Cultural characteristics and molecular confirmation of *Ustilaginoidea virens* the incitant of false smut of rice

ABSTRACT

Aims: *Ustilaginoidea virens*, which causes false smut of rice (synonyms: green smut or pseudo smut) is an emerging grain disease of rice around the world. The main aim of the study is to find out suitable culturable medium, carbon sources and pH for the growth of false smut pathogen and molecular confirmation of *U. virens* isolates.

Place and duration of study: The *in vitro* studies were conducted in the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore between December 2020 to April 2021.

Methodology: To study the growth pattern of *U.virens* five different carbon sources and mediums were used. Standardization of pH was done using PSA medium amended with different pH levels. DNA extraction of all the 13 isolates were done using CTAB method. The phylogenetic tree was constructed using MEGA 12 software

Results: It is a slow growing pathogen and grew well in the potato sucrose agar medium with the mycelial growth of about (54.00 mm) after 30 days and sucrose was found to be a good carbon source. The pH 5 was found to be optimum. PCR reaction was carried out for further validation of 13 isolates using three sets of primer pairs ITS 1/ITS 4, US1-5/US3-3, and US 2-5/US 4-3 with amplified products of 560 bp, 380 bp and 260 bp. Accession numbers were obtained for all the isolates.

Keywords: Potato sucrose agar medium, pH 5, US 2-5/US 4-3, phylogenetic tree

1. INTRODUCTION

Rice production is frequently subjected to a number of biotic challenges, of which diseases including blast, false smut, sheath blight, stem rot, and bacterial blight are vital to limit the crop's potential output. Worldwide, the average yearly losses from rice diseases are between 15 and 20%. A progressive change in a region's disease pattern occurred as a result of the introduction of high-yielding dwarf varieties and the corresponding change in rice farming methods, including increased plant population, greater fertilizer dosages and more irrigation. Several minor diseases have grown to be of considerable concern. The "False Smut Disease" of rice is one such disease that has attracted widespread notice. The disease is favoured by high relative humidity, low temperatures, rain, and overcast days during flowering [1]. The pathogen *U. virens* targets rice blossoms in particular to produce smut balls, an economically significant disease. Cytological analysis has revealed that the infection process occurs in rice flowers at the late booting stage of the rice plant. The spores come into contact with developing spikelets and germinate on their surface, or epiphytically grown hyphae make their way to the surface of developing spikelets. Although the hyphae couldn't enter the spikelet, they might enter its interior through the area between the lemma and the palea [2]. The pathogen first infects the stamen filaments intercellularly [3]. However, during infection, it is impossible to find

infection structures such as the appressorium and haustorium. As they develop, mycelia eventually surround the floral organs emerging from the spikelet, and eventually form a ball-shaped chlamydospores. Stamen filaments are replaced by mycelia in the smut ball or at a late stage of infection, indicating that the ovaries and lodicules may have contributed to the development of the smut balls [3]. Ascospores produced by sclerotia are the initial source of infection for rice plants, although airborne chlamydospores can cause a secondary infection [4]. When a plant is infected, one or more mature grains are replaced with globular, yellowish-green, velvety smut balls. Smut balls explode, releasing powdery black spores [5]. In different parts of India, the disease has grown endemic and has caused losses ranging from 2 to 85 per cent since it first manifested in epidemic form in 2001 and 2002 on cultivar PR 116 [1,6]. There have been reports of disease incidences from Punjab and Tamil Nadu on various cultivars of roughly 10%–20% and 5-85%, respectively. Its emergence in recent years may be related to high-input farming, greater use of hybrid cultivars and climate change [7]. Hence an attempt was made to standardize the medium for growth of the false smut pathogen and identification at molecular level will leads to proper confirmation of pathogen. This molecular identification will eventually leads to sustainable management of false smut disease.

2. MATERIALS AND METHODS

Pathogen *U. virens* (FS 10) accession number (MZ221826) was used in the entire study.

2.1 Standardization of different solid medium on mycelial growth of *U. virens*

Different solid media *viz.* potato dextrose, carrot dextrose, corn meal, oatmeal, and potato sucrose agar were prepared and used for this study. The pathogen, *U. virens* was inoculated at the center of the plates by placing an eight mm actively growing culture disc from a 20 days old culture using a sterilized cork borer. The plates were incubated at (25 ± 2^0C) for 30 days. Three replications were maintained for each treatment. The radial growth of the mycelium was measured on 10^{th}, 20^{th} and 30^{th} days after inoculation.

