex-RT-PCR for detection of PPSMV-1 and PPSMV-2 isolates

In order to differentiate the two species of *Emaraviruses*, a common degenerate forward and reverse primers depicted in Table 1 were used for a conserved nucleocapsid sequence (RNA3) of both PPSMV-1 and PPSMV-2 isolates. These primers that produce two distinct-sized amplicons, were used in all subsequent multiplex RT-PCR studies to detect and differentiate between PPSMV-1 and PPSMV-2 in SMD-infected pigeonpea samples. The diagnostic multiplex RT-PCR involved an initial denaturation at 94 °C for 4 min, followed by 35 cycles at 92 °C for 30s, 52 °C for 40s and 72 °C for 30s and a final extension of 72 °C for 7 min.

Sequence analysis and detection of recombination events

The PPSMV sequences were obtained by Sanger sequencing. The nucleotide homology searches were done with the BLASTN Sequence Analysis of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted in Genbank. Phylogenetic analysis was done using Molecular Evolutionary Genetic Analysis (MEGA) 11 (Kumar *et al*., 2016). Pigeonpea infecting *Emaravirus* and other previously reported isolates were compared using the maximum likelihood method and MUSCLE program with bootstrapping 1000 replications in order to generate the phylogenetic tree, along with evolutionary distance estimates. Furthermore, the per cent identity matrix among PPSMV sequences was determined using Sequence Demarcation Tool 1.2 (SDT v1.2) (Muhire *et al*., 2014).

Recombination breakpoints were detected using various built-in methods of the RDP4 package which includes RDP, GENECONV, BOOTSCAN, MAXIMUM CHI SQUARE, CHIMAERA, SISTER SCAN, and 3SEQ. Partial and complete genomic RNA segment sequences from PPSMV-1 and PPSMV-2 isolates were analyzed using Recombination Detection Programme (RDP4) software. The analyses were conducted with default settings, applying a Bonferroni correction with a p-value threshold of 0.05.

Inoculum maintenance for pigeonpea sterility mosaic virus

Pigeonpea sterility mosaic virus (PPSMV) was maintained on the susceptible pigeonpea cultivar ICP 8863 (a susceptibile check used in the all-India Co-ordinated Research Project trials on pigeonpea) in a glasshouse maintained at 27 ± 1 ℃ with 70-80% relative humidity. For inoculation, the leaf stapling technique employed by Nene and Reddy (1976) was followed on healthy pigeonpea seedlings aged 12 to 15 days.

Transmission of pigeonpea sterility mosaic virus

The seeds of pigeonpea cultivar Vamban (Rg)3 were sown in pots and maintained under insect proof cages in the glass house. The SMD-infected leaflets from cultivar Vamabn (Rg)3 in glasshouse were collected in moist cloth bags from the severely infected grownup plants. The prevalence of a minimum ten number of mites in each leaflet ensured by viewing under stereo zoom microscope. At two to three leafs staged (12 to 15 days old plant) were stapled up on both surfaces of the leaves such that their undersurface of the leaflets coming into contact with each other so as to establish the successful transmission of PPSMV and the symptom expression were observed at 15 days post inoculation of virus.

Statistical analysis

All data on disease incidence, severity, and the percentage of virus-infected plants were analyzed using an Analysis of variance (ANOVA) and correlation analysis was performed using GRAPES software.

Results

Symptomatology and disease incidence of SMD in Tamil Nadu

The survey was conducted from August to December during the Kharif season of 2023-2024 across the districts of Coimbatore, Salem, Dharmapuri, Krishnagiri, Perambalur, and Vellore, encompassing four agro-climatic zones of Tamil Nadu, where pigeonpea is extensively cultivated. As part of the roving survey, three villages were selected from each district, and five fields were surveyed in each village. The results revealed that the incidence of SMD during the *kharif* season of 2023–24 varied across locations, ranging from 12.8 to 71.6%. The lowest disease incidence was observed in North-western zone of Salem district (23.7%) in the vegetative stage, while Dharmapuri, Cauvery Delta zone of Perambalur and western zone of Coimbatore districts documented maximum mean disease incidence of 26.85, 34.33 and 37.04% respectively. The highest mean disease incidence was recorded in North Eastern zone of Vellore district (52.67%) in flowering stage of the crop. LRG 41 and Vamban (Rg)3 have shown the higher disease incidence in Vellore district with monocropping (Table 2). The severe stunting of the infected plants was noticed on 120 days after sowing which leads to complete sterility of the crop (Figure 1). The Correlation matrix studies indicated that, temperature (minimum), relative humidity showed highly significant positive correlation on the SMD incidence whereas maximum temperature showed negative correlation with SMD incidence (Table 3). The severity of the disease was highest in Vellore district, followed by Coimbatore district, which may be attributed to the monocropping system prevalent in these regions. This practice likely supports the continuous survival of the inoculum and sustains populations of the eriophyid mite (*Aceria cajani*), thereby contributing to increased disease severity and virus variability.

