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Detection of citrus yellow mosaic virus in different species of citrus and in different tissues of infected sweet oranges by conventional PCR

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Abstract
Citrus Yellow Mosaic Virus (CiYMV) is the etiologic agent of Citrus Yellow Mosaic Disease (CYMD), which has caused serious economic losses to Indian citrus industry. Detection of Citrus Yellow Mosaic Virus was carried out through PCR by using specific primers CiYMV F and CiYMV R primer set. The detection of CiYMV in different species of citrus showing bright amplification were observed at 726 bp. Similarly, the amplification was observed at 726 bp in different infected tissues of infected sweet orange tree *viz.*, leaf, fruit rind, bark, twig, petiole, midrib leaf, feeder root, tender shoots, except young leaves through a conventional PCR.

Keywords: Tissues of infected, mosaic virus, conventional PCR

Introduction
Citrus is the world's leading tree fruit crop and it is the third largest crop grown in India after mango and banana. Citrus is considered as one of the most important tropical fruit crop of India. Among the different species of citrus sweet orange (*Citrus sinensis* L.) is of great economic importance in Andhra Pradesh. It belongs to the family Rutaceae, having chromosome number 2n=2x=18 and it is indigenous to Southern China. In India, sweet orange is grown in an area of 2, 09,190 ha producing (34, 01, 000 MT) of fruits. Andhra Pradesh is the pioneer in sweet orange cultivation in India, ranking first in both area (70,110 ha) and production (17,52,630 MT), followed by Telangana state, Maharashtra, Madhya Pradesh and Karnataka (Department of Agriculture, Co-operation & Farmers Welfare, 2017). The major sweet orange producing districts in Andhra Pradesh are East Godavari, West Godavari, Anantapuramu, YSR Kadapa, Nellore, Chittoor and Prakasam.

The decline in the citrus production is due to biotic, abiotic and management practices includes disease and pest incidence, climatic extremities, poor fertility of soil, low- quality planting materials, and poor orchard management were found to be positively influencing citrus decline. Diseases are one of the major limiting biotic factors for low citrus production and seriously threaten the citrus industry.

These diseases are caused by fungi, prokaryotes, nematodes, viroids, viruses and virus-like pathogens. In India, bud-wood-transmitted diseases are majorly responsible for the decline of citrus orchards. The *Citrus Yellow Mosaic Virus* (CiYMV) is a non- enveloped bacilliform DNA virus causes a severe mosaic disease of citrus, particularly of 13.75 per cent in Mosambi sweet orange as compared to 5.88 per cent in acid lime and 3.22 per cent in Nagpur mandarin in different citrus growing tracts of Vidharbha. In Andhra Pradesh, the major diseases, that are affecting the crop are citrus greening (36.16%) followed by a *Citrus Yellow Mosaic Virus* (35.18%), canker (35.64%), *Citrus Tristeza Virus* (24.95%), dry root rot (13.22%) and twig blight (13.20%).

CiYMV is one of the major causes of sweet oranges decline in A.P. (Gopi *et al.*, 2010) [8]. Further, these pathogens incur a varying degree of damage to sweet orange plants and make their life span shorter, causing low yield and deterioration of quality and ultimately loss of economy which leads towards the citrus decline in A.P. The main symptoms of the CiYMV in field trees were mosaic pattern with irregular yellow or light green patches alternating with normal green leaf area irregularly distributed all over the leaf without any definitive pattern.

In India it was first described by Murthi and Reddy (1975) [6] and was later studied detail by Ahlawat *et al*. (1996a and 1996b) [1, 2].

The losses caused by the CiYMV disease were apparent in Sathgudi sweet orange orchards in Andhra Pradesh and Karnataka. There are several orchards with trees 4 to 10 years old were abandoned since they were no longer productive. The reduction in fruit yield was 77% in trees 10 years old, and fruit from affected trees had 10% less juice and ascorbic acid. The disease has been recorded in up to 46% of trees in some commercial nurseries at Kodur in Andhra Pradesh. It was described as a new graft transmissible disorder in sweet orange characterized by yellow mottling of leaves and yellow flecking along the veins.