2.2 Growth on different carbon sources

The Czapek's dox agar medium contained various carbon sources, including sucrose, glucose, fructose, sodium carbonate and calcium carbonate in place of the standard carbon source. The different carbon sources amended media was poured into sterilized Petri plates @ 20 ml and allowed to cool. Using a sterilized cork borer, an eight mm *U. virens* actively growing culture disc was cut from a 20-day-old culture and placed in the center of each Petri plate containing the aforementioned solid media separately and three replications were maintained. Each Petri plate was then incubated for 30 days at 25 ± 2 °C. The diameter of mycelium was measured on 10^{th}, 20^{th} and 30^{th} day.

2.3. Growth at different pH levels

U. virens growth was examined on PSA in different pH ranges. Potato sucrose agar medium was prepared and the pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 using 0.1 N HCl or NaOH. The media was then poured into a 250 ml conical flask and sterilized. Under aseptic conditions, the

different pH amended medium was poured into the Petri plates and an eight mm *U. virens* actively growing culture disc was placed in the middle of each Petri plate and incubated for 30 days at room temperature (25 ± 2 °C). For each pH level, three replications were maintained.

2.4. DNA extraction and molecular confirmation

Total DNA of 13 isolates was extracted using the Cetyl trimethyl ammonium bromide (CTAB) method. Pure culture of all the isolates of *U. virens* mycelia was inoculated in PSA broth and incubated at 28°C for 2 weeks. The mycelium was harvested by filtration and allowed to dry. Approximately 1gram of the dried mycelium was placed in a pestle with mortar and ground using liquid nitrogen; 750 µl of CTAB buffer (10 ml of 10 % CTAB, 5.0 ml of 1 M Tris pH 8.0; 2.0 ml of 0.5 M EDTA pH 8.0; 14.0 ml of 5 M NaCl; 20.0 ml of H_2O and final volume was made up to 50 ml) was added to the powdered mycelia and ground. The extract was transferred into eppendorf tubes (700µl) and incubated in water bath at 65°C for 30 min. The tubes were vortexed for 5 seconds throughout the incubation to ensure complete mixing. After incubation, the tubes were added with an equal volume of phenol, chloroform, isoamyl alcohol (25: 24: 1) (v/v) and centrifuged at 13,000 rpm for 15 min. The uppermost aqueous phase was transferred to a new Eppendorf tube. An equal volume of ice-cold isopropanol was added and incubated overnight at −20 °C; followed by centrifugation at 13,000 rpm for 15 min. The DNA pellet was washed with 70% ethanol, air dried and finally dissolved in 30 µl of the double sterile distilled water. The final concentration of DNA was checked using nanodrop and total DNA was confirmed using 0.8% agarose gel.

The ITS region of DNA was amplified using PCR conditions with Universal primers reported by [8]. ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC). The cycle parameters included were: for initial denaturation at 96 °C for 2 min, followed by 30 cycles consisting of denaturing at 96 ºC for 30 seconds, annealing at 58 °C for 30 seconds and extension at 72°C for 30 seconds and final extension for 7 min at 72 °C. Specific internal transcribed spacer (ITS) primers (US 2-5/US 4-3) and (US 1-5/, US 3-3) were used to confirm. *U. virens* [9]. The polymerase chain reaction (PCR) mixture (20 µl) consisted of 0.5 µl of 2.5 mM of dNTPs, 10 pmol of each primer one unit of Taq polymerase, DNA template (20 ng). PCR reaction was carried out for false smut specific primers with an initial denaturation step at 96 °C for 2 min, 30 cycles of amplification (20 s for denaturation at 96 °C, 30 s for primer annealing at 53 °C and 30 s for extension at 70 °C), and one cycle of final extension at 72 °C for 7 min. A Thermocycler was used for the amplification (Eppendorf). Standard agarose gel (1.2 %) electrophoresis was used to examine PCR-amplified products. The gel was documented using a gel documentation device (Bio-rad system), under UV light.

2.5 ITS sequencing and phylogenetic tree

The purified DNA from ITS-PCR was sequenced and were subjected to BLAST analysis on http://www.ncbi.nih.gov/ index.html. The sequences were submitted to NCBI-Gen Bank database and accession numbers were obtained for all 13 isolates and a phylogenetic tree was constructed using MEGA 12 software.