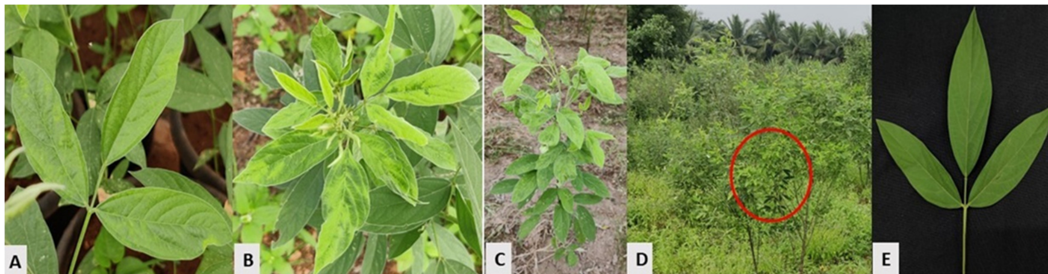


Figure 1. Characteristic symptoms of SMD in pigeonpea a) PPSMV-2: Chlorotic spots on the leaves; b) PPSMV-2: leaves with complete malformation; c) PPSMV-1&2 shows complete sterility with stunted appearance; d) PPSMV-2 depicting partial sterility of the crop; e) Healthy leaves

Table 2. Incidence of pgeonpea sterility mosaic disease in agro-climatic zones of Tamil Nadu during *kharif* 2023-2024

S.No	Agro-climatic zone	Name of the District	Location	Mean average temperature (°C)		Relative humidity (%)	GPS coordinates		Crop Stage	Cultivar	SMD incidence (%)*	Cropping system	PPSMV type
				Minimun	Maximum		Latitude	Longitude					
1.	Western	Coimbatore	TNAU – Pulses Research Farm	25	29.7	73	11.012°N	76.935°E	Vegetative and Flowering	CO (Rg)7	61.8	Monocropping	PPSMV1, PPSMV 2
2.			Devarayapuram				10.994°N	76.812°E	Flowering	CO 8	12.8	Intercropping with cotton	PPSMV 2
Mean	**37.04**												
3.	North Western	Salem	Thindamangalam	23.7	33.3	71	11.737°N	78.015°E	Pod formation	LRG 52	33.0	Monocropping	PPSMV 2
4.			Pappankattur				11.731°N	78.018°E	Vegetative	LRG 52	14.4	Intercropping with groundnut	PPSMV 2
		Mean									**23.7**		
5.		Dharmapuri	Nallampalli	26.3	31.1	74	12.059°N	78.099°E	Flowering	BRG 1	36.6	Monocropping	PPSMV 2
6.			Nathahalli				12.134°N	78.066°E	Flowering	BRG 2	23.4	Intercropping with groundnut	PPSMV 2
7.			Somanehlli				12.236°N	78.101°E	Flowering	Co (Rg)7	25.8	Monocropping	PPSMV1, PPSMV 2
8.			Adagapadi				12.141°N	78.099°E	Vegetative	BRG 2	21.6	Monocropping	PPSMV 2
		Mean									**26.85**		
9.		Krishnagiri	Pochampalli	23	30	68	12.363°N	78.366°E	Flowering	CO 6	15.6	Border crop for tomato	PPSMV 2
10.			Bargur				12.542°N	78.357°E	Pod formation	BRG 1	58.6	Monocropping	PPSMV 2
11.			Paiyur Research Station				12.210°N	78.182°E	Harvesting	Co(Rg)7	51.8	Monocropping	PPSMV 2
	Mean										**42.0**		
12.	Cauvery Delta Zone	Perambalur	Vilamuthur	23	33	72	11.218°N	78.872°E	Flowering	Vamban 2	27.8	Monocropping	PPSMV 2
13.			Siruvachi				11.207°N	78.875°E	Vegetative	CO 6	49.8	Monocropping	PPSMV1, PPSMV 2
14.			Ayyalur				11.182°N	78.904°E	Vegetative	Vamban 2	25.4	Intercropping with cotton	PPSMV 2
Mean	**34.33**												