Materials and Methods

Microcentrifuge tubes (0.2 ml, 1.5, 2.0 ml), 0.1 ml PCR tubes, Vortex, Incubation chamber with shaker, Centrifuge, pH meter, Microwave owen, Water-bath, -20 °C and 4 °C Refrigerators, Micropippets, Microtips (100, 200 µl and 1 ml), Qiagility (liquid handling), NanoDrop 1000 spectrophotometer, Rotor Gene Real-time PCR (Qiagen), Corbett research conventional PCR, Gel documentation, Nitrile gloves, Tissue lyser (Qiagen), Stainless steel beads, Aluminum labels, Permanent markers, Laminar air flow, Petri dishes.

Detection and diagnosis symptomatology

Symptomatology of CiYMV-infected plants and severity of the disease vary from variety to variety and from season to season. Usually, the virus symptoms are severe during winter, when conditions are favourable for multiplication of the virus in citrus, than during the drought and severe heat of summer in the southern part of India. Symptoms induced by CiYMV infection include mosaic on various parts of the plant such as leaves, branches and fruits. Mosaic occurs as irregular yellow or bright green patches against dark green background and yellow flecking along the veins (Dakshinamurti and Reddy 1975) [6]. The mosaic pattern is similar on branches with irregular yellow or bright green patches against the dark green background of the branches and twigs. Trees affected by mosaic the disease not only produce significantly fewer fruits, but also yield less juice and ascorbic acid (Ahlawat *et al*., 1996a) [1]. Then the samples are transferred to laboratory and stored at -20^{0}C until they are processed for DNA extraction.

Extraction of total DNA

The total DNA from the entire tissue of leaves with the CiYMV symptoms and healthy sample was extracted by the method of Sodium Sulphite-Tris EDTA (SS-Tris EDTA Method. The addition of sodium sulphite to Tris-EDTA to reduce the degradation of DNA was followed for extraction of DNA.

Leaf midribs (100mg) were minced using sterile scissors, weighed and transferred to a 2.0 ml micro centrifuge tube for maceration with the Tissue Lyser (Qiagen) at 30Hz with duration of 3 minutes. One ml of extraction buffer was added to the ground tissue in an microcentrifuge tube and incubated at 95ºC for 10 min with occasional agitation. The homogenate was placed on ice for 2 min and centrifuged for 10 min at 12000 rpm. The supernatant was treated with RNase (100 µg/ml) and the DNA was precipitated with 0.6 volume of ice-cold isopropanol. After centrifugation, sterile distilled water was added to the precipitate and incubated to 65ºC to completely dissolve DNA. DNA was re-precipitated with 2 volume of 95% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and incubated at –20 °C for overnight. After incubation centrifuged at 12000 rpm for 15 min. Pellet was washed with 70% ethanol and then centrifuged at 12000 rpm for 5 min and air dried. Finally pellet was dissolved in TE buffer and the samples were used for further studies.

Detection of CiYMV by conventional PCR

In CiYMV 10 µl of NCM eluted extracted was used as DNA template 2µl of DNA obtained by commercial kit was used for PCR detection of CiYMV. PCR was performed in 10 µl of reaction mix containing 0.1 µg each of forward and reverse primer of CYMV (5' GAGCTATTAGAAGGAATCTC, 5' AACCAAGCTCTGATACCA),

Taq DNA polymerase 5U (Promega, Madison, U.S.A), 1 µl of 10Xpcr buffer, DNTPS (Qiagen, Germany). Samples were amplified for 30 cycles, using a Mastercycler (Eppendorf, Germany). Each cycle consisted of denaturation at 94°C (30s), primer annealing at 54°C for CYMV, extension at 72°C (60s), with final extension of 10 min at 72°C. 10 µl of amplified product were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide at a concentration of 0.5 µg mL^{-1} and photographed under illumination with an imaging system (Biorad XR documentation system). All the experiments were repeated at least twice.

Table 1: The components of PCR mixture used for DNA isolation from citrus tree

Reagents	Volume/tube (µl)
Taq buffer	1 µl
dNTP's	1 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Mgcl2 (2.0mM)	0.8 µl
Taq DNA ploymerase	0.2 µl
Template DNA	2 µl
Sterile distilled water	4 µl
Total	10 µl

Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described below. The frame of the gel-casting unit was cleaned and sealed with a tape to form a mould. The frame was placed on flat platform to ensure a flat and level base. The comb was then positioned parallel to the open edge of the frame about 2 mm above the surface. 1 g of agarose was dissolved in 100 ml of (1X) TBE buffer at 65ºC. The solution was allowed to cool up to 50ºC, 4 µl of ethidium bromide was added to solution and poured into the gel frame and allowed for solidify. After setting the gel, it was transferred to the gel tank, which is filled with 1X TBE buffer. 10 µl of PCR product was mixed with 5µl of loading dye (bromophenol blue) and loaded into the wells. The electrophoresis was carried out at 50V for 2 h or when the dye had migrated to the end of the gel and observed under U.V Transilluminator.