3. RESULTS AND DISCUSSION

3.1 Growth on different solid medium

In order to assess the suitability of seven different mediums for the mycelial growth of *U. virens*, FS 10 isolate was used to compare the pathogen's growth. The colony diameter was recorded after incubation at 25 °C for 30 days (Table 1). The findings revealed that Potato Sucrose Agar was found to be optimum for fast growth and yielded the maximum growth in diameter of (54.00 mm). The least growth was recorded in oatmeal agar medium (45.66 mm). The results were corroborated with those of [10,11,12,13,14,15] which have a mean mycelial growth of about 56.05 mm, 67.36 mm, 51.6 mm, 69.7 mm, 40 mm and 68.74 mm respectively in PSA medium after 30 days of incubation. However, [16,17,18,19]reported that potato sucrose agar (PSA) medium was the best medium for the growth of *U. virens.*

3.2 Growth in carbon sources

Presence of carbon in the medium is essential for sporulation, growth and development of fungi. In the current investigation, different carbon sources utilized showed varying degrees of mycelial growth and the results are presented in (Table 2). The results showed that after 30 days of incubation, sucrose (68 mm) was the optimum carbon source for the mycelial growth of *U. virens* followed by fructose (5.30 cm). The results were in line with [13], who reported that fungal growth was maximum when sucrose was used as carbon source (56.7 mm). [16] documented the carbon requirements of *U. virens* indicating that sucrose and starch were the optimum carbon sources. The radial growth rate was 2.6 and 2.2 mm in a day on sucrose and starch, respectively. However, [12] documented that mannitol was the best carbon source.

3.3 Growth at different pH

The pathogen was cultured in a PSB medium at various pH levels to determine the optimum pH for the growth of *U. virens*. Six different pH levels (4.0, 5.0, 6.0,7.0, 8.0 and 9.0) were tested (Table 3). After 30 days of incubation, the maximum the mycelial growth of about (62.5 mm) was recorded at pH 5 followed by pH 6 (51.25 mm). These findings demonstrate that fungus thrives in a pH range of slightly acidic to neutral. The least mycelial growth was observed in pH 8 (31.75 mm). The results were substantiated by [11] who reported that *U. virens* grew well at pH 6 (68.82 mm). [12] reported that the fungus has recorded the highest mycelial dry weight at pH 6.5 (560.0 mg) followed by pH 6.0 (360.0 mg). [13 and 14] documented that the highest mycelial dry weight (132.1-171.5 mg) was obtained at pH 6 compared to pH 5.5 and 6.5, [20 and 21] have also documented that pH 6.0 was optimum for mycelial development.

3.4 Molecular confirmation using ITS and specific primers

The molecular level identity of all the 13 isolates were confirmed using the primers ITS 1 / ITS 4, US1-5/US3-3, and US 2-5/US 4-3. The identification of the thirteen isolates was validated using PCR confirmation. All the isolates used in the investigation had bands with the specific length of

approximately 565 bp, 260 bp and 380 bp respectively (Fig. 1 and 2). The ITS 1/ ITS 4 amplified product of all the isolates was subjected to partial sequencing, and submitted to NCBI and accession numbers were obtained (Table 4). The NCBI blast result showed that the nucleotide sequence of all thirteen isolates had the identity of a minimum of 90.86 to a maximum of 100 per cent with all *U. virens*. [10,22,23] documented that the false smut pathogen was confirmed using ITS 1 and ITS4 with sequence homology of about 98 to 100 per cent, 91 to 99 per cent respectively. The results were corroborated with [13,24] for ITS 1/ ITS 4 with a specific amplification. Similar results for US1-5/US3-3 were documented by [1,9,13,25,26] which also had a specific band for all the *U. virens* isolates at 380 bp. Nevertheless, [1,9,13] have reported that specific band was seen at 230bp while using US 2-5/US 4-3 specific primer.