S.No	**Agro-climatic zone**	**Name of the District**	**Location**	**Mean average temperature (℃)**		**Relative humidity (%)**	**GPS coordinates**		**Crop Stage**	**Cultivar**	**SMD incidence (%)***	**Cropping system**	**PPSMV type**
				Minimun	**Maximum**		**Latitude**	**Longitude**					
15.	North Eastern	Vellore	Virinjipuram	27.8	32.6	78	12.923°N	79.018°E	Flowering	LRG 41, Vamban Rg (3)	71.6	Monocropping	PPSMV1, PPSMV 2
16.			Gudiyetham				12.942°N	78.868°E	Pod formation	LRG 41	22.6	Monocropping	PPSMV 2
17.			Sethuvazhai				12.911°N	79.007°E	Pod formation	LRG 52	63.8	Monocropping	PPSMV1, PPSMV 2
Mean	**52.67**												

Mean of _obserbvations

Table 3. Correlation coefficient of PDI for SMD with weather parameters

S.No	Weather parameters	PDI
1.	Maximum temperature	-0.165
2.	Minimum temperature	0.412
3.	Relative humidity (%)	0.213

Multiplex RT-PCR and RT-PCR detection patterns of PPSMV-1 and PPSMV-2

A total of collected 34 pigeonpea leaf samples exhibiting various symptoms, including chlorotic ring spots, pale green appearance, reduced leaf size, mosaic patterns, mottling and leaf distortion. These samples were obtained from several pigeonpea cultivars, namely CO(Rg)7, CO 8, LRG 52, BRG 2, Vamban Rg (3), LRG 41, and VAMBAN 2, during the flowering, vegetative, and pod formation stages across four agro-climatic zones in Tamil Nadu, India. The purpose of this collection was to investigate the genetic variation of PPSMV in these samples.

The dual primer combinations targeting the RNA3 segment generated amplicons of two discrete sizes; 992nt for PPSMV 1 and 284nt for PPSMV 2. The multiplex-RT-PCR analysis of SMD-infected pigeonpea leaf samples collected from six districts of Tamil Nadu resulted that PPSMV-1 and PPSMV-2 occurred as single and mixed infections. The maximum frequency of mixed infection was occurred in North-Eastern region of Vellore district (Figure 2). RT-PCR targeted the RdRp segment of PPSMV 1 and PPSMV 2 also resulted in amplification of expected size of 332bp as depicted in Figure 3.

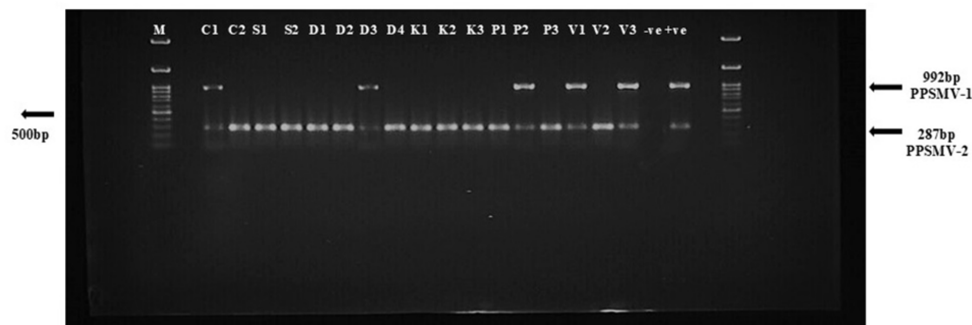


Figure 2. Multiplex RT-PCR for differentiation of PPSMV-1 and PPSMV-2 isolates of RNA3 from SMD-infected pigeonpea: M-Gel Analysis with 100 bp DNA Size Marker; C1-TNAU, C2-Devarayapuram, S1-Thindamangalam, S2-Pappankattur, D1-Nallampalli, D2-Nathahalli, D3-Somanehlli, D4-Adagapadi, K1-Pochampalli, K2-Bargur, K3- Paiyur, P1-Vilamuthur, P2-Siruvachi, P3-Ayyalur, V1- Virinjipuram, V2-Gudiyetham, V3-Sethuvazhai. +ve-Positive control, -ve-Healthy control. Lane: [C1,D3,P2,V1,V3-Mixed infection of both PPSMV-1 and PPSMV-2]