Results and Discussions

Symptomatology

During investigation the symptoms of *Citrus Yellow Mosaic Virus* observed clearly during winter than summer. Symptoms were noticed on leaves and fruits of sweet orange. Symptoms include, mosaic symptoms observed on the leaves (Figure: 3.3.A), the fruits (Figure: 3.3.B), mottling of the leaves (Figure: 3.3.C), reduced in size of the leaves, vein flecking of

the leaves (Figure: 3.3.D).

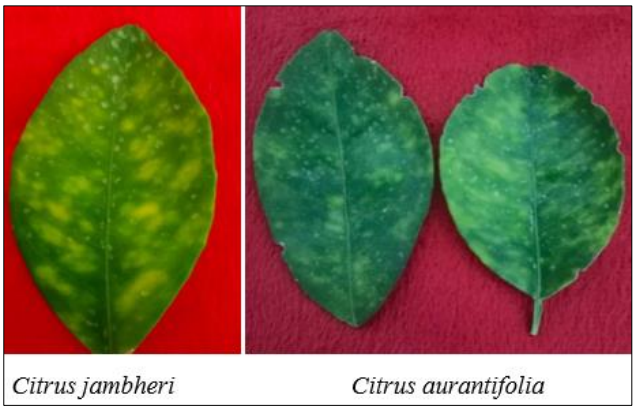
Different species of citrus *viz.*, *Citrus sinensis*, *Citrus jambheri*, *Citrus grandis*, *Citrus aurantifolia*, *Citrus aurantium*, *Citrus paradise* showing different symptoms of CiYMV. (Plate 3.4).

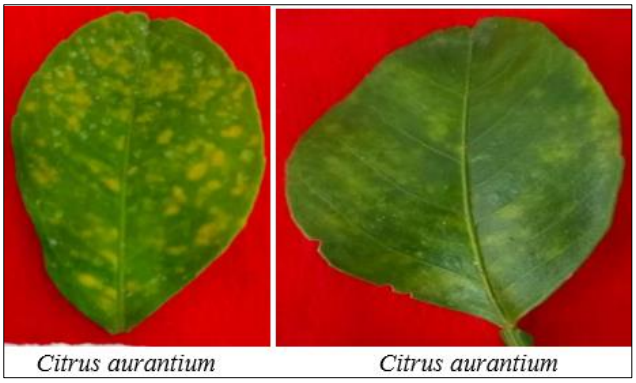
Symptoms induced by CiYMV infection include mosaic on various parts of the plant such as leaves, branches and fruits. Mosaic occurs as irregular yellow or bright green patches against dark green background and yellow flecking along the veins (Dakshinamurti and Reddy 1975) [6]. The mosaic pattern is similar on branches with irregular yellow or bright green patches against the dark green background of the branches and twigs. Trees affected by mosaic the disease not only produce significantly fewer fruits, but also yield less juice and ascorbic acid (Ahlawat *et al*.,1996a) [1].

The field symptoms of the *Citrus Yellow Mosaic Virus* (CiYMV) on Sathgudi sweet orange were mosaic pattern with irregular yellow or light green patches alternating with normal green leaf area. These symptoms were irregularly distributed all over the leaf without any reference to midrib or lateral veins. The affected tree was stunted compared to healthy trees in orchards.

