

Original Research Article

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Multiple Gene Characterization of Rice Orange Leaf (ROL) Phytoplasma Infecting Rice

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ABSTRACT

Phytoplasmas are intracellular obligate prokaryotes that lack cell walls and have very small genomes (680–1,600 kb). Many fungal, bacterial and viral diseases have been characterized and resistant breeding programme is progressing very well. Recently, on the basis of the symptoms and on 16SrDNA sequence identity and phylogenetic relationships the association of ‘*Ca. P. asteris*’ with Rice Orange Leaf phytoplasma disease in South India was confirmed. Rice orange leaf phytoplasma was characterized based on multiple gene systems. The 16S-23S rRNA Spacer region of rice phytoplasma was amplified by the primer pairs p1/p7 and p3/p7 at ~320 bp from all rice samples. The phytoplasma infecting rice samples were confirmed as group I by the group specific primer for 16SrI which amplified DNA fragment at ~1.1 kb in all the samples. The *tuf* gene in phytoplasma (~1000 bp) and phytoplasma in rice belonging to aster yellow (AY) group was confirmed by the primer specific for aster yellow *tuf* gene with amplification at ~940 bp. The primer pair, AYsecYF1 and AYsecYR1 was used which amplified the *secY* gene at ~1.4 kb in all the rice samples which confirmed the phytoplasma infecting rice belonged to aster yellow group. This is the first report to study multiple gene systems of Rice Orange Leaf (ROL) phytoplasma infecting Rice crop.

Keywords

Rice Orange Leaf (ROL) phytoplasma, 16S-23S rRNA Spacer, 16SrI- *tuf* gene, *secY* gene

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Introduction

Phytoplasmas are intracellular obligate prokaryotes that lack cell walls and have very small genomes (680–1,600 kb). Since the first report by Doi *et al.*, (1967), phytoplasmas have been identified as pathogens in different

plant genera and in some cases have caused severe epidemics in major crops (Bertaccini *et al.*, 2014). Phytoplasmas cause complex syndromes with symptoms such as stunting, proliferating auxiliary shoots, forming sterile deformed flowers, virescence and phyllody in several hundred plant species (Lee *et al.*,

2000). Based on phylogenetic analysis of gene sequences (16S rRNA) phytoplasmas were recently assigned to a provisional genus, 'Candidatus (*Ca.*) Phytoplasma' within the class Mollicutes (Anonymous, 2004). The aster yellows (AY) phytoplasma group (16SrI) is the most dominant group comprises with more than 100 economically important diseases worldwide, representing the most diverse phytoplasma group (Lee *et al.*, 2004; Bertaccini *et al.*, 2014). The highly conserved 16S rRNA gene sequence has been used as the primary molecular tool for classification of phytoplasmas. A total of 19 distinct groups, termed 16S rRNA groups (16Sr groups), based on actual RFLP analysis of PCR-amplified 16S rDNA sequences or 33 groups based on *in silico* RFLP analysis have been identified (Lee *et al.*, 1998, 2000; Wei *et al.*, 2007; Bertaccini *et al.*, 2014). They are transmitted from plant to plant by grafting and other vegetative propagation techniques and by phloem-feeding insects, especially leafhoppers, planthoppers and psyllids (McCoy *et al.*, 1989, Weintraub and Beanland, 2006; Olivier *et al.*, 2012). Most phytoplasmas are transmitted from plant to plant by leafhoppers in a persistent manner.

The Poaceae family has the largest number of species associated with phytoplasma diseases worldwide and is also the plant family where the majority of phytoplasma vector species (Delphacidae) are known. Rice yellow dwarf (RYD) and Rice orange leaf (ROL) are the two phytoplasma diseases that have been reported to infect rice (Arocha and Jones, 2010). RYD, a serious problem for rice farmers and has only been detected to date in Asia (Nakashima *et al.*, 1993). The infected rice crop turns pale yellow and gradually starts to decay and produce numerous tillers. ROL phytoplasma is caused by 'Candidatus Phytoplasma asteris' and was widely distributed in South and South-east Asia including Thailand, Malaysia, Indonesia,

China, Sri Lanka and the Philippines (Hibino *et al.*, 1987). ROL phytoplasma is reported to be transmitted by the leafhopper *Recilia dorsalis* Motchulsky in the Philippines in persistent manner (Hibino *et al.*, 1987), which also transmits rice dwarf virus and rice tungro virus (Ling, 1972). Based on the 16S rDNA sequence analysis phytoplasma associated with the ROL belonged to 16Sr I group and RYD belongs to the 16SrXI group (Jung *et al.*, 2002).