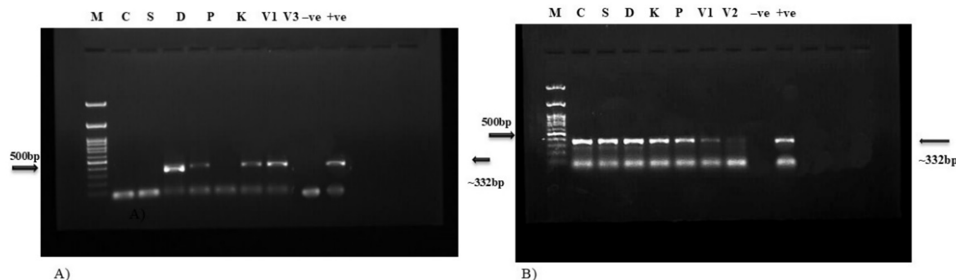
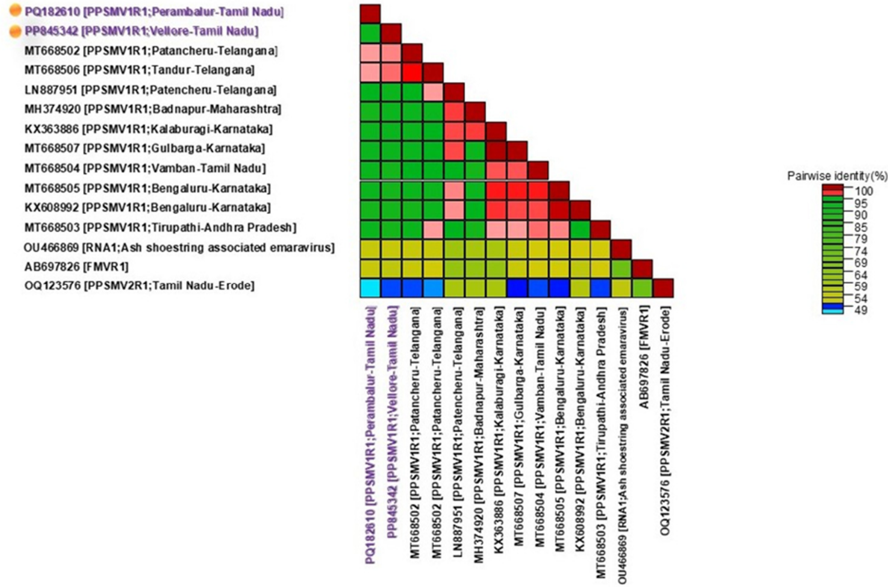


Figure 3. RT-PCR for PPSMV-1 and PPSMV-2 isolates of RNA-1 from SMD-infected pigeonpea: M-Gel Analysis with 100 bp DNA Size Marker; C-Devarayapuram, S-Pappankattur, D-Somanehlli, K-Pochampalli, P-Siruvachi, V1- Virinjipuram, V2-Gudeyetham, V3-Sethuvazhai, +ve-Positive control, -ve-Healthy control, RNA dependent RNA polymerase for PPSMV 1 (A) and PPSMV 2 (B)

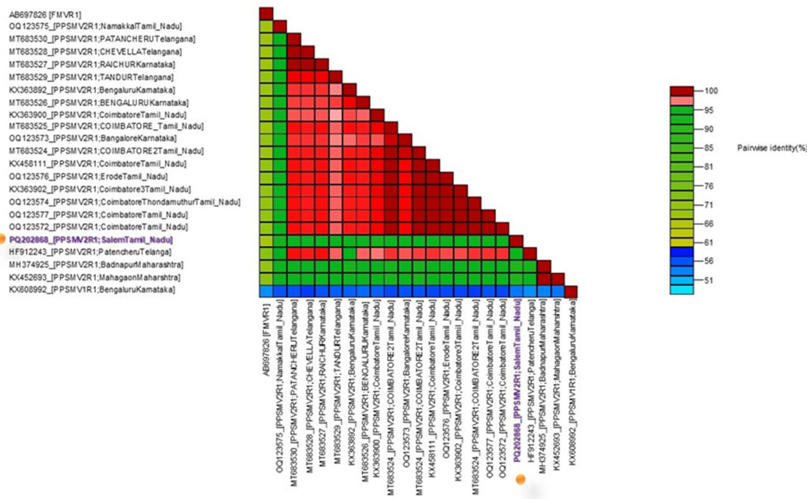
Analysis of RNA 1 and RNA 3 sequences of PPSMV-1 and PPSMV-2 isolates

