

# Phylogenetic analysis identifies a '*Candidatus Phytoplasma oryzae*'-related strain associated with yellow leaf disease of areca palm (*Areca catechu* L.) in India

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Yellow leaf disease (YLD) with phytoplasmal aetiology is a serious disease of arecanut palm in India. The present study was undertaken to characterize the 16S rRNA and *secA* gene sequences of the Indian arecanut YLD phytoplasma for '*Candidatus Phytoplasma*' species assignment and 16Sr group/subgroup classification. Phytoplasma 16S rRNA genes were amplified using three sets of semi-nested/nested primers, 1F7/7R3–1F7/7R2, 4Fwd/3Rev–4Fwd/5Rev and P1/P7–R16F2n/R16R2, producing amplicons of 491, 1150 and 1250 bp, respectively, from diseased samples. The amplicons were cloned and sequenced. A BLAST search showed that the sequences had 99% similarity with sugar cane white leaf phytoplasma (16SrXI) and Napier grass stunt phytoplasma (16SrXI). Phylogenetic analysis based on the 16S rRNA gene revealed the clustering of YLD phytoplasma with the rice yellow dwarf and Bermuda grass white leaf groups. The YLD phytoplasma F2nR2 sequence shared 97.5% identity with that of '*Candidatus Phytoplasma oryzae*' and 97.8% identity with that of '*Candidatus Phytoplasma cynodontis*'. Hence, for finer differentiation, we examined the *secA* gene-based phylogeny, where the YLD phytoplasma clustered with Napier grass stunt and sugar cane grassy shoot phytoplasmas, both belonging to the rice yellow dwarf group. Hence, we are assigning the Indian arecanut YLD phytoplasma as a '*Candidatus Phytoplasma oryzae*'-related strain. Virtual RFLP analysis of a 1.2 kb fragment of the 16S rRNA gene (F2nR2 region) identified the Indian arecanut YLD phytoplasma as a member of 16SrXI-B subgroup. We name the phytoplasma Indian yellow leaf disease phytoplasma, to differentiate it from the Hainan YLD phytoplasma, which belongs to group 16Srl.

## INTRODUCTION

Arecanut palm or betel nut palm (*Areca catechu* L.) is an economically important crop that provides a livelihood to millions of farmers throughout the Old World tropics. Believed to have originated in Malaysia or the Philippines, this palm species is grown extensively in much of the tropical Pacific, Asia and East Africa largely for its fruit, which is used widely for masticatory and religious purposes. Numerous diseases are known to affect arecanut production in India; however, yellow leaf disease (YLD), a

**Abbreviations:** BGWL, Bermuda grass white leaf; NGS, Napier grass stunt; RYD, rice yellow dwarf; RWD, root wilt disease; SCGS, sugar cane grassy shoot; SCWL, sugar cane white leaf; YLD, yellow leaf disease.

The GenBank/EMBL/DDBJ accession numbers for partial 16S rRNA gene sequences of arecanut YLD phytoplasma strains are GU552782, HM215624 and JN967909. The accession number for the partial *secA* sequence of strain Sullia 1 is JX394029.

Two supplementary figures are available with the online version of this paper.

disease largely restricted to the states of Kerala and Karnataka, is widely regarded as the most serious threat to production of this crop (Kurian & Peter, 2007).

Symptoms of YLD begin with a discoloration (yellowing) of leaflet tips on the lowermost (oldest) leaves. Yellowing gradually intensifies to encompass entire leaflets except for portions next to the midrib, which remain green, and progresses to involve successively younger leaves in the mid-crown. As symptoms advance, new leaves that emerge are stunted, leading to a marked reduction in crown size and a tapering of crown girth (Nayar & Seliskar, 1978). Premature shedding of female flowers, buttons and immature fruits accompanies foliar yellowing, resulting in diminished yield (as much as 50%) and quality of fruit within 3 years (Rawther, 1982). There is blackish discoloration of the endosperm of mature and immature nuts, making the nuts unsuitable for human consumption (Fig. 1).

Electron microscope examination of tissues from diseased palms revealed the association of a phytoplasma with the



**Fig. 1.** Arecanut palm showing symptoms of YLD (left) and healthy arecanut palm (right). Endosperm of the diseased nuts (inset) has a blackish appearance.

disease (Nayar & Seliskar, 1978). Kochu Babu *et al.* (2004) obtained positive transmission of phytoplasma from YLD areca palms to oil palm using dodder laurel, *Cassytha filiformis* L. Manimekalai *et al.* (2010b) detected phytoplasma from YLD-symptomatic areca samples using a nested PCR approach. Phytoplasmas are unculturable, wall-less, non-helical prokaryotes that colonize plant phloem and insect vectors. They are characterized and classified based on their 16S rRNA gene sequences.