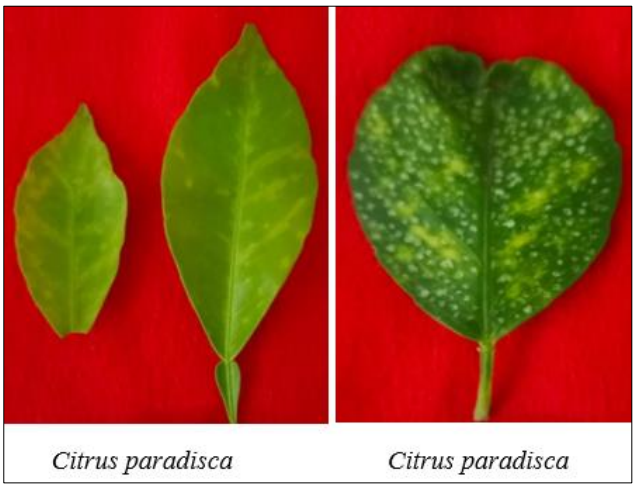
The symptoms of present study were similar to the symptoms reported by Chung *et al* 2012 [5]. CiYMV induces a bright yellow mottling or vein flecking that persists in mature leaves. Yields are sharply reduced in chronically infected 'Sathgudi' sweet orange trees and fruit may also show mosaic symptoms. CiYMV symptoms are easily visible in the field in most citrus cultivars. Symptoms persist in mature leaves, while most other citrus viruses that cause foliar symptoms are masked in older leaves. These results were further supposed by CiYMV causes mosaic and yellow flecking along the veins of the leaves. Trees affected by the disease not only show significant yield reduction, but also a decrease in the quality of fruits (less juice and ascorbic acid). The virus can infect and cause symptoms in almost all Citrus species and cultivars. (Gaddam *et al.*, 2013) [7].

Fig 1: Different types of symptoms produced by CiYMV on sathgudi sweet oranges





a) Mosaic b) Chlrotic spots



a) Yellowing and flecking of the leaves b) Vein flecking

a) Mosaic symptoms of the fruits b) Yellowing of veins

Fig 2: Different species of citrus showing symptoms CiYMV

Table 1: Different species of citrus showing symptoms of CiYMV

Citrus species	Symptoms
1. *Citrus sinensis*	Mosaic, mottling, vein flecking
2. *Citrus jambheri*	Yellow spots, Mosaic
3. *Citrus grandis*	Mild vein flecking and mosaic
4. *Citrus aurantifolia*	Mild mosaic
5. *Citrus aurantium*	Mosaic, Yellow spots
6. *Citrus paradise*	Vein flecking, mosaic
7. *Citrus medica*	Mosaic

Detection and diagnosis

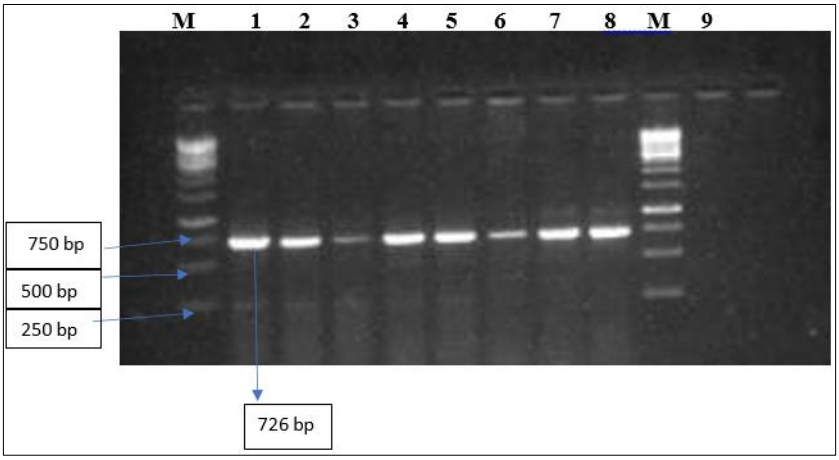
Genomic DNA isolation

DNA was isolated by using sodium sulphite method. DNA was isolated from the infected samples of different species of citrus, showing symptoms of CiYMV infection, However, high levels of polyphenolic and tannins in Citrus leaves generally interfered with obtained good quality DNA and thus affected the reliable detection of virus by PCR, the addition of sodium sulphite in extraction has improved yield of DNA (2036.15 ng/µl), possibly reduced polyphenolics, tannins, improved quality and stability of DNA.

The addition of sodium sulphite provides cleaner and more stable DNA possibly because it was effective in preventing the oxidation of nucleic acids upon the release of sap from cells and prevented degradation during storage as suggested by (Byrne *et al*., 2001) [4].