The assessment of percentage sequence identity among the NC and RdRp sequences from both pigeonpea-infecting emaravirus species with already available PPSMV sequences revealed considerable sequence variability. The PPSMV sequences obtained by Sanger sequencing were submitted to GenBank under accession numbers PQ202868, PQ182610, PP845342, PQ117744, PQ202870, PQ117745, PQ213474, PQ156129, PQ202869 (Supplementary Table 1). The nucleocapsid sequences of PPSMV-1 isolates exhibited nucleotide identity ranging from 91.90% to 99.45%, while those of PPSMV-2 ranged from 91.73% to 95.65%.

Similarly, the nucleotide sequence identity of RdRp among PPSMV-1 isolates varied between 89.78% and 96.10%, and for PPSMV-2, it ranged from 91.12% to 95.56% (Supplementary Table 2,3,4,5; Figure 4).



a)



b)

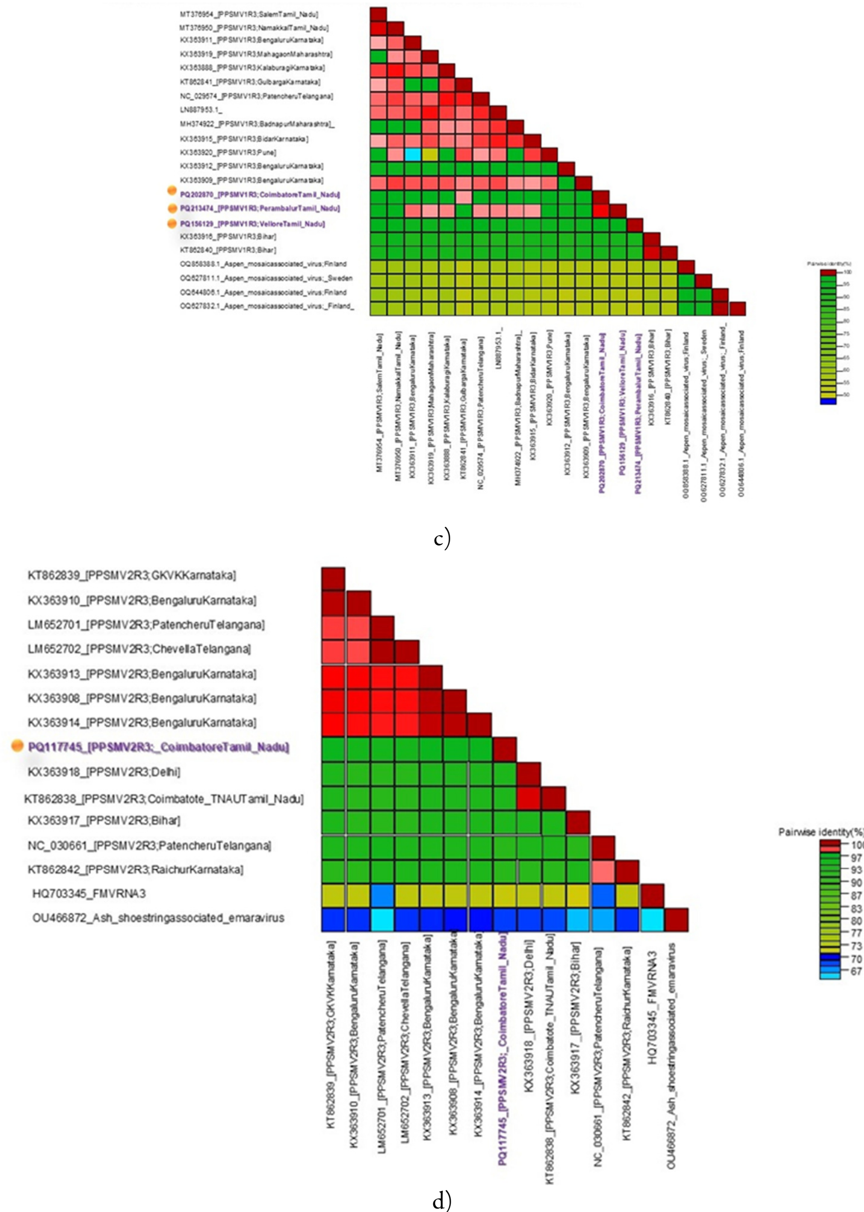


Figure 4. Pairwise sequence identity matrices of of PPSMV-1&-2 components, PPSMV-1 RNA1 (a), PPSMV-2 RNA1 (b), PPSMV-1 RNA3 (c) and PPSMV-2 RNA3 (d)The orange dotted were study isolates

Phylogenetic analysis of PPSMV-1 and PPSMV-2 isolates

Out of the nine sequences, four were obtained for the RNA3 segment and three for RNA1 segment of PPSMV-1 and PPSMV-2 isolates. These nucleotide sequences were phylogenetically subjected with selected emaravirus sequences, the PPSMV-1 and PPSMV-2 isolates segregated into two distinct and separate clusters. Interestingly, RNA3 sequence of *Fig Mosaic Virus* (FMV) was grouped with PPSMV-2 and not with PPSMV-1 isolate. The RNA 1 nucleotide sequence of the Salem isolate had close relationship