The IRPCM Phytoplasma/Spiroplasma working team proposed the candidate genus '*Candidatus Phytoplasma*' to accommodate phytoplasmas. Within this genus, candidate species are identified if organisms share <97.5% similarity among their 16S rRNA gene sequences (IRPCM, 2004). The RFLP profile of the phytoplasma 16S rRNA gene amplified with primers R16F2n/R16R2 forms the basis of phytoplasma group/subgroup classification. Lee *et al.* (1998) differentiated 34 representative phytoplasma strains into 14 major groups and 32 subgroups based on similarity coefficient values of RFLP patterns. This widely accepted classification scheme has identified more groups and subgroups of phytoplasma. Wei *et al.* (2007) introduced computer-simulated restriction digestions to generate virtual RFLP patterns for high-throughput identification and classification of diverse phytoplasmas. Since the 16S rRNA gene is highly conserved, additional genes are being used for better resolution of phytoplasma groups and subgroups. Marcone *et al.* (2000) classified the aster yellows-group phytoplasmas based on combined analyses of 16S rRNA and *tuf* gene sequences, while Lee *et al.* (2006) used *secY* gene sequences for finer differentiation of strains in the aster yellows phytoplasma group. Martini *et al.* (2007) reported congruence between ribosomal protein gene-based and 16S rRNA gene-based phylogenies for finer differentiation and classification of phytoplasmas. Hodgetts *et al.* (2008) used the

less-well-conserved *secA* gene as a phylogenetic parameter to produce an alternative phylogenetic analysis of the phytoplasmas. Mehdi *et al.* (2012) characterized the phytoplasma associated with oil palm stunting disease based on sequence analysis of 16S rRNA and *secA* genes. The present work was designed to characterize the 16S rRNA and *secA* genes of the Indian arecanut YLD phytoplasma for '*Ca. Phytoplasma*' species assignment and 16Sr group/subgroup identification.

## METHODS

**Plant material.** Spindle leaf samples from 15 YLD-symptomatic palms, five each at the early, middle and advanced stages of infection, were collected from Sullia district, Karnataka, India. A healthy sample collected from Kasaragod was used as the negative control.

**Nucleic acid preparation and PCR amplification.** DNA was extracted from 3 g fresh tissue sample using a modified phytoplasma enrichment protocol (Ahrens & Seemuller, 1992) and dissolved in TE buffer (pH 8.0). The DNA concentration was measured in a spectrophotometer and purity was checked on a 0.8% agarose gel. DNA preparations were diluted to 25 ng  $\mu\text{l}^{-1}$  for PCR analysis. Two sets of phytoplasma 16S rRNA gene-specific semi-nested primers, 1F7 (5'-ACGCTTAACGTTGCTCCTGCTA)/7R3 (5'-TTGTAGCCAGAT-CATAAGGGGCA)-1F7/7R2 (5'-GACAAGGGTTGCGCTCGTTTT) and 4Fwd (5'-ACCCCGAGAACGTATTCACCGCGA)/3Rev (5'-TA-AAGAGGGGCTGCGTCACA)-4Fwd/5Rev (5'-AAAGGAGGTGAT-CCATCCCCACCT), were designed for PCR amplification of phytoplasmal DNA. The phytoplasma universal primers P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995) and the nested primers R16F2n/R16R2 (Gundersen & Lee., 1996) were also used in this study. PCR assays were performed in 15  $\mu\text{l}$  volumes containing 50 ng DNA template, 0.2  $\mu\text{M}$  each primer, 150  $\mu\text{M}$  each dNTP, 0.5 U *Taq* DNA polymerase (Bangalore Genei) and 1  $\times$  PCR buffer with 1.5 mM  $\text{MgCl}_2$ . First-round amplification with primers P1/P7, 4Fwd/3Rev and 1F7/7R3 was performed for 35 cycles in a MyCycler thermocycler (Bio-Rad) under the following conditions: initial denaturation at 95  $^{\circ}\text{C}$  for 2 min followed by 35 cycles of 1 min denaturation at 94  $^{\circ}\text{C}$ , 1 min annealing at 60  $^{\circ}\text{C}$  (55  $^{\circ}\text{C}$  for P1/P7) and 1.5 min primer

extension at 72 °C followed by a final extension at 72 °C for 10 min. The products of the first PCR were diluted 1:4 with sterile water and 2 µl of each dilution was used as template for 35 cycles of PCR with nested primer pair R16F2n/R16R2, 4Fwd/5Rev or 1F7/7R2. DNA extracted from grassy shoot-diseased sugar cane was used as a positive control, while the negative control contained sterile water in place of the test DNA.

