

Molecular phylogeny and Secondary structure analysis of Hop Stunt Viroid (HSVd) associated with Mulberry (*Morus alba*) in India

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Abstract

Hop stunt viroid (HSVd), a small, single stranded, circular, non-coding infectious RNA known to cause infection in various economically important crop plants. In the present investigation, a study was conducted in the southern part of Karnataka districts of India to detect the possible association of HSVd infection in mulberry plants. A total of 41 mulberry plants showing typical viroid-like symptoms along with asymptomatic samples were collected and screened using conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using specific set of HSVd-Fw/ HSVd-Re primers. Out of 41 samples, the study confirmed the presence of HSVd in six samples of mulberry collected from Ramanagara (1 sample), Chikkaballapur (3 samples) and Doddaballapura (2 samples) regions with an expected HSVd amplicon size of 300 nucleotides. The mechanical transmission of HSVd was also confirmed on cucumber (cv. Suyu) seedlings through bioassay, which was reconfirmed by RT-PCR. The amplicons were cloned, sequenced, and the representative nucleotide sequences were deposited in the NCBI GenBank. Subsequently, molecular phylogenetic analysis showed that HSVd mulberry isolates from this study were most closely related to grapevine isolates, indicating a common origin. On the other hand, it was shown to belong to a different group from mulberry isolates so far reported from Iran, Italy, Lebanon, and China. The secondary structure analysis of HSVd mulberry Indian isolates exhibited substitutions in the terminal left, pathogenicity, and variable regions comparing to those of the Indian grapevine isolates. As far as this study is concerned, HSVd was detected in some mulberry plants with viral-like symptoms, but the pathogenesis and symptom expression need to be further investigated to establish the relationship between HSVd and the disease symptoms in the mulberry plants.

Introduction

Viroids are the smallest known autonomously replicating infectious plant pathogens, consisting of circular, covalently closed, single-stranded small RNA molecules whose genome length varies between 220–450 nucleotides (nt) (Lee et al. 2022). Viroids have wide host ranges that systemically induce disease on many agricultural and horticultural crops (Diener 2001; Ding 2009). *Hop stunt viroid* (HSVd) is a member of the family *Pospiviroidae* belongs to the genus *Hostuviroid*, consisting of 294–309 nt and was first reported from hops in Japan (Sasaki and Shikata 1977; Sano et al. 1989). HSVd has most diverse host range, having been detected in a wide variety of woody and herbaceous plants and has been frequently pathogenic to few plants such as grapevine, citrus, hops, peach, apple, cucumber, pear, apricot, pomegranate, almonds, fig and mulberry (Yang et al. 2007; Hataya et al. 2019). The infection caused by HSVd appears to be latent in most of the hosts studied (Polivka et al. 1996). Several viroid infections have been reported in India of which, PSTVd on potato and tomato, CEVd on citrus, CSVd and CChMVd on chrysanthemum, CLVd on *Nematanthus* and grapevine associated AGVd, HSVd, GYSVd diseases are important (Mishra et al. 1991; Singh et al. 2010; Adkar-Purushothama et al. 2013; Singh and Kaur 2014; Jeevalatha et al. 2015; Kumar et al. 2015; Walia et al. 2021; Shilpa et al., 2022).

Mulberry (*Morus alba*) is one of the most economically important perennial cash crop cultivated in India and is being cultivated in 242 thousand hectares with an annual production of 24,000 MT in 2021 as

source of silkworm feed (Anonymous 2021). In India, the state of Karnataka is the leading raw silk producer, with 11,000MT of raw silk produced in fiscal year 2022 to feed silkworm (Anonymous 2022). Recently, HSVd infection has been reported from mulberry in Italy, Lebanon, Iran and China (Elbeaino et al., 2012; Mazhar et al. 2014; Lu et al. 2023). In India, mulberry plants showing typical viral-like symptoms have been observed in different mulberry growing regions of Karnataka state during 2020–2022. Hence, the present investigation was undertaken to assess the presence of HSVd in mulberry plant by employing molecular phylogenetic and secondary structure analyses.

Materials and methods

Collection of viroid samples

To investigate the presence of HSVd in mulberry plants, a field survey was conducted during December 2020 to April 2022 in many mulberry growing regions of Bangalore rural, Chamrajanagara, Chikkaballapur and Ramanagara districts of Southern Karnataka (India). Mulberry leaf samples (41 samples) displaying viral-like symptoms such as vein clearing, leaf curling, yellow speckle and leaf deformation were collected along with asymptomatic samples. The collected samples were thoroughly screened for HSVd infection using RT-PCR and bioassay methods.