with the Namakkal isolate of PPSMV-2 group, while Vellore and Perambalur isolates were related with Patancheru (MT668502) and Tandur (MT668506) isolates, respectively amid PPSMV-1 group. Similarly, when RNA 3 segment was subjected to phylogeny analysis, isolate ofPPSMV-1 from Vellore, Perambalur and Coimbatore districts were clustered with Salem (MT376954) isolate along with reference isolate of Bihar (KX363916), whereas PPSMV-

2 of Coimbatore isolate was grouped with a Bengaluru (KX363913) and a Bihar (KX363917) isolate (Figure 5).

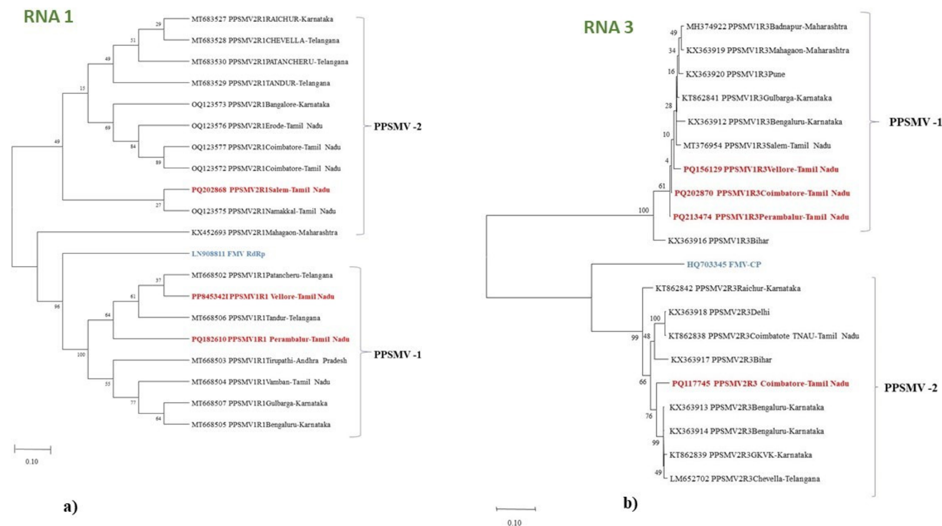


Figure 5. Phylogenetic dendrograms of RdRp-RNA1) (a) and nucleocapsid protein-RNA 3 (b) identified in this study

The numbers at the nodes indicate the percentage bootstrap values of 1,000 replicates. The scale bars represent the genetic distance. Viral genomes identified in this study are marked in red color

Recombination of Nucleocapsid protein gene of PPSMV-1 and PPSMV-2 isolates

Recombination analysis of the PPSMV isolates indicated that the nucleotide sequence of the RNA 3 of PPSMV-1component had developed through intra-specific recombination between two isolates KX363912-Bangalore as a major parent and a PQ202870-Salem isolate as a minor parent with a breakpoint between 43-113 nucleotide positions. Comparatively, PPSMV-2 of Coimbatore isolate exhibited recombination between KX363918-Bangalore isolate as a major parent and NC030661-Patancheru as a minor parent (Table 4). Conversely, no recombination breakpoint was detected in RNA1 segment of PPSMV-1 and PPSMV-2 (Figure 6).