3.5 Construction of phylogenetic tree

The phylogenetic tree was constructed using MEGA 12 software based on partial sequencing. In addition, 12 other isolates of *U. virens* sequences were collected from NCBI and true smut *Ustilago tritici* was used as an outgroup (Fig. 3) The phylogenetic position of *U. virens* isolates were compared with different isolates of *U. virens* and true smut fungi which depicted that all the false smut isolates come under one clade and *Ustilago tritici* in a separate clade. The clade was further subdivided into different subclades showing that there was genetic variability among the isolates of *U. virens.*

[27] constructed phylogeny based on sequences at specific SNP-rich locations and found that there were considerable genetic variations across various regional groups of *U. virens*. [26] reported that using the phylogenetic analysis of ITS sequences, all the isolates were categorically divided into two groups, with 97–100% sequence similarity, and all *U. virens* were placed into a single cluster. [28] constructed the tree using ITS 1 and ITS 2 sequences and reported that three isolates came under the same clade and one isolate was in a separate clade. [29] have demonstrated that all *U. virens* strains constituted a sub-clade within the Clavicipitaceae clade and clearly distinguished true smut fungi from other *U. virens* strains. [14,26,30] constructed phylogeny tree based on RAPD primer sequences and confirmed the variability among the isolates.

4. CONCLUSION

The present study concluded that the suitable medium for the mycelial growth of *U. virens* was potato sucrose agar medium under laboratory condition. The *U. virens* grew luxuriantly in sucrose as the carbon source and at pH 5. The molecular confirmation of all 13 isolates revealed that all were *U. virens.* The phylogenetic tree constructed revealed that as the ascomycetes fungus that causes false smut disease of rice, was different from the true smut fungus that belongs to the basidiomycetes family and all the thirteen isolates of *U. virens* have considerable genetic variability.

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Table 1. Effect of different medium on the mycelial growth of *U. virens*

S.No.	Name of the medium	Radial mycelial growth (mm)		
		10th day	**20th day**	**30th day**
1	Potato dextrose agar	24.00^{ab} (29.331)	36.00^{ab} (36.862)	50.00^{b} (45.000)
2	Carrot agar	17.00^{e} (24.326)	25.66^{c} (30.433)	46.00^{bc} (42.705)
3	Potato sucrose agar	26.00^{a} (30.646)	38.00^{a} (38.049)	54.00^{a} (47.295)
4	Oatmeal agar	18.66^{de} (25.596)	27.33^{c} (31.510)	45.66^{c} (42.510)
5	Cornmeal agar	22.66^{bc} (28.422)	33.33^{ab} (35.239)	47.00^{bc} (43.279)

6	Potato carrot agar	21.00^{cd} (27.271)	32.00^{b} (34.443)	46.66^{bc} (43.088)
CD (P= 0.05)		**1.819**	**2.847**	**1.944**

* Values are mean of three replications. Figures in parentheses represent arcsine transformation.

Means in a column followed by same superscript letters are not significantly different according to DMRT at P ≤ 0.05.

Table 2. Effect of different carbon sources on the mycelial growth of *U. virens*

S.No.	Carbon sources	Radial mycelial growth (mm)		
		10thday	**20th day**	**30th day**
1	Sucrose	27.33^{a} (31.510)	44.60^{a} (41.930)	68.00^{a} (55.573)
2	Fructose	22.30^{b} (28.179)	43.00^{a} (40.972)	62.66^{a} (52.348)
3	Glucose	21.00^{b} (27.247)	35.33^{b} (36.463)	53.33^{b} (46.913)
4	Sodium carbonate	6.00^{c} (14.049)	12.33^{c} (20.496)	19.33^{c} (26.055)
5	Calcium carbonate	4.66^{c} (13.340)	6.66^{d} (14.898)	10.33^{d} (18.720)
CD (P= 0.05)		**3.121**	**3.584**	**3.324**

* Values are mean of three replications. Figures in parentheses represent arcsine transformation.

Means in a column followed by same superscript letters are not significantly different according to DMRT at P ≤ 0.05.