Total RNA extraction

To extract low molecular weight RNAs (LMW-RNA) from infected and healthy leaf samples 2X CTAB based 2M lithium chloride (LiCl) precipitation method described by Adkar-Purushothama et al. (2015) was performed with brief modification. About 100 mg of viroid suspected and healthy mulberry leaf tissues were ground into a fine powder in a sterile mortar and pestle using liquid nitrogen and was homogenized with an appropriate volume of pre-heated 2X CTAB buffer [0.1M Tris–HCl (pH 9.5), 1.4 M NaCl, 0.02M EDTA (pH 7.0), 2% CTAB, and 0.05% 2-mercaptoethanol]. To the homogenate, an equal volume of phenol: chloroform (1:1) was added and nucleic acid was extracted followed by centrifugation at 13,000 rpm for 5 min. at 4°C. The aqueous phase was collected and total nucleic acids was precipitated by adding 2.5 volumes of 99.5% ethanol, incubated overnight at – 30°C, and centrifuged at 13,000 rpm for 10 min. The pellets were air-dried at room temperature and re-suspended in nuclease-free water followed by the addition of equal volume of 2 M LiCl with gentle shaking and allowed to stand on ice for 4 h. High molecular weight RNAs was precipitated by centrifugation at 13,000 rpm for 10 min., and 2 M LiCl soluble nucleic acids was recovered by ethanol precipitation from the resultant supernatant, followed by *DNase1* treatment for 30 min. at 37°C. LMW-RNA was ethanol precipitated by centrifuging at 13,000 rpm, and the resultant residues were washed with 70% ethanol and air-dried. Finally, the obtained RNA pellets were suspended in 100 µl of nuclease-free water. Further obtained RNA was quantified using NanoDrop Lite Spectrophotometer (Thermo Scientific™, USA), and stored at – 20°C for further analysis.

Reverse transcription (RT)

The first-strand complementary DNA (RT solution) was synthesized according to Adkar-Purushothama et al. (2015) in a 25 µl of total volume of reaction mixture. Where 2 µg of purified RNA sample containing 10 mM dNTPs, 1µl of random hexamer (100 µM) was used for the preparation of RT-solution. The reaction mixture was thoroughly mixed and incubated for 5 min. at 95°C on a Thermal Cycler and immediately chilled on ice for 5 min. Approximately 5µl of denatured RNA was added to cDNA synthesis reaction mixture containing M-MuLV reverse transcriptase (200 U/µl) (Invitrogen, Carlsbad, CA, USA), 5×first strand buffer, 0.1M DTT, RNase inhibitor (20U/µl). The RT reaction was incubated for 60 min. at 37°C. After incubation, the reaction mixture was heated at 72°C for 5 min. on a Veriti 96- Well Thermal cycler (Applied Biosystems) and chilled on ice for 2 min. to inactivate the reverse transcriptase. The reaction mixture without template RNA served as a negative control.

RT-PCR amplification and cloning

To detect HSVd, a conventional PCR was performed by using a resultant RT-solutions in a 25µl PCR reaction mixture. The PCR master mixture containing 2 units of Taq DNA Polymerase (Takara Bio, Shiga, Japan), 10×Taq PCR buffer, 2 mM dNTP, 25 mM MgCl₂, and 20 pmol each of HSVdF1-5'-GGGGCAACTCTTCTCAGAATCC-3' and HSVdR1-5'-GGGGCTCCTTTCTCAGGTAAGTC-3' (Bernad and Duran-Vila 2006) and remaining volume was made up with nuclease free water. Similarly, samples were also prepared from healthy and infected mulberry plants. The reaction mixture was thoroughly mixed before carrying out PCR in a thermal cycler with the following PCR cycling profile: Initial denaturation at 95°C for 4min., followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 55.3°C for 1min., and extension at 72°C for 1min., and final extension was performed at 72°C for 10min. The amplified PCR products were separated by 1.5% agarose gel electrophoresis with ethidium bromide (0.5 µg/ml), in 1X TAE buffer at 100 V for 35 min., and visualized under UV transilluminator using Gel Documentation apparatus (Major Science, USA). The amplified PCR products were purified using a GeneJET Purification Kit from Thermo Scientific (Thermo Scientific, USA) as per the manufacturer's instructions. Further, the purified DNA fragments were cloned into pGEM®-T Easy vector (Promega, Madison, USA) and performed bacterial transformation process as per Promega (USA) protocol.

Biological indexing of HSVd

Biological indexing was performed to confirm the pathogenicity of HSVd on cucumber (*Cucumis sativus*, cv. 'Suyo') indicator plants. The 100 ng of small RNAs (inoculum) from infected and healthy mulberry plants was prepared by homogenizing with 0.5M sodium phosphate buffer (pH 7.0) supplemented with bentonite solution (40 µg/ ml) (Sano et al. 2004). Inoculations were carried out on 25-days old three cucumber seedlings at two-leaf stage by gentle rubbing with 50 µl of the RNA inoculum on each leaflet, which were previously dusted with carborundum-600 meshes (HiMedia) in greenhouse conditions (Verhoeven et al. 2010a). The inoculated leaflets were left for 2min. and gently washed with sterile distilled water. Cucumber seedlings inoculated only with sterile distilled water served as control. All the inoculated seedlings were maintained under controlled conditions (25°C – 30°C) to screen the infection by HSVd, and the experiments were repeated twice. The HSVd infections in all the symptomatic plants were further confirmed by RT-PCR product and sequencing.