Semi-nested primers *cocsf* (5'-GACGAAGGAAGAACGCCTTAAT)/*cocsr* (5'-TAGCAGTTCCTGTCATGCCTG)-*cocsnf* (5'-TATATGG-ATGCTAATCGTTTTGC)/*cocsr* were designed based on available *secA* gene sequences of Napier grass stunt (NGS) phytoplasma (GenBank accession no. EU168750) and Malaysia Bermuda grass white leaf (BGWL) phytoplasma (FJ755004) using the Fast PCR software. The *secA* gene of the Indian arecanut YLD phytoplasma was amplified using these primers. The PCR conditions followed were as mentioned above; the annealing temperature was 50 °C. The final PCR products were analysed in 1% agarose gel in 1 × TBE buffer (90 mM Tris/borate, 2 mM EDTA, pH 8.0) containing ethidium bromide. The gels were documented using an image analyser (Gene Genius; Syngene).

**Cloning and sequencing.** PCR fragments of the expected sizes were excised from the gel, purified using a Qiagen gel extraction kit, cloned into pTZ57R/T vector (MBI Fermentas Inc.) and sequenced with M13 forward and reverse primers. Vector sequences were removed from the target nucleotide sequences using VecScreen (Altschul *et al.*, 1997) and trimmed sequences were subjected to similarity searches using the local alignment search algorithm BLASTN (Altschul *et al.*, 1997).

**Phylogenetic analyses.** Phylogenetic analysis of the R16F2n/R16R2 amplification product from the 16S rRNA gene and the *cocsnf/cocsr* amplification product from the *secA* gene was employed for 'Ca. Phytoplasma' species assignment. 16S rRNA gene sequences from 31 strains of 'Ca. Phytoplasma' were retrieved from GenBank, trimmed to correspond to the F2nR2 region and aligned using CLUSTAL W. Similarly, the *secA* gene sequences of 42 other phytoplasma strains were retrieved from GenBank, trimmed and aligned. Phylogenetic trees were reconstructed using the neighbour-joining method with MEGA software version 4 (Tamura *et al.*, 2007). *Acholeplasma laidlawii* JA1 (16S rRNA gene) and *Bacillus subtilis* 168 (*secA*) were taken as the outgroup to root the phylogenetic trees. Bootstrapping was performed 1000 times.

**16Sr group/subgroup assignment.** 16Sr group/subgroup classification of the Indian YLD phytoplasma was based on virtual RFLP profiles of the 16S rRNA gene. The YLD phytoplasma sequence corresponding to the F2nR2 region was subjected to *in silico* restriction enzyme digests and virtual gel plotting using the pDRAW32 program (AcaClone Software; <http://www.acaclone.com>). Virtual RFLP analysis included 16 other phytoplasma 16S rRNA gene sequences. Sequences were aligned and trimmed. The sequences were digested with 17 restriction enzymes universally accepted for phytoplasma classification (Lee *et al.*, 1998). The virtual RFLP patterns for the 17 sequences produced by the 17 restriction enzymes were compared and analysed using the software NTSYS-pc version 1.70 (Exeter Software). The similarity matrix showing pairwise similarity coefficients was plotted and clustering was done using the SAHN routine. Further RFLP classification was undertaken with six representatives of the 16SrXI group for subgroup assignment of YLD phytoplasma.

To validate the results of *in silico* restriction digestion studies of the Indian YLD phytoplasma, actual RFLP was performed on R16F2n/R16R2-amplified products of the 16S rRNA gene. Seven restriction enzymes, *EcoRI*, *TaqI*, *KpnI*, *BamHI*, *HinfI*, *HpaII* and *AluI*, were used separately to digest the 1250 bp 16S rRNA gene fragment. The RFLP products were run on 3% agarose gel in 1 × TBE.