Recently in India, on the basis of the symptoms, 16Sr DNA sequence identity and phylogenetic relationships, association of 'Ca. Phytoplasma asteris' with ROL disease in South India was confirmed (Valarmathi *et al.*, 2013). Hence an attempt was made to identify the associated phytoplasmas upto using multiple gene systems for the first time in India.

Materials and Methods

Extraction of DNA from rice samples

Modified CTAB method (Warokka *et al.*, 2006) was used for the extraction of total DNA from leaf samples of rice for detecting phytoplasma. Infected sample (0.4 g) was initially ground in liquid nitrogen using a precooled mortar and pestle and then incubated for 10 min in 2 ml of Phytoplasma Grinding Buffer, PGB in a centrifuge tube. Then add 1.25 ml more of PGB. The homogenate was centrifuged for 5 min at 420 g. The supernatant of each sample was transferred into clean tubes and centrifuged for 25 min at 9677 g. The pellet was dissolved in 1 ml CTAB buffer. After one-hour incubation at 65 °C, the nucleic acids were purified by chloroform-isoamyl alcohol (24:1) extraction and an equal volume of ice cold isopropanol was added to the drawn aqueous phase and then incubated on ice for 1 h. After centrifugation at 6720 g for 10 min, the pellet

was dissolved in 400 ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) to which 40 ml of 3 M sodium acetate and 0.9 ml of 95 % ethanol were added. After incubation for 2 h at -20 °C, the mixture was centrifuged for 10 min at 6720 g. Once the supernatant was eliminated, the tubes were washed with 80 % ethanol, the pellet containing the DNA was dissolved in 35 µl of sterile water. The DNA was checked by 0.8 % agarose gel electrophoresis.

Amplification of 16S rRNA gene of phytoplasma by nested PCR

The nested PCR approach was followed to detect the phytoplasma present in the disease infected rice samples. Six sets of primer pair as described were verified for the specificity in amplifying 16S rRNA gene of phytoplasma. All the PCR assays were performed in a thermal cycler (TECHNE TC 5000, Germany). One microlitre of DNA was used for first round amplification with primer pair p1/p7 and 0.5 µl of first round product was used as template in nested-PCR without dilution with phytoplasma specific primers R16F2n/R16R2. Aliquots of 10 µl PCR products were analyzed by horizontal gel electrophoresis (Bangalore Genei) on 1.5 % agarose with ethidium bromide (0.5 µg/ml) using TAE (Tris buffer, EDTA, Glacial acetic acid and water) as the running buffer. The sample was mixed with 2 µl of gel loading buffer (0.25 % Bromophenol blue + 40 % Sucrose in 100 ml of water) then loaded with gel slots. The gel was visualized with a UV transilluminator and photographed in the gel documentation system (Alpha Innotech Corporation, USA).

Detection of phytoplasma based on multiple gene systems

The PCR approach was followed to detect the phytoplasma present in the infected rice samples based on multiple gene systems. Four

sets of primer pair as described in the Table 1 were verified for their specificity in detecting phytoplasma based on the group I of phytoplasma, aster yellow group, elongation factor *tuf* gene and secretion Y gene.

Results and Discussion

Detection of phytoplasma based on multiple gene systems

The detailed result of characterizing rice phytoplasma based on multiple gene systems were furnished in Table 2. The primer, p4/p7 PCR products amplified ~ 530 bp in length which include the 16S-23S rRNA Spacer Region and part of 16S rRNA gene from all rice samples (Plate 1a). The 16S-23S rRNA Spacer Region of rice phytoplasma was amplified by the primer pairs, p1/p7 and p3/p7 at ~ 320 bp from all the rice samples (Plate 1b).