Sequencing and Phylogenetic analysis

The amplified PCR and cloned DNA products were sequenced in an automated DNA sequencing system (Applied Biosystems, ABI Prism 310, USA) by Sanger sequencing method. The obtained sequences were aligned and analyzed using BioEdit sequence alignment editor (version 7.2.6.0) and ClustalW software. Further, the sequences were subjected to nBLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and analysis using NCBI-BLAST program to compare with the related nucleotide sequences available in the NCBI GenBank. The obtained consensus nucleotide sequences were assembled and compared for similarities to identify the closest match. A phylogenetic tree was constructed with the representative homologous viroid sequences retrieved from the NCBI GenBank (<https://www.ncbi.nlm.nih.gov/>) employing the Neighbour-Joining method (NJ) of MEGA 11 version (Tamura et al. 2021) with 500 bootstrap replications to assess the reliability of the constructed evolutionary tree. The evolutionary distances were calculated using the Kimura-2-parameter method (Kimura 1980).

Secondary structure analysis of HSVd

The sequences of isolated HSVd (Acc. No. OP313761) and representative HSVds available in GenBank (Acc. No. AB742225) were obtained, and multiple sequence alignments was performed using the ClustalW program. The possible secondary structure was predicted to identify the variations and mismatches at the central conserved, terminal, variable and pathogenicity regions. In addition, the base-pairing probabilities were also predicated among the aligned sequences using RNA structure Version 6.3- Mathews Lab Computational Biology of RNA (<https://rna.urmc.rochester.edu>) and Mfold (Zuker 2003) with the lowest free energy at a folding temperature of 37°C and MFOLD (circular version) program from the GCG package and RNA viz (Zuker 1989) for prediction of all the five domains of HSVd.

Results

HSVd symptoms and Molecular detection through RT-PCR

It was observed that, mulberry plants showed the viral-like symptoms, such as leaf curling, yellow speckle, yellow mottling, vein thickening, clearing and banding, leaf deformation and stunted growth (Fig. 1). Of the 41 symptomatic mulberry leaf samples from different locations, six from Ramanagara (1 sample), Chikkaballapur (3 samples) and Doddaballapura (2 samples) regions tested positive for HSVd with an expected PCR amplicon size of ~ 297 nt for HSVd (Fig. 2). However, none of the other healthy samples showed amplicons for HSVd. The amplified full-length DNA fragments were recovered from gel purification and subsequently cloned.

Biological indexing

After 6–7 weeks of post-inoculation, all the three small RNA inoculated cucumber plants displayed typical HSVd symptoms such as leaf curling, stunted growth, yellowing and leaf thickening. Whereas water-inoculated cucumber plants remained healthy under greenhouse conditions even after 7 weeks of

post-inoculation (Fig. 3). Bioassay results were confirmed by RT-PCR, where sample collected from Ramanagra, Doddaballapura and Chikkaballapura samples showed an expected amplicon size of ~ 290–300 nt by confirming the association of HSVd.

Sequencing and Phylogenetic analysis

A clone from each positive isolates (~ 290–300 nt) were selected and sequenced from both the directions for further confirming HSVd infection. The nBLAST analysis, revealed the association of HSVd in mulberry by showing 99.0% homology with HSVd sequence from *Vitis* spp. isolated from Russia (Acc. No. OL799308), China (Acc. No. AB219944), followed by 98.7% homology with the Indian isolate of grapevine (Acc. No. AB742225), Korean isolate of Japanese apricot (Acc. No. KY445746) and Chinese isolate of Japanese plum (Acc. No. EF076834). A similar co-efficient of 98.0% identity with HSVd infecting hop (Acc. No. AB039270) and peach fruit (Acc. No. D13765) in Japan was observed in the study. The assembled nucleotide sequences of HSVd isolates obtained in the study and the details of the accession numbers of the deposited sequences of the present work along with reference sequences are provided in Table 1. The obtained representative consensus sequences were deposited in NCBI GenBank with the Acc. No. OP313761, OP313762 and OQ784657 (Table 1). The sequence of OP313761 and OQ784657 was 100% matched with each other, but OP313762 was one nucleotide difference at the position 86, however, this variation was considered to be an artifact because it was located within the PCR primers used.

The representative nucleotide sequences were considered for constructing a phylogenetic tree. The HSVd sequences from various hosts among the world were selected from NCBI database. In order to simplify the tree, closely related or the representative sequences of the HSVd isolates were considered along with previously deposited sequences of HSVd from grapevine and citrus in India. The phylogenetic analysis among these HSVd sequences from mulberry, grapevine, hop, citrus, peach and others including the present sequences formed several distinct clades. Detailed inspection revealed that the Indian mulberry sequences belong to the grapevine group, which authenticating the close evolutionary relationship, but different from those isolated from mulberry in Italy, Lebanon, Iran, and China (Fig. 4).

Table 1

Accession numbers of the deposited mulberry-HSVd sequences along with closet reference sequences from NCBI GenBank.

