Int.J.Curr.Microbiol.App.Sci (2018) *7*(7): 171-176

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 07 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.707.021

Natural Occurrence of Phytoplasma Associated with Chickpea Phyllody in Andhra Pradesh, India

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Keywords

16SrDNA, Chickpea, *Candidatus Phytoplasma aurantifolia* and Nested PCR

Article Info

Accepted:
04 June 2018
Available Online:
10 July 2018

A B S T R A C T

Phytoplasma disease symptoms were observed on chickpea growing fields in Kurnool district, Andhra Pradesh, India. The symptoms included phyllody, pale green leaves, bushy appearance and excessive axillary proliferation. The causal agent of the phyllody 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 1,200 bp size products were amplified in first round PCR and nested-PCR respectively. The PCR product was cloned, sequenced and compared with the reference phytoplasma sequences collected from the database (NCBI). 16S rDNA sequences of Andhra Pradesh chickpea phyllody phytoplasma shared the highest nucleotide identity i.e., 98% with Sesame phyllody phytoplasma 16SrII-D (KP297862), this is the first report of a '*Candidatus Phytoplasma aurantifolia'* of the 16SrII-D group infecting chickpea from Andhra Pradesh.

Introduction

Chickpea (*Cicer arietinum* L.) is the world's third important legume crop after French bean (*Phaseolus vulgaris* L.) and peas (*Pisum sativum* L.) and ranks fifteenth among the top grain crops in the world. It is grown in tropical, subtropical and temperate regions (Anon. 2009). Chickpea is a highly nutritious pulse and cultivated throughout the world. In India, the chickpea phyllody was first reported during 1988 in Tamil Nadu and was confirmed by electron microscopy (Ghanekar *et al.,* 1988). The phytoplasma is mainly transmitted by leaf hopper; the vector leaf hopper has a wide host range in addition to chickpea (Akhtar *et al.,* 2009). The plants infected with phytoplasma exhibit the symptoms are phyllody (green leaf like structure instead of flowers), proliferation of shoots resulting in sterility of flowers, witches broom symptoms, leaf curling, yellowing,

phloem necrosis and stunting (Bertaccini, 2009). Phytoplasmas are associated with plant diseases in several plant species, including important fooed, vegetable, fruit crops, ornamental plants and timber and shade trees (Bertaccini, 2009). 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 allowed for detection of phytoplasma (Rao *et al*, 2011). 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).

The chickpea phyllody was also reported from Australia and Pakistan and the disease was confirmed to be caused by phytoplasma of the 16S rII group by sequencing of 16S rDNA (Saqib *et al.,* 2006: Akhtar *et al.,* 2008).

Materials and Methods

Leaf samples of phytoplasma infected chickpea plants showing typical symptoms of phyllody and healthy chickpea plants used as a control were collected from farmer chickpea field at Kurnool district, Andhra Pradesh during 2014. Nucleic acids were isolated from infected and healthy leaf samples by using modified CTAB method (Murray and Thomson, 1980). 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) followed by nested PCR was done using 2 µl of diluted standard PCR product with phytoplasma specific primers R16F2n/R16R2 (Gundersen and Lee.1996). 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 denaturation, 56°C for 1 min primer annealing (55°C for 1 min for nested PCR), 72°C for 2 min primer extension 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 using gel documentation system. The PCR amplified 1250bp DNA from gel slices was extracted using the ultra clean gel kit as per the manufacturer's protocol.

Results and Discussion

Isolation of total DNA and amplification by nested PCR

The phytoplasma infected chickpea samples show phyllody and production of little leaves (Fig. 1). These samples were collected from Kurnool district of Andhra Pradesh. DNA was isolated from phytoplasma infected chickpea samples 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 chickpea samples were collected from Kurnool district were amplified by PCR using 16S rDNA specific primers R16F2n/R16R2 and obtained 1250 bp product in all isolates (Fig. 2).

Fig.1 A. Phytoplasma infected chickpea plant, B. Healthy chickpea plant

Fig.2 Amplification of phytoplasma 16S rDNA by nested PCR using the phytoplasma specific primers R16F2n/R16R2 from infected plants. Lanes: M- gene 1kb ruler (0313) 1 and 2- phytoplasma infected chickpea 3 and 4- healthy chickpea