Detection of CiYMV by conventional PCR

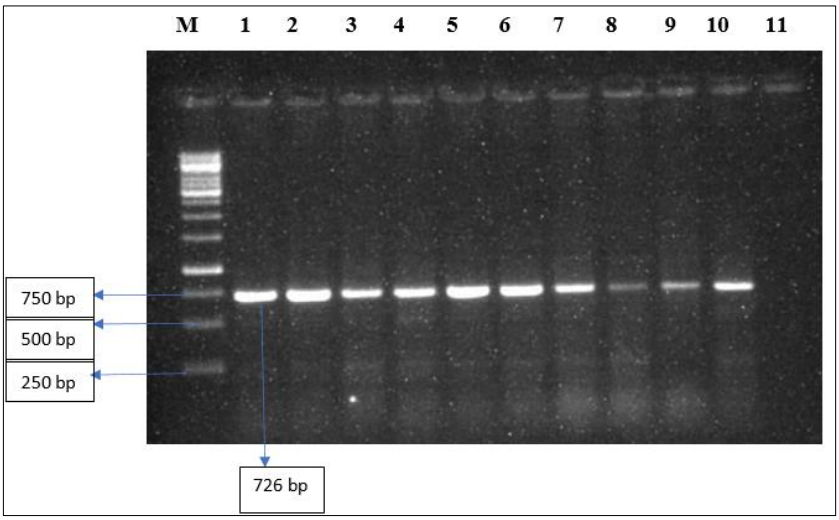
Detection of *Citrus Yellow Mosaic Virus* was carried out through PCR by using specific primers CiYMV F and CiYMV R primer set (Table 3.1.) The detection of CiYMV in different species of sweet orange viz., *Citrus sinensis*, *Citrus jambheri*, *Citrus grandis*, *Citrus aurantifolia*, *Citrus aurantium*, *Citrus paradise*, *Citrus medica* showing bright amplification was observed at 726 bp. (Fig. 3.3).



M- 1 kb ladder, Lane 1: *Citrus sinensis*, lane 2: *Citrus jambheri*, lane 3: *Citrus grandis*, lane 4: *Citrus aurantifolia*, lane 5: *Citrus aurantium*, lane 6: *Citrus paradise*, lane 7: *Citrus medica*, lane 8: positive control, lane 9: Negative control

Fig 3: The detection of *Citrus Yellow Mosaic Virus* by PCR in different tissues of infected sweet orange

Similarly the amplification was observed at 726 bp in infected tissues of sweet orange tree *viz.*, leaf, fruit rind, bark, twig, petiole, midrib leaf, feeder root, tender shoots, senescent leaves through a conventional PCR (Fig.3.4.)



M- 1 kb ladder, Lane 1: leaf, lane 2: fruit rind, lane 3: bark, lane 4: twig, lane 5: petiole, lane 6: Midrib leaf, lane 7: feeder root, lane 8: Tender shoots, lane 9: Senescent Leaves, lane 10: Positive control, lane 11: Negative control

Fig 4: The detection of *Citrus Yellow Mosaic Virus* by PCR in different tissues of sweet oranges

Sensitive detection of CiYMV in different infected parts of citrus plant that include fruit-juice, fruitrag, fruit-rind, twig bark and leaf was recently carried by Gopi *et al.* (2010) [8] through a conventional PCR and ELISA.

Gupta *et al.* (2017) [9], detected CiYMV by PCR using leaf tissue. The disease is graft transmissible and demonstrated that the CYMV can be detected by PCR in bark and bud in addition to leaf tissue of infected citrus plants.

A region of the genomic nucleic acid extracted from CMBV virions was successfully amplified by PCR using the badnavirus-specific oligonucleotide primer pair Badna 2 and Mys 3', which is known to prime amplification of all mealybug- transmitted badnaviruses (Ahlawat *et al.*, 1996a) [1].

An amplicon of 400 bp of ORF II of CiYMV was obtained in PCR amplification from the full-length pUC 18–CYMV clone. (Kumar *et al.*, 2018) [10].

BLAST analysis

The sequenced 726 bp partial DNA sequence of CiYMV was deposited in genebank database with accession no. OR536587. The current CiYMV Tirupati isolate sequence was searched using BLASTn algorithm. It showed a maximum identity of 100% with CiYMV complete genome and 98% with CiYMV JNTU isolate.