Sl. No.	HSVd isolates			BLAST Closest Match	Country of origin	Identity (%)
	Code	Obtained Amplicon (nt)	Accession number			
1	MGBV7	297	OP313761	OL799308	Russia	98.99
				OL332772	India	98.99
				AB219944	China	98.99
2	MGBV8	297	OP313762	MN695313	Iran	98.66
				AB742225	India	98.65
				KY445746	Korea	98.65
3	MGBV10	297	OQ784657	EF076834	China	98.65
				AB039270	China	97.98
				D13765	Japan	97.98

Secondary structure analysis

The MultAlin analysis of HSVd (Acc. No. OP313761) sequence assembled with closely related previously reported representative Indian isolate of HSVd sequences from grapevine species (Acc. No. AB742225) revealed that there were not much differences among the nucleotide sequences studied. The secondary structure analysis by mfold thermodynamically predicted a rod-shaped structure with 5 structural domains (Fig. 5A). The predicted structure revealed the presence of 27 loops with a slight difference compared to the reference structure predicted from an Indian grapevine sequence (Acc. No. AB742225; Fig. 5B). It was also noted that, the HSVd from mulberry isolate showed 4 substitutions in the positions 58 ($U_{58} \rightarrow G$), 126 ($C_{126} \rightarrow U$), 253 ($C_{253} \rightarrow U$) and 272 ($U_{272} \rightarrow C$) and shared pairwise identity score of 98.7%. Of them, a $U_{58} \rightarrow G$ change closed an internal loop and a $U_{272} \rightarrow C$ change enlarged the other internal loop in the predicted secondary structure. The other two changes do not seem to cause structural changes between HSVd isolate from mulberry and grapevine in India.

Discussion

Hop stunt viroid belongs to the species of the genus *Hostuviroid* (Di Serio et al. 2021). HSVd contains central conserved regions and terminal conserved hairpins, which are similar to that of Cocadviroids, but lacks a terminal conserved region and other structural hallmarks (Flores et al. 1998; Marquez-Molinset al. 2021). HSVd is known to infect broad range of natural hosts and thus posing a potential threat to agricultural crops worldwide. It was first identified in hop plants showing abnormal dwarfing of vines and known as hop stunt disease (Sasaki and Shikata 1977). The disease was first described in Japan during

1950s and became the biggest concern for hop growers in the 1970s causing important huge economic losses (Hataya et al. 2017). Since then hop stunt epidemics have been sporadic for some years, but in the 2000s, another epidemics began to emerge in the United States (Eastwell and Nelson 2007), China (Guo et al. 2008) and Slovenia (Radišek et al. 2012). Apart from hops, HSVd has a wide host range, infecting fruit bearing citrus, malus, prunus, plum, peach, apricot, sweet cherry, apple, fig and *Vitis* spp., flower bearing *Hibiscus* spp. (Marquez-Molins et al. 2021) and recently reported on economically important jujube and mulberry plants (Elbeaino et al. 2012). In most cases, HSVd infection is symptomless, but in some cases, it has been reported to cause cachexia in citrus, cracking and stunted growth in fruit bearing plants (Marquez-Molins et al. 2021). Mulberry is widely grown in India as a source of silkworm feed and has been affected by few viral diseases. HSVd infection has been reported from Italy, Lebanon, Iran and China (Lu et al. 2023). HSVd has been detected from mulberry trees characterized by mosaic dwarf followed by vein clearing, leaf deformation, yellow speckle, chlorotic speckling and irregular spots over the leaves, however, the etiology awaits for further analysis.

In India, HSVd has been detected in grapevine (Sahana et al. 2013) and citrus (Roy & Ramachandran 2003). However, HSVd infection on mulberry has not been diagnosed and reported hitherto. The preliminary RT-PCR identified the HSVd infection for the first time in six mulberry samples collected from various fields of Karnataka state of India. The sequence data confirmed the HSVd association with mulberry with the high percentage homology with HSVd-grapevine, isolated from Russia, China, Indian (Sahana et al. 2013; Singhal et al. 2019), and widely around the world. The phylogenetic analysis of the HSVd Indian mulberry isolates showed an evolutionary relationship with those from grapevine, plum, peach, apple and hop reported from various countries including India; i.e., “grapevine” or “grapevine-hop” group (Amari et al. 2001; Sano et al. 2001). Which was somewhat different from those reported from Italy–Lebanon, Iran, and China, wherein mulberry isolates clustered together with those of “citrus” group, “plum” group, and “hop” group, respectively (Elbeaino et al. 2012; Amiri Mazhar et al. 2014; Lu et al. 2023). These results indicate that the HSVd mulberry isolates reported so far from Italy-Lebanon, Iran, China, and now from India belong to distinct groups depending on the region, and do not shows host specificity. Incidentally, while the PWIS between Indian mulberry and Indian grapevine was 99% (4 nucleotides differences), the PWIS between Indian mulberry and Italian-Lebanese mulberry, Iranian mulberry, and Chinese mulberry was ~ 95.7% (13–16 nucleotides differences), 89.0% or less (33 or more nucleotides differences), and ~ 94.6% (17–18 nucleotides differences), respectively (Fig. 4). In particular, the isolates from Indian grapevine and mulberry in this study differ by only 4 nucleotides, and the secondary structure analysis revealed the presence of 27 loops in the Indian mulberry isolate, which was slightly different in two loops located in the TL and P domains compared to those of Indian grapevine, suggesting a close relationship between the two isolates.