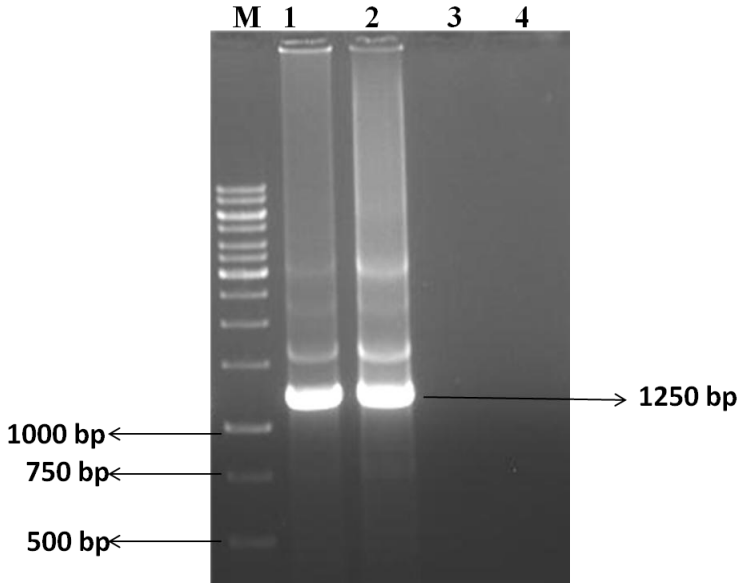
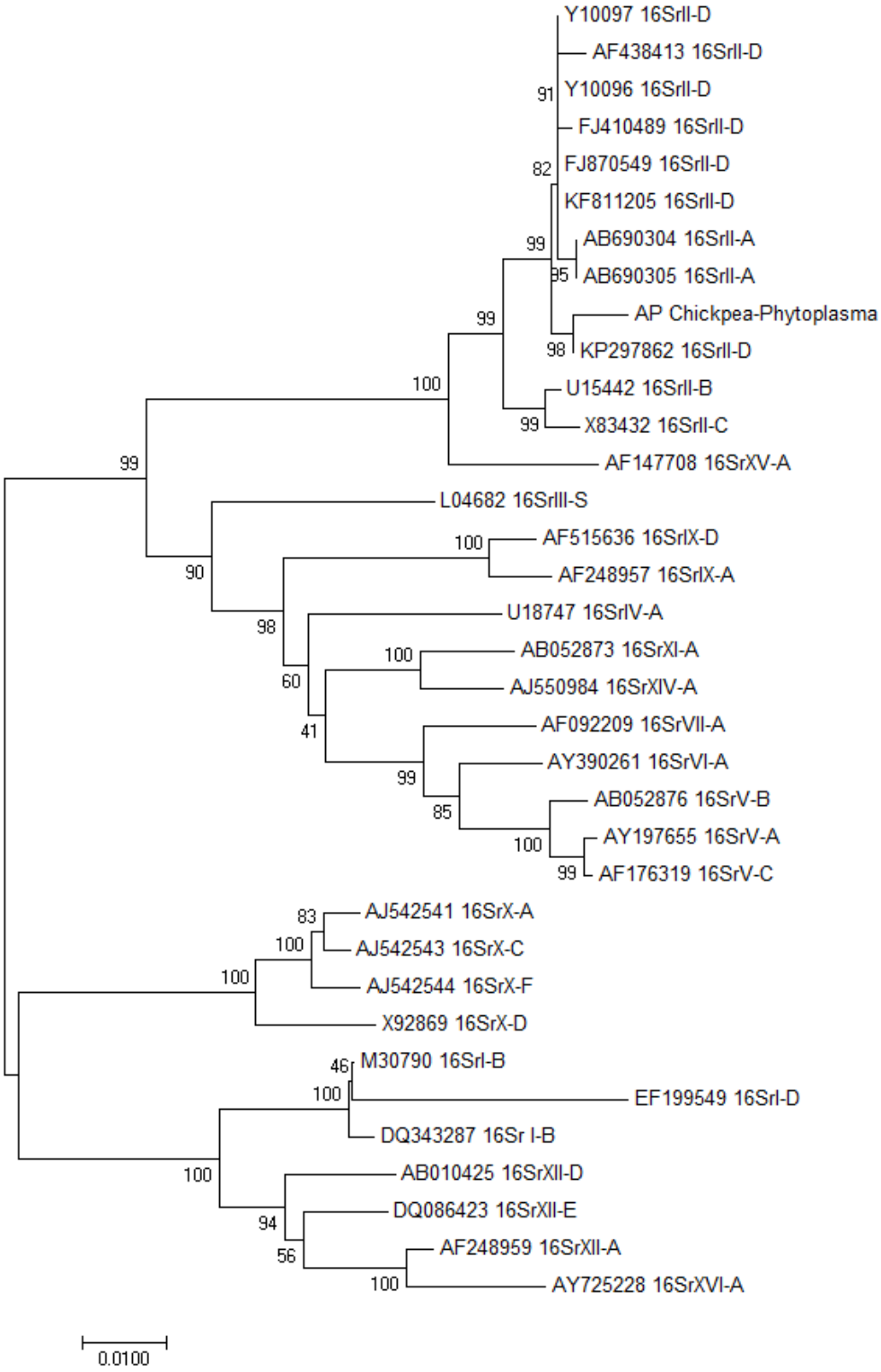


Fig.3 Phylogenetic tree showing the genetic relationship of AP chickpea phyllody phytoplasma to other phytoplasmas based on 16S rDNA sequences



The 1250bp product was eluted from agarose gel was cloned into a pTZ57R/T vector (Fermentas, USA) and sequenced and the sequence was submitted to GenBank (KP899064)

In this study, based on 16S rDNA sequences, it was shown that the chickpea phyllody 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 sesame phyllody from Oman. Phylogenetic analysis (Fig. 3) using MEGA version 7.0 evidenced that the AP chickpea phyllody is closely related to phytoplasmas associated with *Candidatus Phytoplasma aurantifolia*. This is the first report of a phytoplasma of the 16SrII-group infecting chickpea from Andhra Pradesh.

Pallavi *et al.,* (2012) eluted causal agent of chickpea phyllody is '*Candidatus Phytoplasma aurantifolia*' based on 16S rDNA sequence >98% similar to the members of the 16S rII group and Akthar *et al.,* (2008) identified casual agent of chickpea phyllody is belongs to the group of 16Sr II phytoplasma. Saady *et al.,* (2006) in Oman and Saqib *et al.,* (2005) in Australia identified phytoplasma associated with chickpea is a member of 16 Sr II group.

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How to cite this article:

Vijay Kumar Naik, D., B.V. Bhaskara Reddy, J. Sailaja Rani, R. Sarada Jayalakshmi Devi and Hari Prasad, K.V. 2018. Natural Occurrence of Phytoplasma Associated with Chickpea Phyllody in Andhra Pradesh, India. *Int.J.Curr.Microbiol.App.Sci.* 7(07): 171-176.
doi: https://doi.org/10.20546/ijcmas.2018.707.021