The phytoplasma infecting rice samples were confirmed as group I by the group specific primer for 16Sr I which amplified DNA fragment at ~ 1.1 kb in all the samples (Plate 2a). The elongation factor (*Tuf* gene), which is a well-conserved gene with a central role in translation for phytoplasma were analyzed by the group specific primer *tuf* revealed amplification at ~ 1000 bp from all the rice samples (Plate 2b). The phytoplasma in rice belonging to aster yellow (AY) group was confirmed by the primer specific for aster yellow *tuf* gene with amplification at ~ 940 bp (Plate 3a). The *secY* gene which encodes a protein involved in protein secretion used for differentiation of AY group phytoplasma were studied by the *secY* primer results in amplification of DNA fragment at ~ 1.4 kb in all the samples (Plate 3b).

Some additional tools for phylogenetic analyses and finer strain differentiation of phytoplasmas such as *as rp*, *secY*, *tuf*, *groEL* genes and the 16S-23S rRNA intergenic

spacer region sequences have been used as supplementary tools selecting those providing the most useful and reliable taxonomic information in combination with 16S rDNA.

The phylogenetic relationships of sugarcane grassy shoot phytoplasma strains to each other and to closely related phytoplasmas infecting mainly gramineous plant worldwide, at both 16S rRNA gene and 16S-23S rDNA spacer region sequence level were examined (Rao *et al.*, 2007). Similarly the 16S-23S rRNA Spacer Region and part of 16S rRNA gene was studied in the rice samples which amplify at ~ 530 bp.

The phytoplasma 16S-23S rRNA intergenic spacer region contains a portion that codes for the highly conserved tRNA^{Ile}. However, the flanking sequences that extend from the tDNA^{Ile} to 16S rDNA and to 23S rDNA are variable among various phytoplasmas.

The ISR can serve as a useful tool for differentiation of phytoplasma groups and subgroups. Overall, the ISR is comparable to the 16S rRNA gene sequence in its capacity for use in delineating distinct phytoplasma lineages (Smart *et al.*, 1996). To increase the sensitivity of PCR, the primer pairs p1/p7 and p3/p7 designed to amplify the 16/23S spacer region approximately 320 bp in length in the aster yellow group by Wang and Hiruki (2001). Similarly the 16S-23S rRNA Spacer Region was studied in the rice samples which amplify at ~ 320 bp by the use of primer pairs, p1/p7 and p3/p7.

A specific primer pair, designed by Lee *et al.*, (1994) for the amplification of a ribosomal DNA fragment from strains belonging to the 16SrI group and named R16 (I) F1/R1, is presently used for the amplification from strains related to 'Ca. Phytoplasma asteris'. A specific phytoplasma product of ~ 1.1 kb was obtained

after nested amplification using R16 (I) F1/R1 primer pairs in DNA samples collected from bulb scales and leaves of all diseased lilies and periwinkle plants infected by the phytoplasma belonging to aster yellow group (Kaminska and Dziekanowska, 2002).

A specific phytoplasma product of ~ 1.1 kb was obtained after nested PCR amplification using R16 (I) F1/R1 primer pairs in DNA samples collected from garlic and onion infected by the phytoplasma belonging to aster yellow group (Khadhair *et al.*, 2002).

With the references to the above study, similar results were obtained from the rice samples by the use of primer R16 (I) F1/R1 to amplify DNA fragment of ~1.1 kb confirming the phytoplasma infecting rice belonged to aster yellow group.

The *tuf* gene, encoding the elongation factor Tu (EF-Tu), is a well - conserved gene with a central role in translation (Schneider *et al.*, 1997) and there is a single copy of this gene in the phytoplasma genome.

This gene has often been used in phylogenetic studies for other bacteria. The primers amplified products of the expected size (~1000 bp) for 16SrI AY, 16SrIII green valley X and vaccinium witches'-broom and 16SrXII stolbur (STOL) groups but failed to amplify from 16SrII faba bean phyllody and 16SrX AP (Schneider *et al.*, 1997). It has been subsequently found that these primers also fail to amplify from the 16SrIV coconut lethal yellowing (LY) and 16SrXXII coconut lethal decline phytoplasmas.

The main use of *tuf* gene primers has therefore been to establish subgroups within the 16Sr groups, particularly within the 16SrI AY group (Marcone *et al.*, 2000) and the 16SrXII 'Ca. Phytoplasma australiense' group (Streten and Gibb, 2005).