Amiri Mazhar et al. (2014) specifically detected HSVd from mulberry trees exhibiting vein-clearing and yellow speckle symptoms, and showed that the mulberry disease in question is transmitted from mulberry to mulberry by grafting the buds of the diseased tree onto the mulberry seedlings and by mechanical inoculation of the mulberry seedlings with the sap (but not purified preparation) from the diseased leaves. Since HSVd was detected again from inoculated seedlings, they concluded that vein

clearing and deformation of mulberry leaves is a transmissible disease associated with HSVd. HSVd in this study was also detected only from samples showing viral-like symptoms, although the detection frequency was not very high. The mechanical inoculation experiment confirmed HSVd infection in indicator cucumber (cv. Suyo) plant with the expression of typical HSVd-inducing symptoms, however, it will need to analyze the relationship between HSVd and viral-like symptoms by conducting infection experiments on mulberry seedlings. Since the outbreaks of HSVd associated crops have become widespread in recent years, and it often remains symptomless in nature which act as a reservoir for potentially dangerous viroid to susceptible crop plants. Studies also showed that agricultural tools could transmit HSVd during trimming, mechanical stripping and by grafting infected scions. Hence, it is necessary to investigate the mechanism of transmission and expression to establish the relationship between host-pathogen. Since Mulberry is an important cash crop, its leaf is used as feed for silkworms and is an unreported host of HSVd in India. Hence, it is important to further analyze the origin and its potential role as a source of infection to other horticultural plants. A further investigation is also required to find out the exact source of the HSVd infection in mulberry as it was observed that nucleotide sequence divergence may result from molecular evolution from a common source under various selective pressures.

Declarations

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Author contributions

SN: Conceptualized the work and carried out analysis, drafting the manuscript, TS and TN: Analyzed data, contributed new methods or models and JGR: overall monitoring of the work and fund acquisition for the work. All authors were involved in drafting and editing of the manuscript.

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Data availability statement

Genome sequences are submitted to NCBI and available.

Conflict of interest

The authors declare no conflict of interest.

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Figures

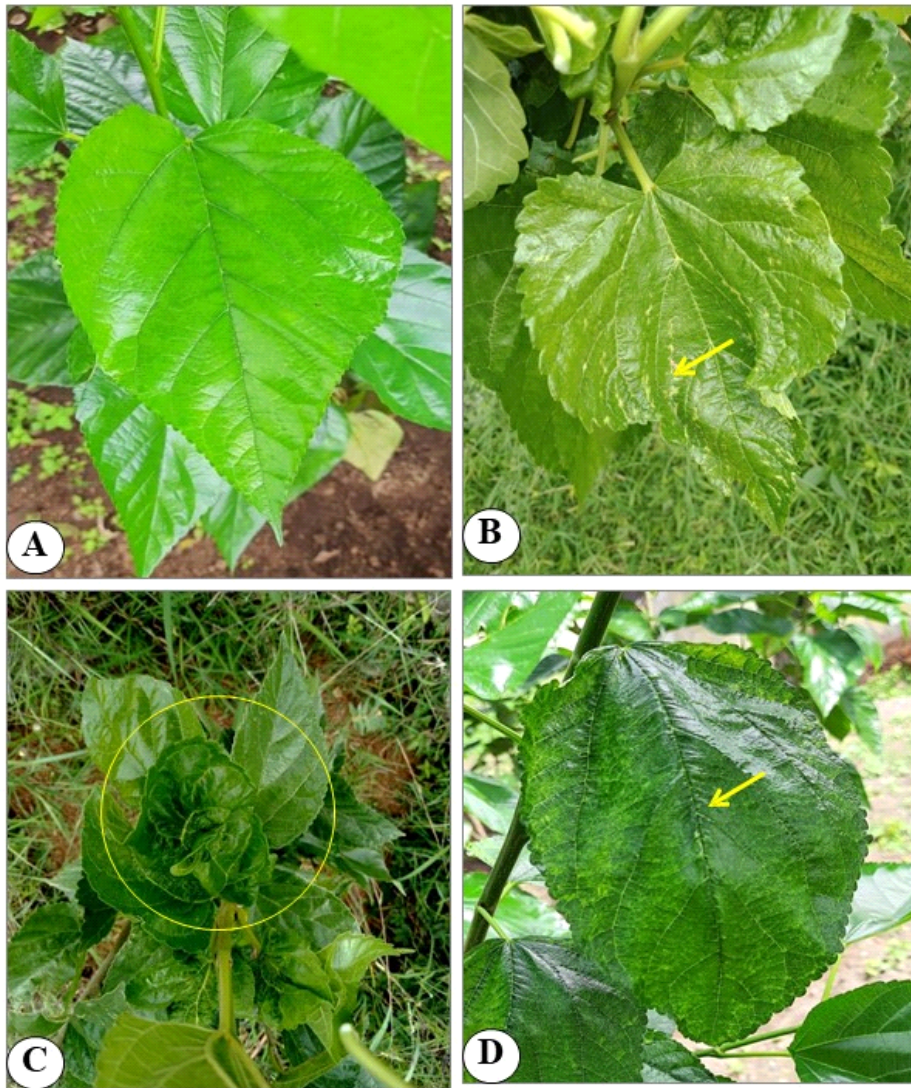


Figure 1

Mulberry leaf samples showing viral-like symptoms. A: Healthy leaf; B: Yellow spots; C: Leaf curling and leaf curling with leaf rugosity; D: Leaf thickening.

