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Research Article

Detection and characterization of 16SrI-B phytoplasmas associated with yellow leaf disease of arecanut palm in India

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Abstract

During a survey on arecanut palms in Sullia district of Karnataka, yellowing of leaves and shedding of both mature and immature fruits were observed in June 2013. The disease association with phytoplasmas was confirmed using nested polymerase chain reaction with phytoplasma specific ribosomal primers, which amplified the 16*S rRNA* gene in leaves, roots and rachis tissues of symptomatic arecanut palms. BLAST analysis of ~1.2kb sequence of amplicon from symptomatic leaf samples revealed 99% sequence identity with 16S rDNA sequences of '*Candidatus* Phytoplasma asteris' (16SrI). Phylogenetic analysis based on 16S rDNA also confirmed the clustering of areacanut yellow leaf disease phytoplasma with members of 16SrI group. Real and virtual RFLP analysis suggested that the arecanut phytoplasmas in the present study belongs to 16SrI-B subgroup. The identification of 16SrI-B subgroup phytoplasma in yellow leaf disease of arecanut is the first report from India.

Keywords: '*Candidatus* Phytoplasma asteris', 16Srl-B, arecanut

Introduction

Arecanut (*Areca catechu L.*), commonly known as betel nut, is a tropical palm cultivated for its nuts that are chewed as a masticator by about 400 million people around the world. The nut also has various medicinal uses. Today, the major arecanut growing countries are India, Sri Lanka, Bangladesh, Malaysia, Indonesia and the Philippines, with India leading the world production. In India the cultivation of arecanut is mostly confined to states of Karnataka, Kerala and Assam. It is also cultivated to a small extent in states of Tamil Nadu, West Bengal, Maharashtra, Andhra Pradesh, Meghalaya, Goa, Tripura, Puduchery, Mizoram, Andaman and Nicobar Islands. The area under cultivation and production in states of Karnataka, Kerala and Assam covers around 83% of the total Indian production. Karnataka stands first both in terms of area and production followed by Kerala and Assam (Ramappa, 2013). Much of the arecanut growing areas in Kerala and Karnataka are located in the humid tropics of Western Ghats with high rainfall and hilly topography. Palms in most of these areas suffer from a disease called "yellowing in arecanut" (Nampoorhiri, 2000) also named yellow leaf disease (YLD) that is one of the most important diseases of arecanut (Kurian and Peter, 2007). Earliest report of yellow leaf disease in India is traced back to 1914. The most important symptoms of the disease are yellowing of leaves, reduction in crown size, premature shedding of female flowers accompanied with deterioration of fruit quality (Nayar and Seliskar, 1978; Rawther, 1982; Manimekalai *et al.*, 2013). It reduces the yield as much as 50% over a period of 3

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years after its appearance (Nampoothiri, 2000). Phytoplasma association with YLD of arecanut palms in India has been confirmed based on electron microscopy, dodder transmission, PCR assays and ribosomal gene sequence (Nayar and Seliskar, 1978; Kochubabu *et al*., 2004; Purushothama *et al*., 2007; Manimekalai *et al*., 2010; 2013). Manimekalai *et al*. (2010) identified and characterized 16SrXI group phytoplasmas associated with root wilt disease in India, and Mehdi *et al*. (2012) reported the association of 16SrI-B subgroup phytoplasmas with oil palm stunting disease from Godavari district of Andhra Pradesh, India. However, 16SrI-B phytoplasmas were also associated with YLD of arecanut from Hainan Province of China (Zhou *et al*., 2010). Hence, in the present work, investigation on possibility of association of different groups and subgroups of phytoplasmas with YLD of arecanut in Karnataka, India was carried out.

Materials and Method

During survey in the month of June 2013, yellowing of crown leaves of arecanut palms was observed in Sullia district of Karnataka. Samples of leaves, rachis and roots were collected from symptomatic and asymptomatic arecanut palms showing characteristic leaf yellowing symptoms and kept at -80ºC for molecular analysis for phytoplasma detection. DNA extraction was performed by a CTAB method (Ahrens and Seemüller, 1992) utilizing one gram of arecanut palm plant tissues of leaf, root and rachis from both symptomatic and non symptomatic plants. Amplification of phytoplasma ribosomal DNA (rDNA) was done with the universal phytoplasma primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by nested primers, R16F2n/R16R2 (Gundersen and Lee, 1996). The DNAs extracted from leaf, rachis and root tissues of non symptomatic arecanut palms were used as negative controls. DNA extracted from leaves of sugarcane infected with sugarcane grassy shoot phytoplasma belonging to 16Sr XI group and maintained in greenhouse was used as positive control (Rao *et al*., 2014).