Dendrogram

A dendrogram was constructed with the current isolate to find the phylogenetically closest neighbours. The dendrogram was constructed using MEGA 11 software program with kimura-2-parameter model. The bootstrap test was performed using 1000 replicates through NJ method.

Comparision of CiYMV CRS isolate with other isolates of CiYMV

The phylogenetic tree constructed based on nucleotide sequences revealed very close relationship with *Citrus Yellow Mosaic Virus* complete genome sweet orange isolate, Accession number: NC 003382.1 (Fig.3.3.)

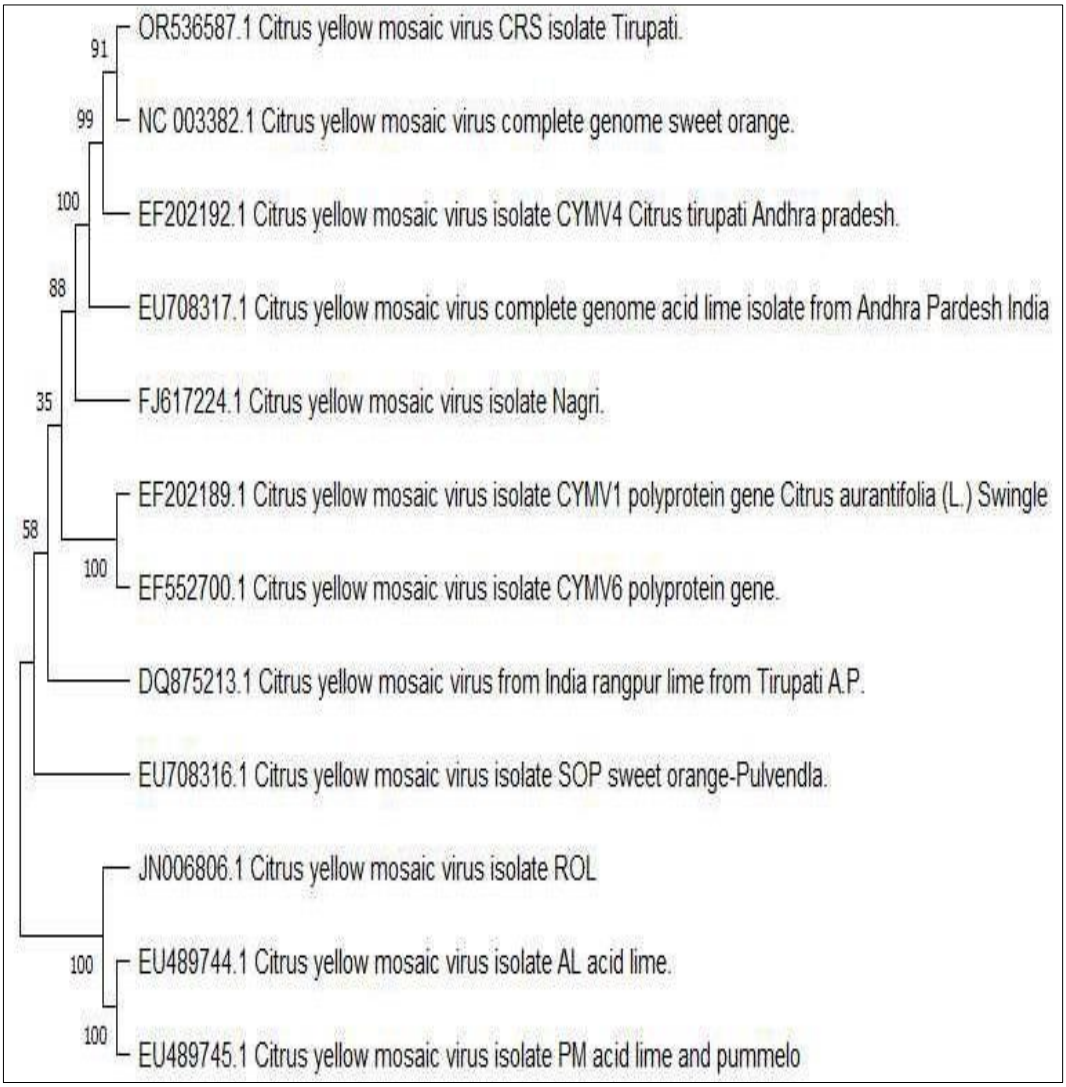


Fig 5: Phylogenetic analysis of *Citrus Yellow Mosaic Virus* CRS isolate. The dendrogram was constructed using MEGA 11 software program with kimura-2- parameter model. The bootstrap test was performed using 1000 replicates through NJ method.