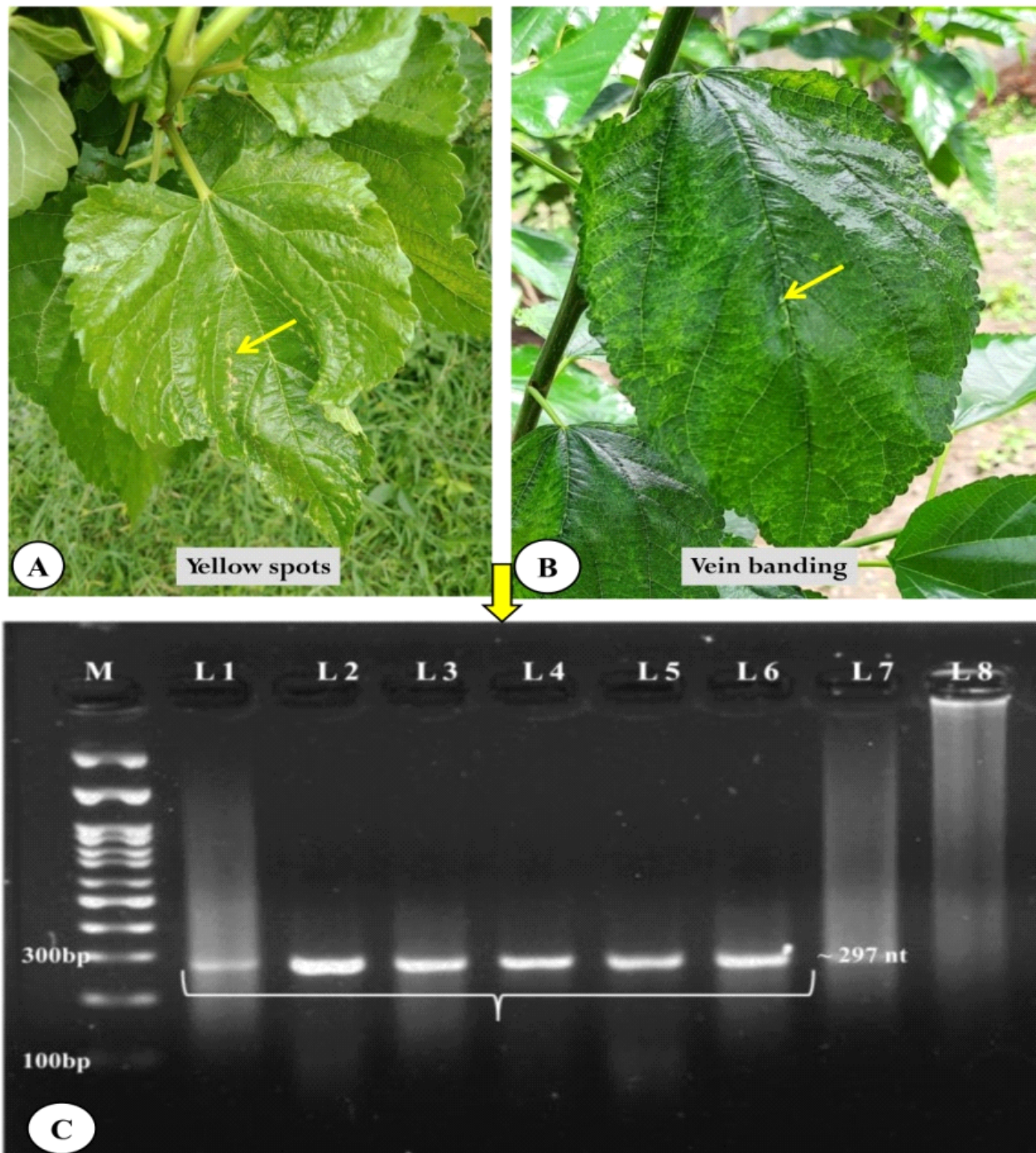


Figure 2

Amplification of HSVd (~297 nt) from mulberry samples showing amplified products on 1.5% agarose gel. Lane M: 100 bp marker; Lane 1: Ramanagara sample; Lane 2-3: Chikkaballapur sample; Lane 4-6: Dodaballapura sample; Lane 7-8: Control.