Table 4. Recombination event for nucleo capsid protein of PPSMV 1 and PPSMV 2

Viral sequence	Recombinant	Breakpoint position (in nt)	Major parent	Minor parent	RDP	GC	BS	MC	CHI	33	3 Seq
PPSMV 1 CP	PQ156129-Vellore	43-113	KX363912-Bangalore	PQ202870-Salem	-	-	-	-	-	4.799 x 10-03	-
PPSMV2 CP	PQ117745-Coimbatore	671-932	KX363918-Bangalore	NC030661-Patencheru	-	-	-	-	>1.0	1.048x10-03	-

Recombination detection methods abbreviated: GC-GENCONV; BS-BOOTSCAN; MC-MAXCHI; CHI-CHIMERA; SS-SISCAN

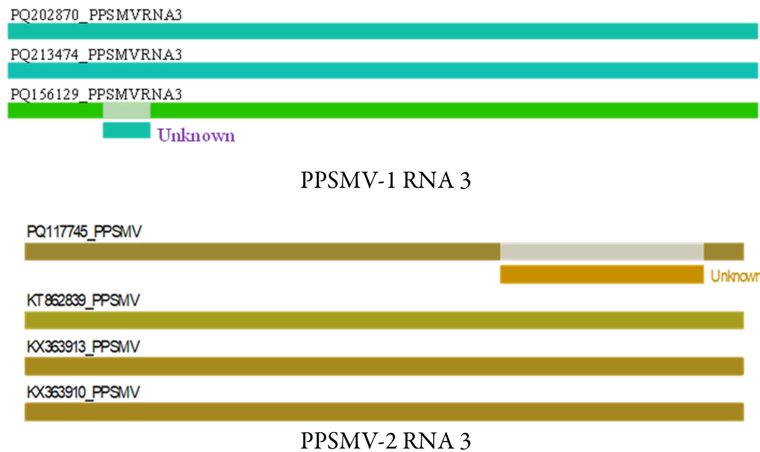


Figure 6. Recombination analysis for Nucleocapsid protein (RNA3), PPSMV-1 and PPSMV-2

SMD virus transmission by leaf stapling method

The maximum number of the inoculated pigeonpea seedlings (92%) exhibited chlorotic mottling and mosaic symptoms at 15-20 days post inoculation in Vamban (Rg)3 cultivar, demonstrating that *Aceria cajani* mites had been successful in transmitting PPSMV-2 and as mixed infections but not PPSMV-1 alone depicted in Figure 6. RT-PCR testing examined the presence of both PPSMV-1 and PPSMV-2 in inoculated and newly emerged leaves of test and healthy plants (Figure 7).

Figure 7. Virus transmission by leaf stapling method

a. Leaf stapled on healthy pigeon plant with viruliferous mite
b. Post inoculation of virus at 5 days after leaf stapling
c. Initiation of symptoms at 15-17 days post inoculation with chlorotic ring spot
d. Severe symptom expression of PPSMV showing leaf mosaic and leaf mottling

Discussion

In the present investigation, the overarching objective to examine the variation of PPSMV isolates within a particular geographic region, focusing on the four agro-climatic zones in Tamil Nadu where pigeonpea is extensively cultivated envisaged that PPSMV-1 and PPSMV-2 were prevalent throughout North Eastern and Western region, with mixed infections. the Vellore district under North Eastern region recorded the maximum SMD incidence ranged from 22.6% to 71.6% in the varieties such as LRG 41, ICP 8863 and LRG 52 under rain fed conditions. The findings align with the research conducted by Mediga *et al.,* (2020), which revealed that LRG-41, LRG-52, BRG-2, BRG-3, LRG-41, CO-7, VAMBAN-2, and CO-1 exhibited the highest disease occurrence in Vellore district, both in monoculture and when intercropped with groundnut. The earlier investigations have recorded varying levels of SMD incidence in Tamil Nadu. Kannaiyan *et al*., (1984) reported a maximum incidence of 37.50% was found in Pudukkottai district (Cauvery Delta Zone (CDZ). Recently,

