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First report of 16S rII group phytoplasma "*Candidatus* phytoplasma aurantifolia" associated with little leaf disease of Pigeonpea in India

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Abstract
The phytoplasma disease symptoms was observed on pigeon pea in experimental plots at Regional Agricultural Research Station, Tirupati, Andhra Pradesh, India. The causal agent of the little leaf disease was identified based on symptoms, amplification of 16S rDNA of the phytoplasma by nested PCR with primers P1/P7 and R16F2n/R16R2 and 1,800 bp and 1250 bp size products were amplified in first round PCR and nested-PCR respectively. The 1250 bp PCR product was cloned, sequenced and compared with the reference phytoplasma sequences collected from the database (NCBI). The 16S rDNA sequences of Andhra Pradesh isolate of little leaf of pigeon pea shared the highest nucleotide identity i.e., 98% with blackgram phyllody from Tirupati (KJ540943), which has been identified as 16SrDNA II group of phytoplasma. This is the first report of associate of '*Candidatus* Phytoplasma aurantifolia'of the 16Sr II group identifying pigeon pea from India.

Keywords: 16SrDNA, Pigeon pea, '*Candidatus* Phytoplasma aurantifolia' and Nested PCR

Introduction
The plants infected with phytoplasma exhibit typical symptoms of phyllody (green leaf like structure instead of flowers), proliferation of shoots resulting in sterility of flowers, witches broom, leaf curling, yellowing, phloem necrosis and stunting (Bertaccini, 2009) [2]. Phytoplasmas are associated with plant diseases in several plant species, including important food, vegetable, fruit crops, ornamental plants and timber and shade trees (Bertaccini, 2009) [2]. In India previously the identification of phytoplasma was based on microscopic methods including Transmission Electron Microscopy (TEM), light microscopy and DAPI fluorescence microscopy technique, however in last few years the application of DNA based technology was used for detection of phytoplasma (Rao *et al*, 2011) [10]. The application of PCR to diagnosis of phytoplasma diseases has greatly facilitated the detection and identification of phytoplasmas in different plant species in India. (Rao *et al*, 2011) [10].
Pigeon pea (*Cajanus cajan*) is a protein rich, major cultivated pulse crop in India. The infected pigeon pea plants exhibit the phytoplasma disease symptoms like little leaf and stunted growth, bushy appearance. In India, the little leaf of pigeon pea was first reported during 2006 in an experimental field at NBRI, Lucknow and the disease was confirmed to be caused by phytoplasma of the 16S rI group by sequencing of 16S rDNA (Raj *et al*. 2006) [8].

Materials and Methods
Leaf samples of phytoplasma infected pigeonpea plants showing typical symptoms of little leaf and bushy appearance and healthy pigeonpea plants used as a control were collected from experimental plots of Regional Agricultural Research Station, Tirupati, Andhra Pradesh, India during 2014. Nucleic acids were isolated from infected and healthy leaf samples by using modified CTAB method (Murray and Thomson, 1980) [7]. The isolated DNA samples were stored at -20 ^{0}C for further use.
The total isolated DNA used as a template in first round PCR for amplification with P1/P7 primers (Deng and Hiruki. 1991; Smart *et al*.1996) [3, 11] followed by nested PCR with phytoplasma specific primers R16F2n/R16R2 (Gundersen and Lee.1996) [5]. The first round PCR and nested PCR were carried out sequentially in a final volume of 25 µl reactions containing 2.5 µl of (10X) PCR buffer, 2.0 µl (25 mM) $MgCl_2$, 0.5 µl (10 mM each) dNTPs, 1.0 µl (10 µM) each primers, 0.2 µl Taq DNA polymerase (5 u/ µl), and 2 µl template DNA (50 ng/ µl). The DNA was amplified by an initial denaturation of 94°C for 4 min followed by 35 cycles of 94 °C for 30 seconds, 56 °C for 1 min (55 °C for 1 min for nested PCR), 72 °C for 2 min and final extension at 72 °C for 10 min. The PCR products were analysed by

Electrophoresis in 1% (w/v) agarose gel. The DNA fragments in the gel were recorded in gel documentation system. The PCR amplified 1250bp DNA from gel slices was extracted using Gene JET Gel Extraction kit (Thermo scientific) as per the manufacturer's protocol. The eluted 1250bp product was cloned into a pTZ57R/T vector and sequenced.

Results and Discussion

Isolation of total DNA and amplification by nested PCR

The phytoplasma infected pigeon pea plants showing little leaf, stunted growth and bushy appearance (Fig.1) were collected from experimental plots of Regional Agricultural Research Station, Tirupati, Andhra Pradesh, India. DNA was isolated from phytoplasma infected pigeon pea leaves by CTAB method. The amount of DNA and purity of DNA (260/280 ratio) was measured in Nanodrop spectrophotometer. This DNA used as template in nested PCR with universal primers P1/P7 and R16F2n/R16R2.