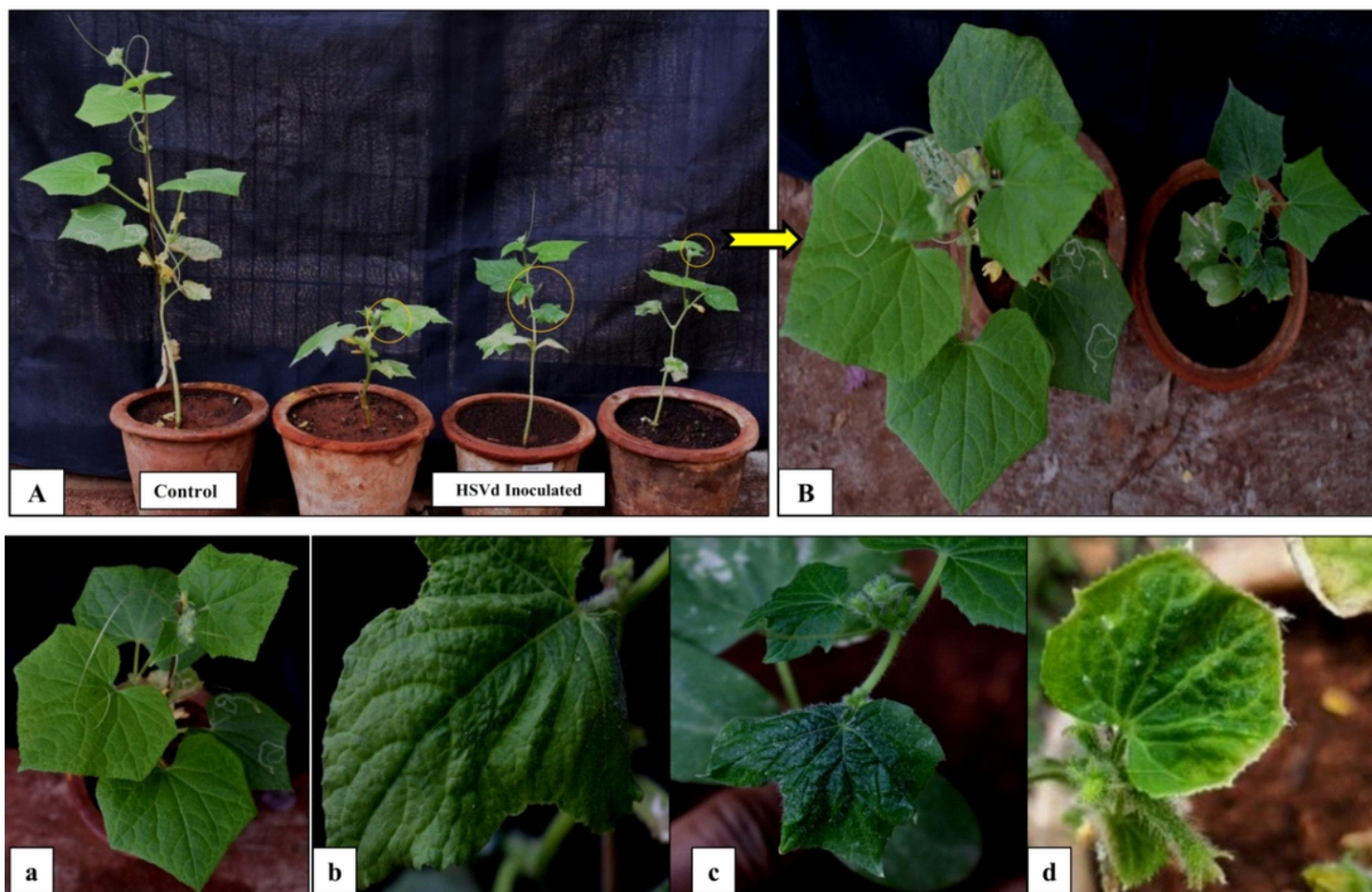


Figure 3

Bioassay studies with mulberry-HSVd on indicator plant. A-B: Inoculated and healthy Cucumber indicator plants under greenhouse conditions; a: Healthy plant; b-d: HSVd inoculated plants exhibiting symptoms of stunted growth, curling and small leaves with yellow spots.