Table 3. Effect of different pH on the mycelial growth of *U. virens*

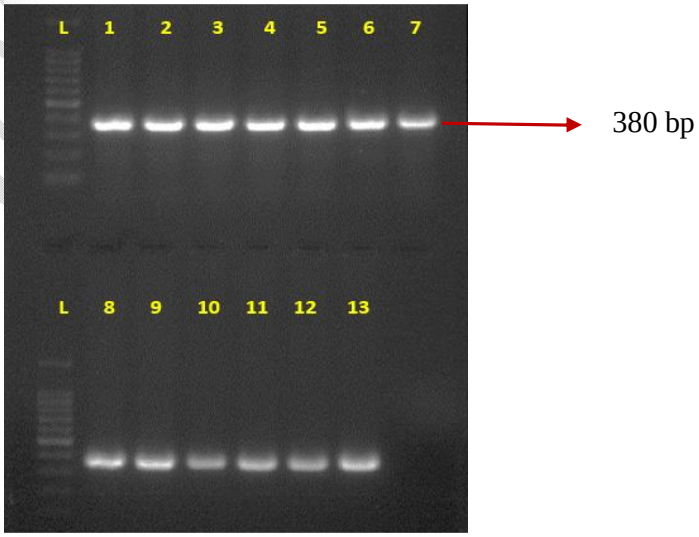
S.No.	pH levels	Radial mycelial growth (mm)		
		10thday	**20th day**	**30th day**
1	4	14.25^{cd} (22.164)	33.25^{c} (35.213)	42.25^{a} (40.541)
2	5	24.25^{a} (29.498)	45.25^{a} (41.986)	62.50^{b} (52.242)
3	6	20.25^{b} (26.742)	36.50^{b} (37.166)	51.25^{b} (45.718)
4	7	19.50^{b} (26.203)	32.50^{c} (34.756)	40.75^{c} (39.666)
5	8	15.50^{c} (23.182)	24.50^{d} (29.662)	31.75^{c} (34.291)
6	9	13.75^{d} (21.744)	25.50^{d} (30.324)	35.00^{d} (36.254)
CD (P= 0.05)		**1.141**	**0.973**	**2.143**

* Values are mean of three replications. Figures in parentheses represent arcsine transformation.

Means in a column followed by same superscript letters are not significantly different according to DMRT at P ≤ 0.05.

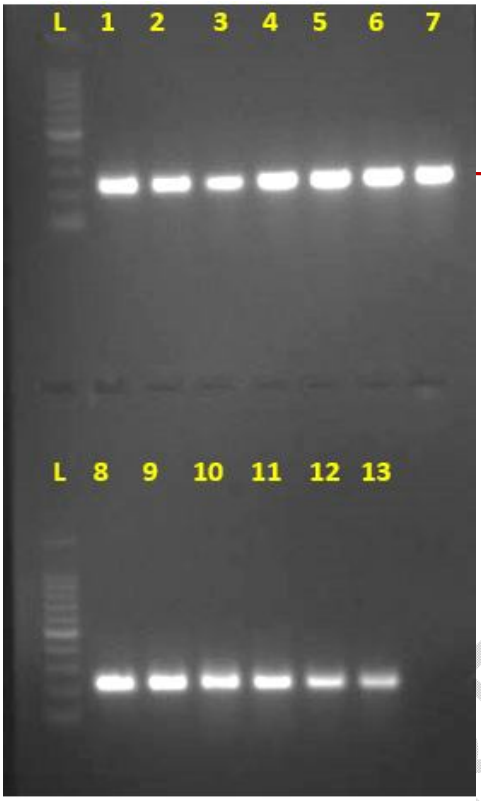
Table 4. Molecular confirmation of *U. virens* using ITS 1 / ITS 4 primers

Isolates	Acession number	Identified species	Identity (%)
FS 1	MZ157262	*Ustilaginoidea virens*	99.16
FS 2	MZ605380	*Ustilaginoidea virens*	100
FS 3	MZ157263	*Ustilaginoidea virens*	99.83
FS 4	MZ133725	*Ustilaginoidea virens*	98.39
FS 5	MZ157280	*Ustilaginoidea virens*	99.49
FS 6	MZ158322	*Ustilaginoidea virens*	95.74
FS 7	MZ646028	*Ustilaginoidea virens*	91.79
FS 8	MZ221827	*Ustilaginoidea virens*	91.75
FS 9	MZ646301	*Ustilaginoidea virens*	93.87
FS 10	MZ221826	*Ustilaginoidea virens*	99.24
FS 11	MZ662821	*Ustilaginoidea virens*	100
FS 12	MZ221831	*Ustilaginoidea virens*	100
FS 13	MZ646351	*Ustilaginoidea virens*	90.58



L- 100 bp DNA marker, 1 – FS1, 2- FS 2, 3- FS 3, 4- FS4, 5- FS 5, 6- FS 6, 7- FS7, 8- FS8, 9- FS9, 10- FS 10, 11- FS 11, 12- FS 12, 13- FS 13