SMD incidence was observed in Vamban-2, CO6 variety with varying range from 3.6% to 21.3% across Tamil Nadu by Baskar *et al*. (2020). Meanwhile Sayiprathap *et al*. (2020) has reported the highest incidence of 16.25% in Krishnagiri district and Mediga *et al*., (2024) reported the disease incidence which varied from 0 to 71.40% in *Kharif* 2021–22 and 0 to 25.50% in *Kharif* 2022–23. The high incidence in Vellore district might be due to the molecular variability of PPSMV-1 and PPSMV-2 and large-scale cultivation of susceptible cultivars. Similar studies were conducted by Patil *et al*. (2017) have revealed that molecular variability due to reassortment of RNA4 segment from PPSMV-1 to PPSMV-2 from Bangalore and Coimbatore isolate, which may indicate that PPSMV-2 isolates are more versatile for reassortment and is compatible with the RNA4 segment of PPSMV-1.

In this study, a diagnostic multiplex RT-PCR method was optimized to distinguish PPSMV isolates based on the nucleocapsid protein by utilizing previously designed primers (Sayiprathap *et al*., 2020; Patil *et al*., 2017) to assess infection frequency and geographic distribution. Occurrence of PPSMV-2 was more prevalent with high frequencies across Tamil Nadu, and mixed PPSMV-1/PPSMV-2 infections with maximum SMD were observed in the Vellore and Coimbatore districts. These findings were in accordance with Patil *et al*. (2017), who documented that prevalence of mixed infections of PPSMV-1 and PPSMV-2 isolates using multiplex RT-PCR for the RdRp segment and found that PPSMV 2 was predominant in the locations of Coimbatore and Bangalore. Further, this information could be instrumental in developing effective management strategies for pigeonpea SMD, particularly through RNA-interference technology, which is sequence-specific in nature (Gandhi *et al*., 2021; Karthikeyan *et al*., 2023).

The analysis of sequence identity among PPSMV-1 and PPSMV-2 isolates revealed significance variability associated with SMD. Precisely, the PPSMV-1 isolate from Vellore exhibited the greatest divergence among other PPSMV-1 sequences analyzed in this study and variability in the Vellore isolate was also evident in the phylogenetic analysis of their RNA segments. Regarding the recombination breakpoints (RdRp) examination of RNA3 segment of PPSMV-1 and PPSMV-2. the PPSMV-1 isolates displayed a broader range of sequence identities compared to PPSMV-2 isolates. The findings of the present study are in agreement with the previous reports of Baskar *et al*., (2021); Elbeaino *et al*., (2015); Kumar *et al*., (2017); Patil *et al*., (2017) and Sayiprathap *et al*., (2020), who suggested that these two emaraviruses infecting pigeonpea have followed two independent evolutionary paths.

The successful transmission of PPSMV-2 and prevalence of mixed infections in pigeonpea was in accordance with the earlier reports of Kulkarni *et al*., (2002) and Baskar *et al*., (2020). The emergence of mixed infections throughout years is probably due to the dissemination of the virus to these regions by its eriophyid mite vector, *Aceria cajani*. Under natural circumstances, mites are the sole means of transmitting the SMD causal agent to pigeonpea, as it is not dispersed through seed, pollen, or soil (Seth 1962; Reddy *et al*., 1998; Kulkarni *et al*., 2002; Jones *et al*., 2004; Kumar *et al*., 2004; Patil and Kumar 2015).

Conclusions

This current investigation revealed significant variation of SMD incidence in pigeonpea across Tamil Nadu, ranging from 12.8% to 71.6%, with the maximum incidence in Vellore district. Genetic analysis has exclusively shown the notable diversity among isolates, with phylogenetic clustering of PPSMV-1 and PPSMV-2 into distinct groups. Furthermore, molecular insights confirmed the presence of PPSMV-1 and PPSMV-2, both as single and mixed infections, by RT-PCR and multiplex RT-PCR. Recombination events were detected in the nucleocapsid (RNA 3) segment, but not in RNA-dependent RNA-polymerase (RNA1) segment. Thus, it is clear from these findings that the genetic diversity and widespread nature of PPSMV-1 and PPSMV-2 will contribute to the development of better diagnostic tools and disease management strategies in the future.

Authors' Contributions

RR: Conceptualization; Writing- original draft; Editing. VS: Writing- review, Investigation. GK: Methodology, Writing- review. BLP: Data analysis. MR: Data analysis. MJ: Editing, Investigation. MD: Editing, Visualization. All authors read and approved the final manuscript

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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