PCR reactions were performed in a Minicycler (Eppendorf) and the cycling protocol used for the direct and nested PCR assays was as reported by Rao *et al*. (2014). Ten microlitres of each PCR product was subjected to electrophoresis in a 1.0% (w/v) agarose gel, stained with ethidium bromide and observed under UV transilluminator. A P1/P7 PCR amplified product from symptomatic arecanut (~1.8 kb) was purified using the Wizard^{R} SV Gel and PCR Clean-up System (Promega) and was directly sequenced in both directions. The 16S rDNA sequences were aligned using CLUSTAL W method of Bio-Edit software (Bio-edit Sequence Align Editor). Aligned consensus sequence was used in BLAST search. The sequence generated from the present study and 25 reference phytoplasma strain sequences retrieved from GenBank were used to construct phylogeny through MEGA 5.0 version software (Tamura *et al*., 2011) employing the neighbor joining method with default values and 1,000 replications for bootstrap analysis.

The ~1.2 kb 16S rDNA sequence of YLD phytoplasma was subjected to *in silico* restriction and virtual gel plotting using *iphy*classifier online tool (Zhao *et al*., 2009). In virtual gel plotting with restriction endonucleases *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau3*AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I profiles were obtained and compared with the virtual RFLP gel from a reference phytoplasma of 16SrI subgroup (GenBank accession number M30790, Lee *et al*., 2004). Actual restriction fragment length polymorphism (RFLP) analysis of the ~1.2 kb nested PCR product was performed with five endonucleases *BamH*I, *Bfa*I, *Hae*III, *Hha*I and *Taq*I (Fermentas, USA) and compared with RFLP profiles reported in literature (Lee *et al*., 1998). Restriction fragments were separated by electrophoresis through 2.8% agarose gels in 1 X TAE buffer, stained with ethidium bromide and visualized under UV illumination.

Results and Discussion

During the survey yellowing of leaves and shedding of both mature and immature fruits were observed. The yellowing started from the tips of the leaflets to the outer leaves gradually extending to the middle of the lamina. Blackening of the kernel of the YLD affected arecanut palms was also observed (Figure 1). The affected leaves often developed necrosis from their tips. In advanced stages, the leaves were reduced in size and become stiff and pointed. The crown size gets reduced and ultimately falls off. Tips and absorbing regions of young roots become dark and gradually rotted.

PCR amplification of ~1.8 kb DNA bands was only observed with symptomatic arecanut leaf samples in direct PCR assay along with positive control (data not shown). However, 1.2 kb amplicons were obtained

Figure 1. YLD affected arecanut palm: (a) showing initial of yellowing of crown leaves, (b) close up of yellowing and drying of the crown leaves, (c: inset) blackening of the kernel of the YLD affected arecanut.

with all the leaf, root and rachis tissues from symptomatic arecanut palms along with positive control in nested PCR assays with R16F2n/R16R2 primers (Figure 2). No DNA was amplified from the template DNA isolated from the non symptomatic leaf, root and rachis tissues.

The 1247 bp sequence obtained from the sequencing of one of the direct PCR products from symptomatic areacanut leaf tissues was submitted to GenBank with accession number KF728948. BLASTn analysis of arecanut YLD phytoplasma *16SrRNA* gene showed 100% sequence identity with the same sequences of sesame phyllody (JF706215) from India,

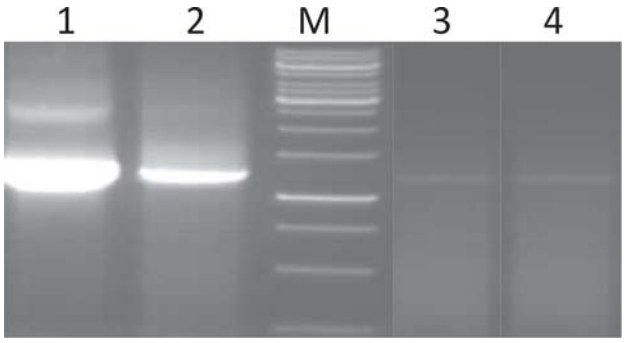

Figure 2. Agarose gel showing PCR results from symptomatic arecanut samples amplified with R16R2n/R16F2 primer pair: lane 1: positive control (sugarcane grassy shoot phytoplasma); lane 2: arecanut leaf; lane M: 1 kb DNA Ladder; lane 3: arecanut root; lane 4: arecanut rachis.

and 99% identity with other phytoplasmas strains of 16SrI group, such as onion yellows (AP006628) from Japan, *Zanthoxylum schinifolium* aster yellows (KC354961), mulberry dwarf (AB693124) and bitter gourd little leaf (AB74163) from South Korea, coneflower phyllody (EU333394) from USA, *Lactuca sativa* leaf rot (KJ668578) from China and aster yellows (KC312703) from India.