Comparision of CiYMV isolate with other banaviruses

CiYMV CRS isolate shared 73 per cent and 99% per cent nucleotide sequence homology with *Taro bacilliform virus*, *Agalonema bacilliform virus* isolate minneosta. (Fig 3.4.).

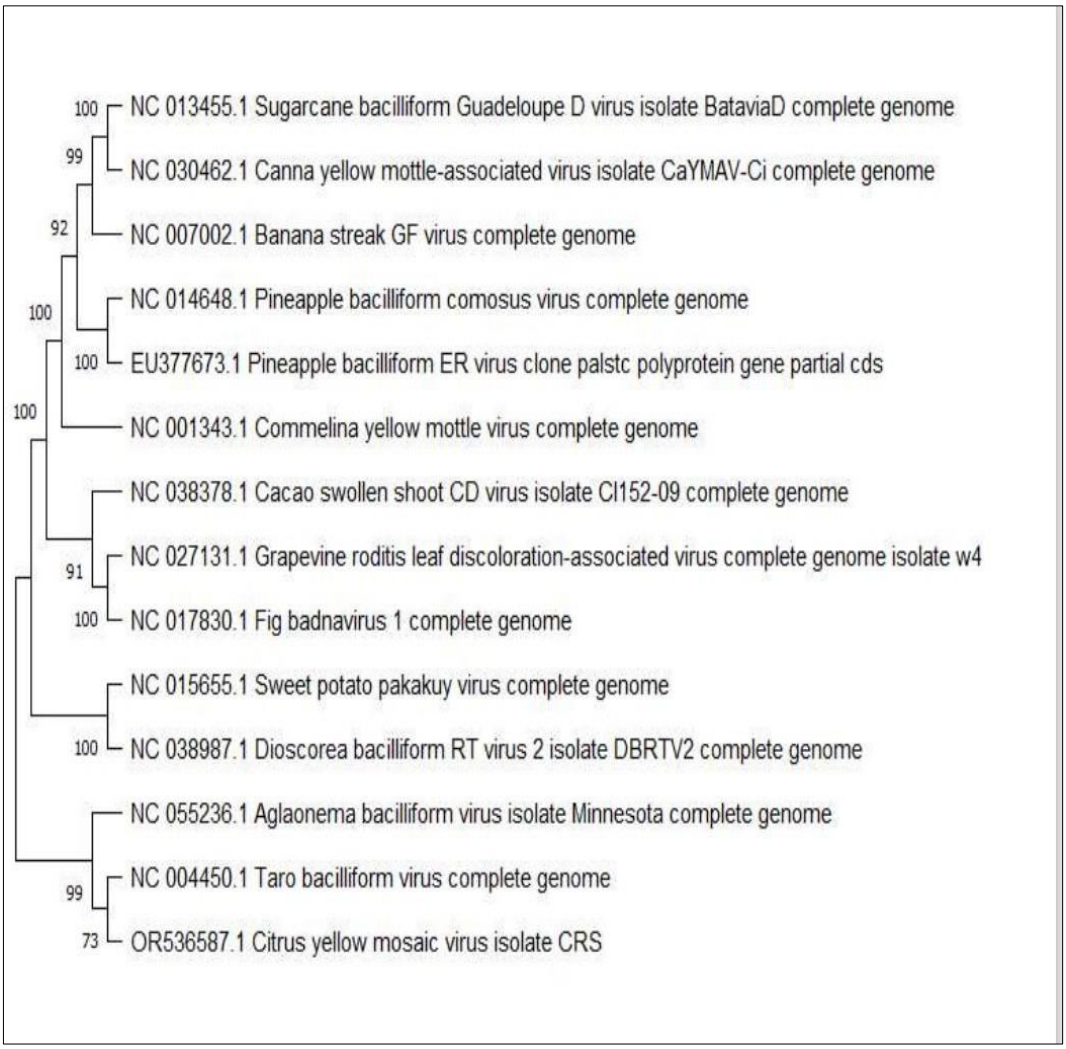


Fig 6: Phylogenetic analysis of *Citrus Yellow Mosaic Virus* CRS isolate comparison with the other badna viruses

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