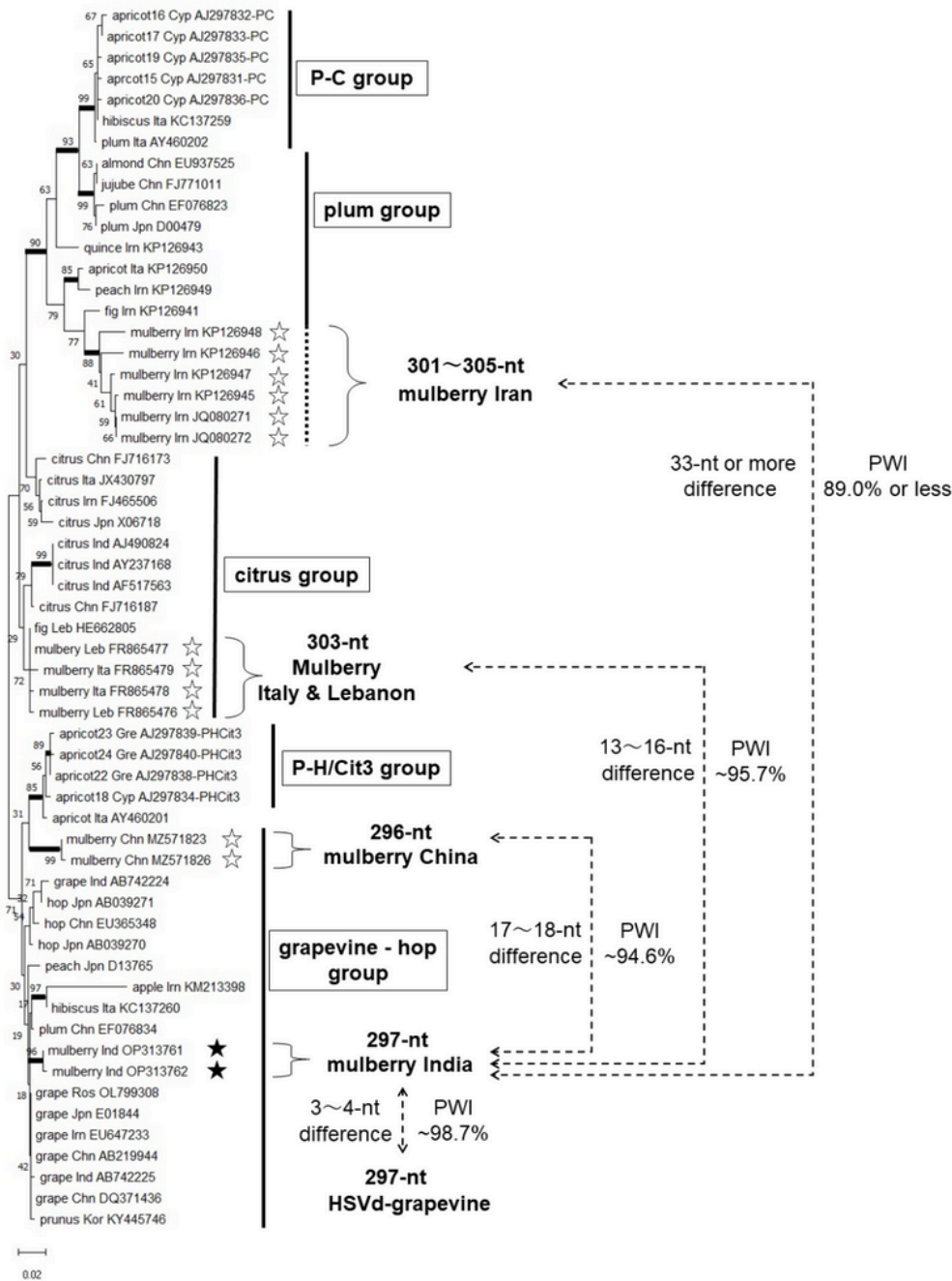


Figure 4

Molecular phylogenetic relationships of HSVd-mulberry isolates from mulberry in India in this study, mulberry in Italy, Lebanon, Iran, and China, and major HSVd isolates from various crops reported so far. Mulberry isolates detected in this work are marked with a black star, and mulberry isolates in other countries are marked with a white star. Five representative groups (P-C, plum, citrus, P-H/Cit3, grapevine-hop) found in HSVd isolates are shown. The number of nucleotide differences and PWIS (pairwise

identity scores) between mulberry isolates from India and those from Italy, Lebanon, Iran, China, and grapevines are shown in the right side of the figure.

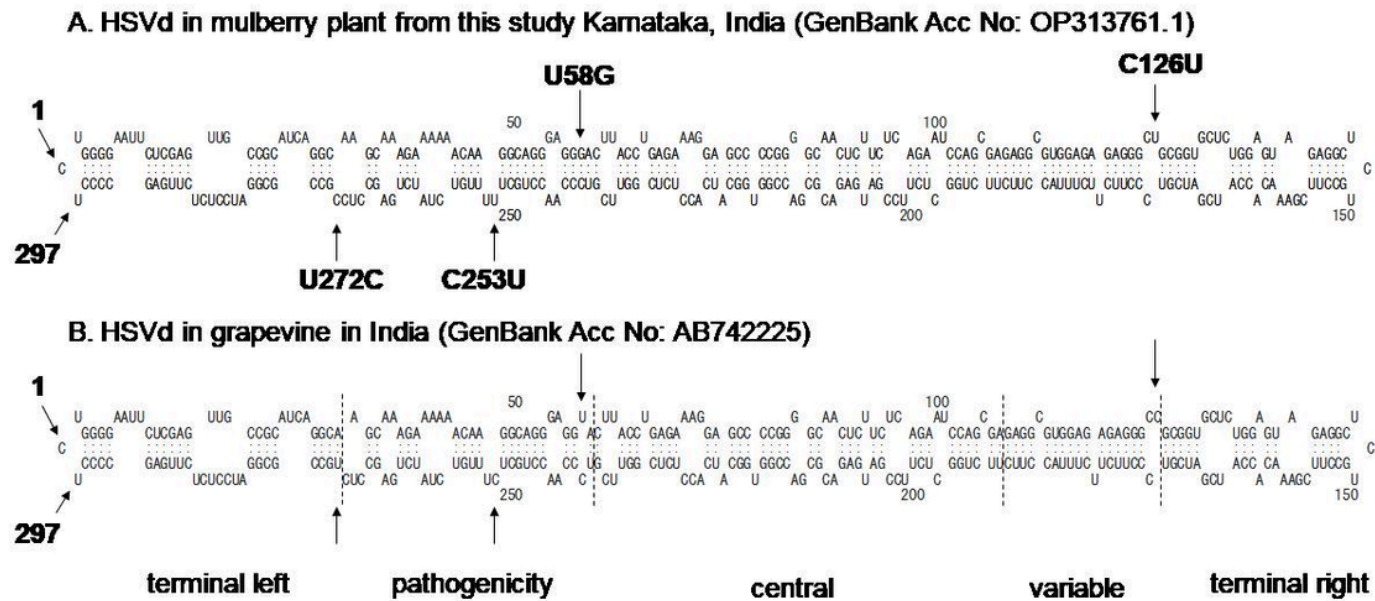


Figure 5

Predicted secondary structures of the HSVd isolate of the present study along with reference sequences predicated through RNA structure 6.0. A: Secondary structure of HSVd isolates (Acc. No. OP313761) from this study; B: Secondary structure of previously reported Indian HSVd reference sequence (Acc. No. AB742225).