The *16SrRNA* gene-based phylogenetic relationships of the areacanut YLD phytoplasma revealed that it clustered with phytoplasmas associated with the arecanut yellow leaf (FJ694685; FJ998269) and mulberry yellow dwarf (GQ249410) from China, rice orange leaf (JQ965688) from Thailand, aster yellows (AY265206) and blueberry stunt (AY265213) from USA, lethal wilt of oil palm (JX681021) from Colombia and sesame phyllody (JF706215) from India, all belonging to 16SrI group of phytoplasmas (Figure 3).

Virtual analysis of R16F2n/R2 sequence of YLD phytoplasma confirmed that it was referable to the phytoplasma of 16SrI subgroup B (M30790, Lee *et al.*, 2004) and identified it as a member of 16SrI-B subgroup phytoplasmas (Figure 4). In actual restriction digestion of the YLD phytoplasma with *Bam*HI, *Bfa*I, *Hae*III, *Hha*I and *Taq*I, the banding (Figure 5) was identical to virtual RFLPs of 16SrI-B subgroup phytoplasmas confirming the 16SrI-B phytoplasma presence in arecanut YLD in Karnataka India.

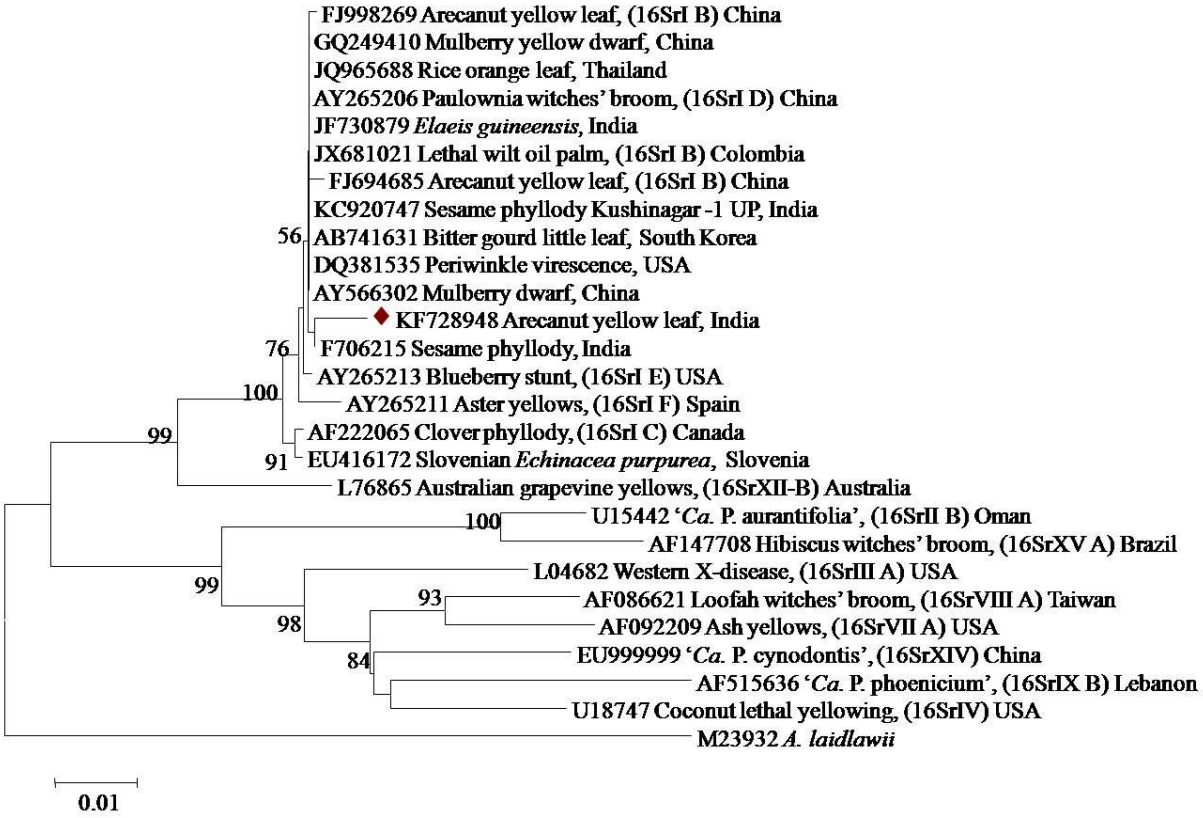

Figure 3. Phylogenetic tree built by the neighbour-joining method using MEGA 5.0 software using 16S rDNA sequences and showing phylogenetic relationships of the Indian arecanut YLD phytoplasma with other phytoplasmas using *Acholeplasma laidlawii* as outgroup.