Fig. 1 Polymerase chain reaction for US1-5/US3-3



L- 100 bp DNA marker, 1 – FS1, 2- FS 2, 3- FS 3, 4- FS4, 5- FS 5, 6- FS 6, 7- FS7, 8- FS8, 9- FS9, 10- FS 10, 11- FS 11, 12- FS 12, 13- FS 13

Fig. 2 Polymerase chain reaction for US2-5 / US4-3

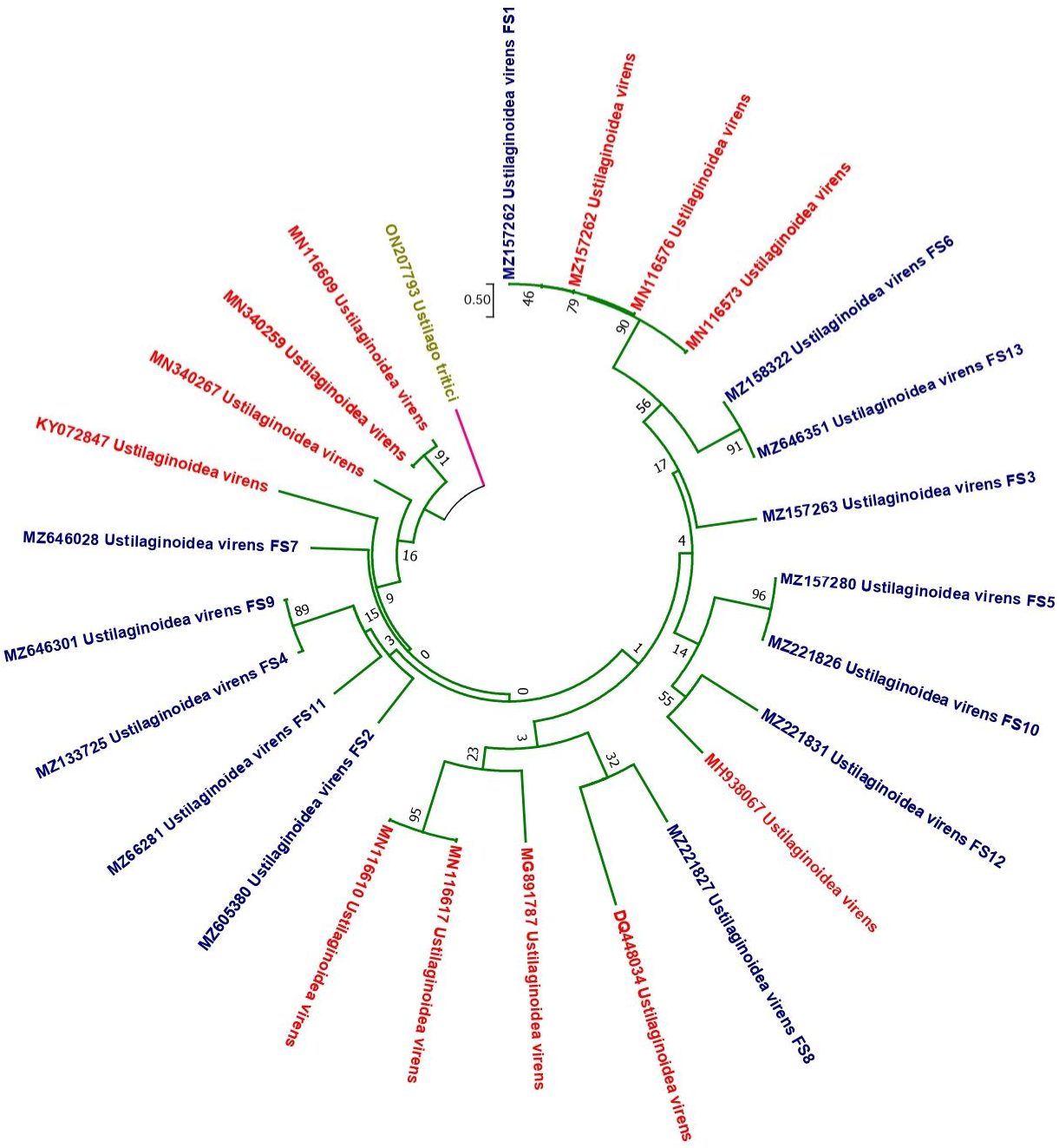


Fig.3 Phylogenetic tree showing genetic variability among different *U. virens* isolates