Cloning and sequencing of phytoplasma 16S rDNA

16S rDNA from pigeon pea samples collected from experimental plots of Regional Agricultural Research Station, Tirupati were amplified by PCR using 16S rDNA specific primers R16F2n/R16R2 and obtained 1250 bp product in all isolates (Fig.2). The 1250bp product was eluted from agarose gel was cloned into a pTZ57R/T vector and sequenced and the sequence was submitted to GenBank (KP271167).

In this study, based on 16S rDNA sequences, it was shown that the little leaf disease of pigeon pea from Andhra Pradesh was caused by phytoplasma. The sequence obtained in this study was compared with those of known phytoplasmas in the database (NCBI) and found to be 98% similar to the members of the 16S rII group, *Candidatus* Phytoplasma aurantifolia, that contains phytoplasmas associated with black gram phyllody from Andhra Pradesh (KJ540943). Phylogenetic analysis (Fig. 3) using MEGA version 7.0 evidenced that the little leaf disease of pigeonpea from Andhra Pradesh is closely related to phytoplasmas associated with *Candidatus* Phytoplasma aurantifolia.

Raj *et al*. (2006) [8] identified causal agent of little leaf disease of pigeon pea as '*Candidatus* Phytoplasma asteris' based on 16S rDNA sequence data and sequence shows the 99%

Similar to the members of the 16S rI group and Rao *et al.* (2017) [9] identified causal agent of pigeonpea little leaf is '*Candidatus* Phytoplasma phoenicium' (16SrIX-C) but in our investigation the association of little leaf of pigeon pea in Andhra Pradesh shows 98% similar to the members of the 16S rII group '*Candidatus* Phytoplasma aurantifolia'. To our knowledge,this is the first report of a phytoplasma of the 16SrII-group associated with little leaf disease of pigeon pea from India.

Fig 1: A. Phytoplasma infected pigeon pea plant

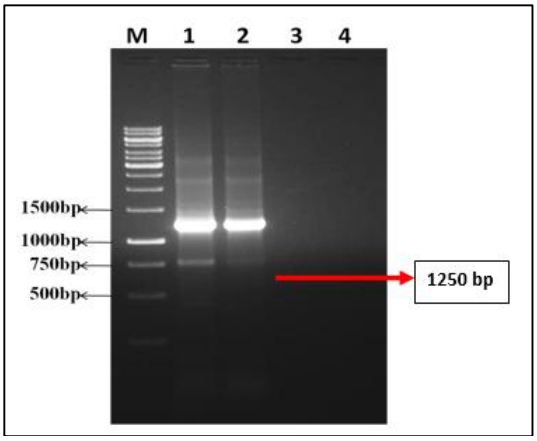

Fig 2: Amplification of phytoplasma 16S rRNA gene with R16-F2n/R16R2 from infected redgram samples. Lanes: M. 1 Kb DNA ladder, 1 & 2 infected redgram samples, 3 & 4 healthy redgram.

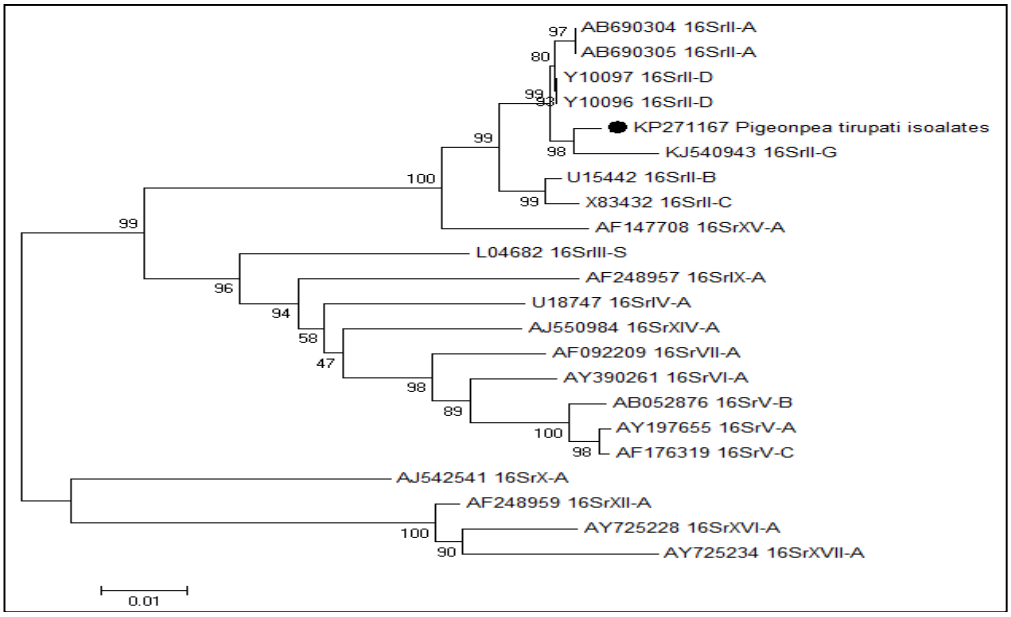

Fig 3: Phylogenetic tree showing the genetic relationship of AP little leaf disease of pigeon pea to other phytoplasmas based on 16S rDNA sequences

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