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First report of Rice orange leaf disease phytoplasma (16 SrI) in rice (*Oryza sativa*) in India

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Abstract Rice (*Oryza sativa* L.,) is one of the most important cereal crops worldwide. In this study, phytoplasmas have been detected in symptomatic leaves of four varieties of rice using nested-PCR with primer pairs P1/P7 and R16F2n/R16R2, which amplified a 1.2 kb fragment of the 16S rRNA gene. The nucleotide sequence analysis of the fragment had 100 % identity among the sequences from the four varieties (GenBank Accession numbers: JX290545, JX290546, JX290547 and JX290548) and 99 % nucleotide sequence identity with 16S rRNA gene sequences of the Rice orange leaf disease phytoplasmas from the Philippines and Thailand, Maize bushy stunt phytoplasma and Wheat blue dwarf phytoplasma belonging to the '*Candidatus* Phytoplasma asteris' (16SrI) group. This is the first report of 16SrI group phytoplasmas infecting rice in India.

Keywords Rice · Orange leaf phytoplasma · Nested PCR

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 such as grapevine, sugarcane and coconut (Weintraub and Beanland 2006). 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* (IRPCM 2004). The aster yellows (AY) phytoplasma group (16SrI) comprises AY and numerous related phytoplasmas that are associated with more than 100 economically important diseases worldwide, representing the most diverse phytoplasma group (Lee et al. 2004)

The Gramineae have the largest number of species associated with phytoplasma diseases worldwide, and are also the plant family where the majority of phytoplasma vector species (Delphacidae) have been found. Rice yellow dwarf (RYD) and Rice orange leaf (ROL) are the two phytoplasma disease that have been reported to infect rice. RYD, a serious problem for rice farmers, has only been detected to date in Asia, where it has been recorded from most rice-growing countries (Nakashima et al. 1993). Infected rice turns pale yellow and gradually starts to decay and produce numerous tillers. ROL has also only been found in Asia to date and the symptoms are typified by orange-coloured leaves, which later roll inward and desiccate. Despite the fact that infected plants die 2–3 weeks after the symptoms appear, diseased plants are generally distributed sporadically in the field so the disease does not as yet cause serious yield losses (Hibino et al. 1987). Phytoplasma have been associated with ROL in Thailand, Malaysia, Indonesia and the Philippines based on electron microscopy evidence (Hibino et al. 1987). Based on the 16S rDNA sequence similarity between ROL from the Philippines and Onion Yellows (OY) (99.9 %), along with other aster yellows subgroup members (98.9–99.8 %), it is reasonable to classify the ROL phytoplasma in the AY 16SrI group, thus distinguishing it from RYD, which belongs to the 16SrXI group (Jung et al. 2002).

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The occurrence of orange leaf phytoplasmas in India has not previously been reported. However, recently the occurrence of phytoplasmas in the Cauvery Delta areas and other rice growing areas were noticed for first time. An extensive survey has been conducted in the Cauvery Delta, Lower Bhavani project Delta, Parambikulam Aliyar Delta, Periyar Vaigai Project Delta and Thamiraparani Delta zones. The plants exhibited typical symptoms with orange yellow discoloured leaves which were distributed sporadically in the field (Fig. 1). The symptoms were suspected to be phytoplasma as testing for Rice Tungro Disease (RTD) with PCR gave negative results and the bacterial blight pathogen could not be isolated. Subsequently we attempted to identify the causal agent by using nested PCR using phytoplasma specific primers.

Leaf samples were collected from four symptomatic plants of each of the rice varieties ADT 43, CO 39 and White Ponni (collected from TNAU farms, Coimbatore district) and BPT 5204 (from the Erode district) and used for the present study. A modified CTAB method (Warokka et al. 2006) was used for the extraction of total DNA from leaf samples of rice for detecting phytoplasmas. Nested-PCR assays with the universal primer pair P1/P7 followed by the universal primer pair R16F2/R2, designed to amplify a portion of the 16S rRNA gene (Lee et al. 1993; Gundersen and Lee 1996) were employed. 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. A total of 35 thermal cycles were carried out which included denaturation for 1 min (2 min for first cycle) at 94 °C, annealing for 2 min at 50 °C and extension for 3 min (10 min in final cycle) at 72 °C. DNA fragments ca. 1.2 kb in size were amplified by nested-PCR (Fig. 2) from DNA extracts of symptomatic leaves from the infected plants but not from the DNA of healthy leaves. The nested-PCR was repeated thrice using the same samples. The DNA fragments were gel purified using a gel extraction kit (Qiagen, New Delhi, India) and cloned in plasmid (pGEMT® vector- Promega). The Plasmid DNA was directly sequenced in both orientations at SciGenom Labs Pvt Ltd, Kerala, India and

Fig. 1 Symptoms of Rice orange leaf (ROL) phytoplasma showing rolling of infected leaves towards the inner side (*left*) and typical orange coloured leaves (*right*)