Table.1 Primer sequences and PCR conditions adapted for PCR to detect different genes of phytoplasma used in the study

Primer pair	Designation	Primer Sequences	Annealing Condition	References
Group specific primer-I ^a	R 16 (I)F1 & R16(I)R1	5'- TAAAAGACCTAGCAATAGG-3' 5'- CAATCCGAACTGAGACTGT-3'	50°C for 2 min	Lee <i>et al.</i> , 1994
Group specific primer (<i>tuf</i> gene) ^a	f Tuf AY r Tuf AY	5' -GCTAAAAGTAGAGCTTATGA- 3' 5'- CGTTGTCACCTGGCATTACC- 3'	55°C for 30sec	Schneider <i>et al.</i> , 1997
AY specific primer (<i>tuf</i> gene) ^a	f Tuf 1 r Tuf 1	5' -CACATTGACCACGGTAAAAC -3' 5'-CCACCTTCACGAATAGAGAAC-3'	45°C for 30sec	Schneider <i>et al.</i> , 1997
<i>secY</i> gene primer ^a	AYsecYF1 & AYsecYR1	5'- CAGCCATTTTAGCAGTTGGTGG- 3' 5'- CAGAAGCTTGAGTGCCTTTACC- 3'	66°C for 1 min	Lee <i>et al.</i> , 2006

^aThe other PCR conditions are 95°C for 1 min followed by 35 cycles of 95°C for 30 sec, appropriate annealing condition and 72°C for 1 min with final extension of 72°C for 10 min

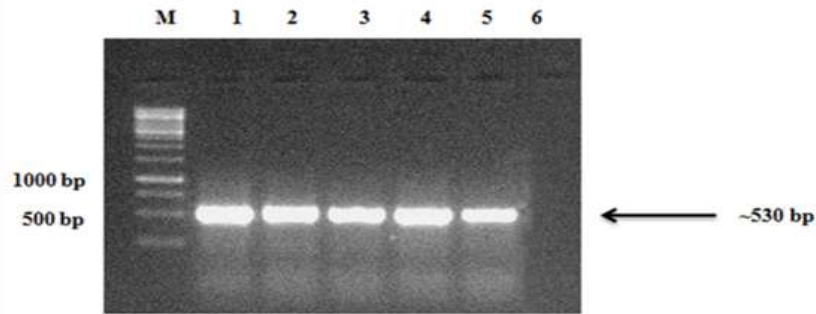
Table.2 Characterization of Rice Orange Leaf (ROL) phytoplasma based on multiple gene based systems

Samples	16S-23S rRNA Spacer Region and part of 16S rRNA gene	Intergenic spacer region (16S- 23S rRNA)	Group specific primer-I	Group specific primer (<i>tuf</i> gene)	AY-specific primer (<i>tuf</i> gene)	<i>secY</i> gene
Coimbatore-ADT 43	√	√	√	√	√	√
Coimbatore-CO 39	√	√	√	√	√	√
Coimbatore-White Ponni	√	√	√	√	√	√
Erode-BPT 5204	√	√	√	√	√	√
Negative (healthy sample)	X	X	X	X	X	X

× Absent

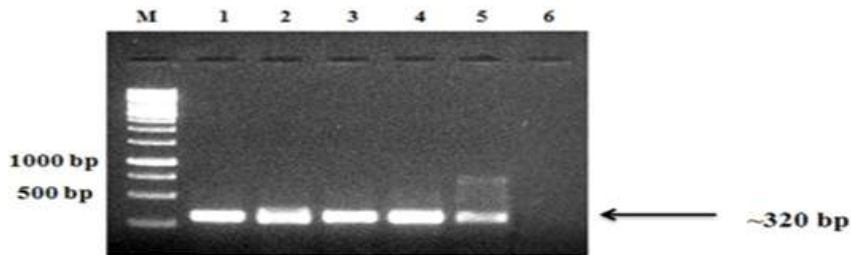
√ Present

Plate 1a. Detection of ROL by p1/p7 and p4/p7



- M- 1 kb DNA ladder
- 1- Rice (Erode- BPT 5204)
- 2- Rice (Coimbatore-White Ponni)
- 3- Rice (Coimbatore- CO 39)
- 4- Rice (Coimbatore- ADT 43)
- 5- Positive control (Infected brinjal)
- 6- Negative control (Healthy rice sample)