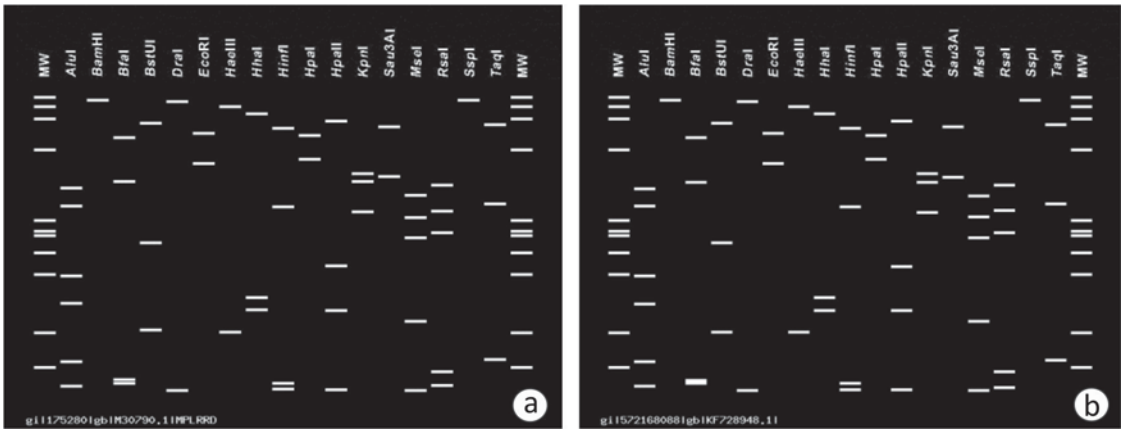

Figure 4. Comparison of virtual RFLP patterns derived from *in silico* digestions of 1.2 kb 16S rDNA sequences of (a) '*Ca.* P. asteris' (M30790) and (b) arecanut YLD phytoplasma (KF728948) indicating that the arecanut YLD belongs to 16SrI-B phytoplasma subgroup.

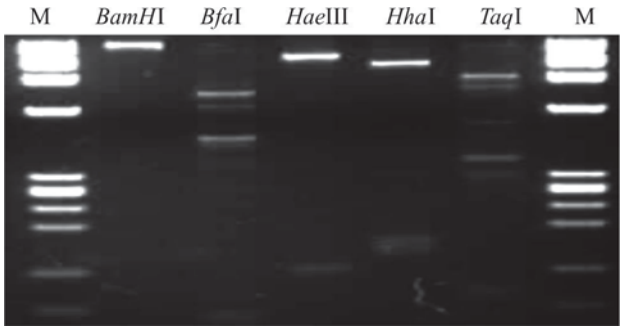

Figure 5. RFLP profiles of the amplicon obtained from nested PCR with primers P1/P7 and R16R2n/R16F2 of arecanut YLD phytoplasma, M, ϕX174 DNA-*Hae*III digested marker.

Discussion

YLD of arecanut is a major production constraint faced by arecanut farmers in south India, especially in the states of Kerala and Karnataka. Detection of phytoplasmas associated with YLD symptomatic arecanut trees in the same area has been reported earlier in India through *16S rRNA* and *secA* gene sequences and RFLP comparison and indicated the presence of RYD- and BGWL group phytoplasmas (16SrXI-B) (Manimekalai *et al.*, 2011; 2013). On the other hands the arecanut YLD phytoplasma reported from China (FJ998269 and FJ694685) belongs to group 16SrI-B (Zhou *et al.*, 2010) and its 16S rDNA sequence shares 99% nucleotide identity with the sequence of this YLD phytoplasma from India. Mehdi *et al.* (2012) reported association of a 16SrI-B subgroup phytoplasma with stunting disease of oil palm in India. The results in this study confirmed the occurrence of 16SrI-B subgroup phytoplasmas in YLD of arecanut palms in India. These findings support the conclusion that the YLD in India is associated with phytoplasmas belonging to different groups related to '*Candidatus* Phytoplasma asteris' and '*Ca.* P. oryzae'.

So far in India only few hosts like periwinkle (Kumar and Byadgi, 2012), okra (Kumar *et al.*, 2012), sugarcane (Kumar *et al.*, 2014) and *Portulaca oleracea* (Chaube *et al.*, 2014) have been identified up to subgroup B level under the group 16SrI. The present study reports arecanut palm as a new host of this subgroup based on ribosomal DNA sequence analysis and RFLP profile patterns. This study represents the first evidence of association of phytoplasmas related to '*Ca.* P. asteris' belonging to 16SrI-B with YLD of arecanut palms.

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