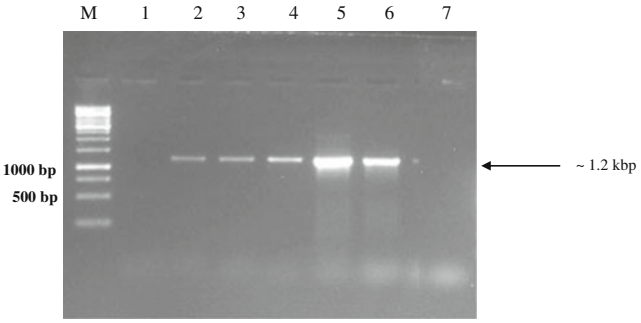

Fig. 2 Detection of phytoplasma through nested PCR using primers - P1/P7 and R16F2n/R16R2. Lanes M - 1 kbp DNA ladder; (1) Negative control (Healthy rice sample); (2) Rice (Erode- BPT 5204): (3) Rice (Coimbatore-White ponni): (4) Rice (Coimbatore- CO 39); (5) Rice (Coimbatore- ADT 43); (6) Positive control (Infected brinjal); (7) Non template control

the subsequent 1,179 bp of the sequence were aligned with Clustal W (Thompson et al. 1994). The NCBI BLAST program was used to scan the sequence data against other phytoplasma 16S rRNA gene sequences. A phylogenetic tree was constructed by the neighbour joining method using the MEGA software version 5 (Saitou and Nei 1987; Tamura et al. 2011).

The 1179-bp nucleotide sequences had 100 % identity among the sequences from the four varieties (JX290545, JX290546, JX290547 and JX290548) and 99 % nucleotide sequence identity with 16S rRNA gene sequences of 16SrI ('*Candidatus* phytoplasma asteris') phytoplasmas infecting the graminae family including rice orange leaf phytoplasma from the

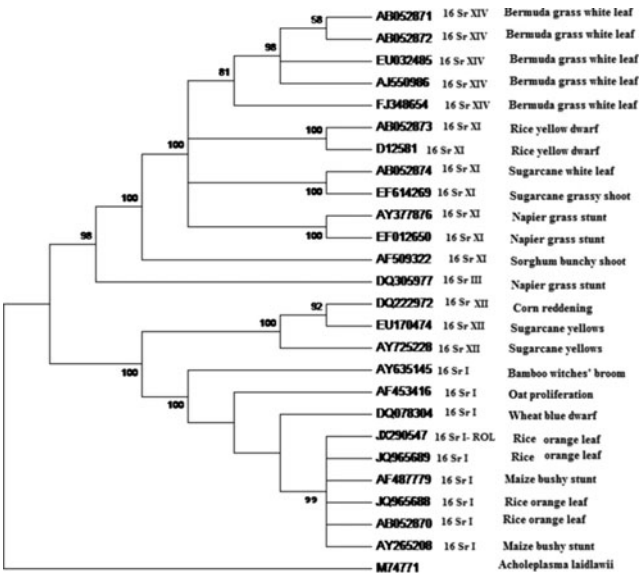

Fig. 3 Dendrogram constructed by the Neighbor-joining method, showing the phylogenetic relationships between the Rice orange leaf phytoplasma with different groups of phytoplasmas constructed based on 16S rRNA sequences. The numbers refer to the GenBank Accession numbers. For the ROL sequences, JX290547 is a sequence from India (this study), AB052870 from the Philippines, and JQ965688 and JQ965689 are from Thailand

Philippines (AB052870), Maize bushy stunt phytoplasma (AY265208), Oat proliferation phytoplasma (AF 453416) and Wheat blue dwarf phytoplasma (DQ078304) (Fig. 3)

There are previous reports of phytoplasmas belonging to the '*Ca*. Phytoplasma asteris' 16SrI group in India including, Crotalaria witches' broom phytoplasma (FJ 185141) (Baiswar et al. 2010), Chrysanthemum little leaf (DQ 431842) (Raj et al. 2007), Chilli phytoplasma (DQ 343288) (Khan and Raj 2006) and Black pepper phytoplasma (FJ 462798) (Adkar-Purushothama et al. 2009). First reports of Sesame phyllody (DQ 431843) and Pigeon pea little leaf disease (DQ 343287) caused by phytoplasmas belonging to the aster yellow group have also been reported from India (Khan et al. 2007; Raj et al. 2006).

The symptom analysis and phylogenetic analysis in this study has confirmed the presence of Rice orange leaf phytoplasma (ROL) in India. As the 16S rDNA sequence similarity is greater than 97.5 %, the ROL phytoplasma clearly belongs to the 16SrI '*Ca.* Phytoplasma asteris' group as ROL-associated phytoplasmas from the Philippines and Thailand. '*Candidatus* Phytoplasma asteris' group phytoplasmas have been reported in many crops in India, but to our knowledge, this is the first report of 16SrI aster yellows group phytoplasma infection of rice (*Oryza sativa* L.,) in India.

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