Plate 1b. Detection of ROL by p1/p7 and p3/p7



- M- 1 kb DNA ladder
- 1- Rice (Erode- BPT 5204)
- 2- Rice (Coimbatore-White Ponni)
- 3- Rice (Coimbatore- CO 39)
- 4- Rice (Coimbatore- ADT 43)
- 5- Positive control (Infected brinjal)
- 6- Negative control (Healthy rice sample)

Plate 2a. Detection of ROL by group specific primers for 16S rI

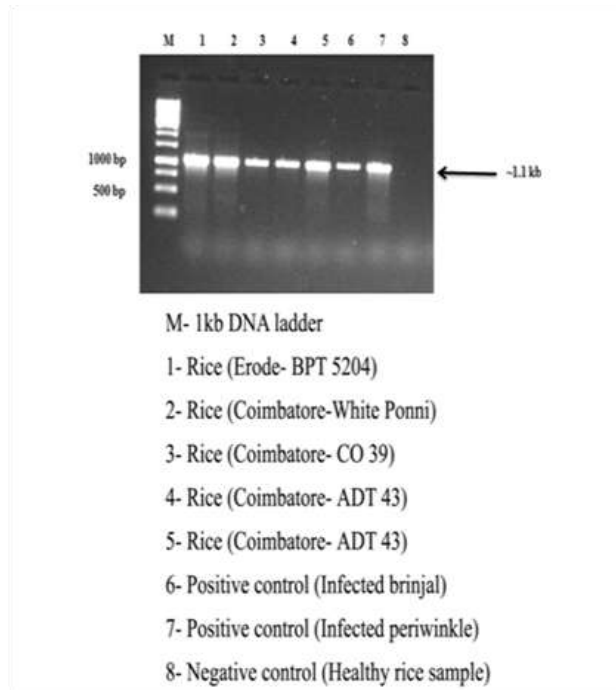


Plate 2b. Detection of ROL by group specific primers for *tuf* gene

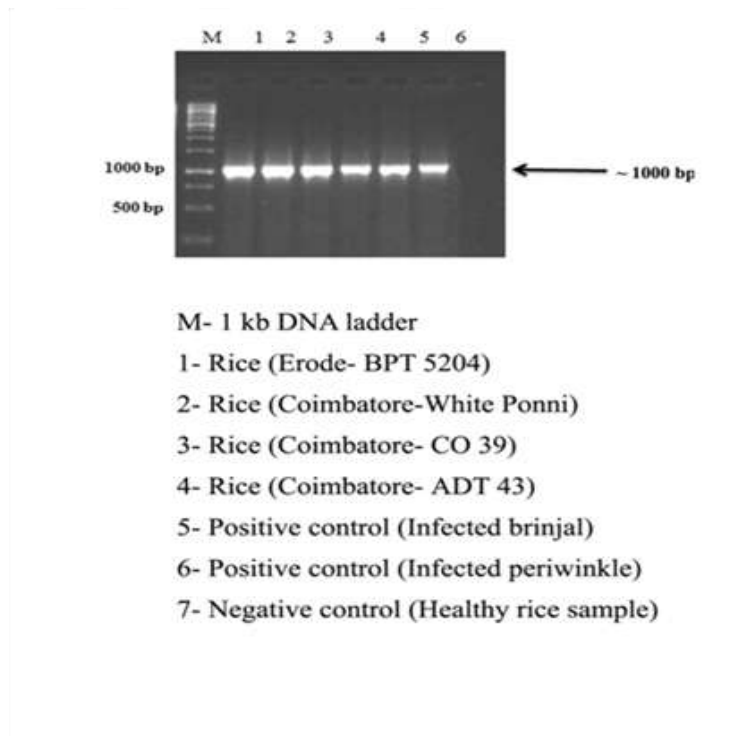


Plate 3a. Detection of ROL by Aster yellow group for *tuf* gene

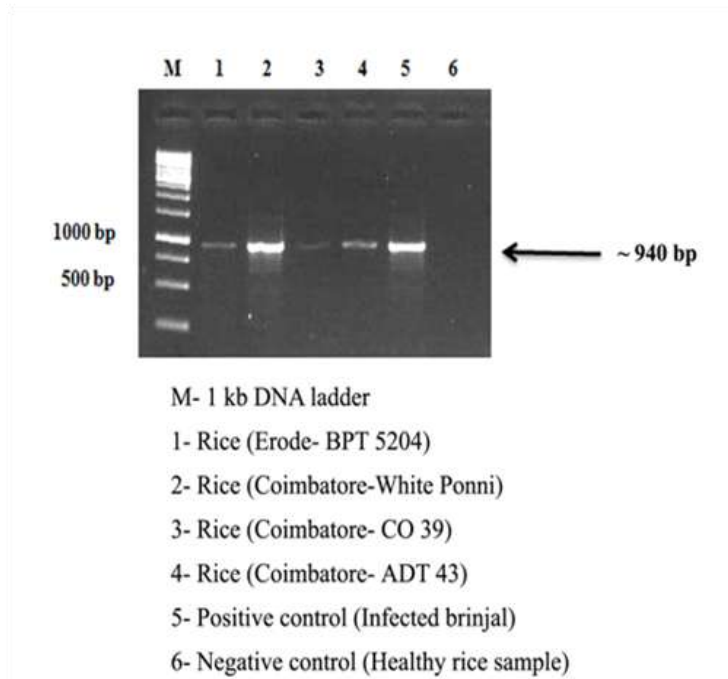
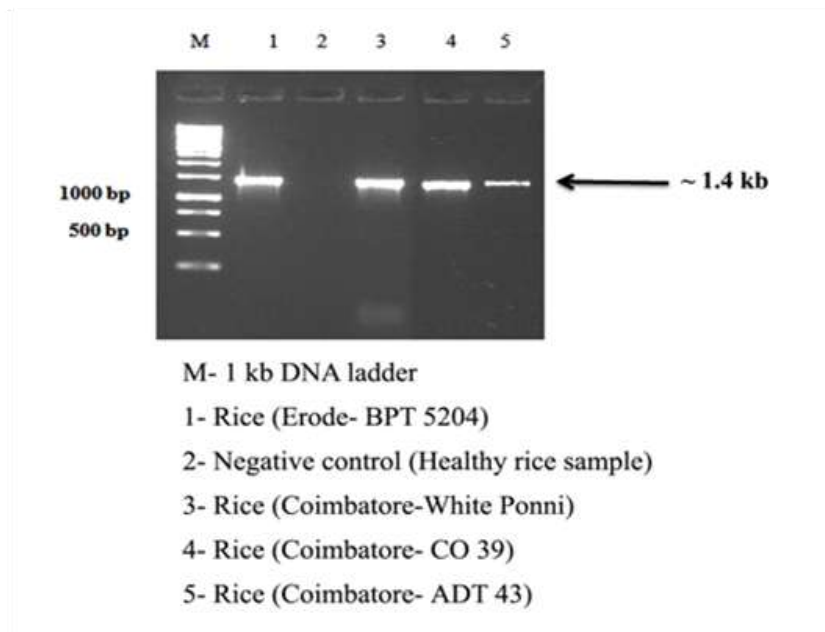


Plate 3b. Detection of ROL by Aster yellow group sec gene



In a study on the AY group, the AY-specific primers fTufAy (5' GCT AAA AGT AGA GCT TAT GA 3') and rTufAy (5' CGT TGT CAC CTG GCA TTA CC 3') (Schneider *et al.*, 1997), which amplify ~ 940 bp product, were used on 70 phytoplasma isolates in conjunction with the 16S rRNA gene primers (Marcone *et al.*, 2000). The *tuf* gene in phytoplasma (~ 1000 bp) and *tuf* gene of AY group (~ 940 bp) has been confirmed in the rice samples by our study. These results were in agreement by the findings of Schneider *et al.*, (1997) and Marcone *et al.*, (2000).

The *secY* gene, which encodes a protein involved in the protein secretion mechanism from bacteria and has also been used for differentiation of the AY group phytoplasmas (Lee *et al.*, 2006). Primers were designed based on the published AY and OY sequences to amplify a 1.4 kb near-full-length *secY* gene for AY group phytoplasmas. In our study, we used the primer pair, AYsecYF1 and AYsecYR1 to amplify the *secY* gene at ~ 1.4 kb in all the rice samples which confirms the phytoplasma infecting rice belonged to aster yellow group.

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