## RESULTS

### PCR amplification and sequence analysis

In the nested PCR, primers 1F7/7R2 (490 bp amplicons) (Fig. S1, available in IJSEM Online), 4Fwd/5Rev (1150 bp) and R16F2n/R16R2 (1250 bp) gave positive results for 13, 11 and four samples, respectively. The small number of positives with the universal primers necessitated the design of two new primer sets. Of these, the primer set 1F7/7R3–1F7/7R2 gave more positive results; 13 of 15 symptomatic samples tested positive. The healthy and water controls showed no amplification in the first or nested PCR. BLASTN analysis of the Indian YLD phytoplasma 16S rRNA gene showed 99% nucleotide identity with the 16S rRNA gene sequences of sugar cane white leaf (SCWL) (GenBank accession no. HM215624), sugar cane grassy shoot (SCGS) (GQ850122), coconut root wilt disease (RWD) (GQ850122), NGS (AY736374) and BGWL (EF444485) phytoplasmas, i.e. phytoplasmas belonging to groups 16SrXI and 16SrXIV.

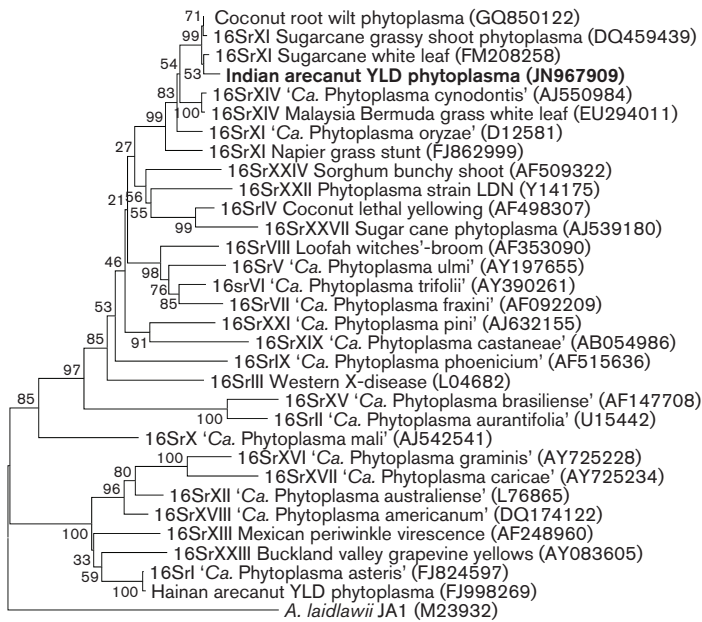
Semi-nested primers *cocsf/cocsr*–*cocsnf/cocsr* amplified a 425 bp fragment of the *secA* gene from three of nine diseased samples tested. The partial *secA* sequence (JX394029) showed 99% nucleotide identity to the *secA* sequence of SCGS phytoplasma (GenBank accession no. DQ459440), 88% identity to the sequence of NGS phytoplasma (EU168750) and 87% identity to the sequence of Malaysian BGWL phytoplasma (FJ755004).

### Phylogenetic analyses

Phylogenetic relationships of the Indian arecanut YLD phytoplasma to other phytoplasmas are shown in Figs 2 and 3. In the 16S rRNA gene-based phylogeny, the Indian YLD phytoplasma clustered with the rice yellow dwarf (RYD)- and BGWL-group phytoplasmas. However, within the subcluster, the Indian YLD phytoplasma grouped with the SCWL, SCGS and RWD phytoplasmas, all belonging to the RYD group (Fig. 2). In contrast, the Hainan YLD phytoplasma sequence clustered with 'Ca. Phytoplasma asteris'. Moreover, the Indian YLD phytoplasma 16S rRNA gene sequence (GenBank accession no. JN967909) shared 97.5% nucleotide sequence identity with the 'Ca. Phytoplasma oryzae' reference strain (D12581) and 97.8% nucleotide sequence identity with 'Ca. Phytoplasma cynodontis' (AJ550984). For finer differentiation, we examined the *secA* gene-based phylogeny. Here, the Indian YLD, SCGS, NGS, RWD and Malaysian BGWL phytoplasmas were found to diverge from the same parental node, and Indian YLD, SCGS, NGS, RWD formed a subcluster separate from the Malaysian BGWL phytoplasma (Fig. 3). The Indian YLD phytoplasma clearly clustered with the NGS and SCGS phytoplasma, both belonging to the RYD group. Hence, we assign the arecanut YLD phytoplasma as a 'Ca. Phytoplasma oryzae'-related strain.

### 16Sr group/subgroup assignment

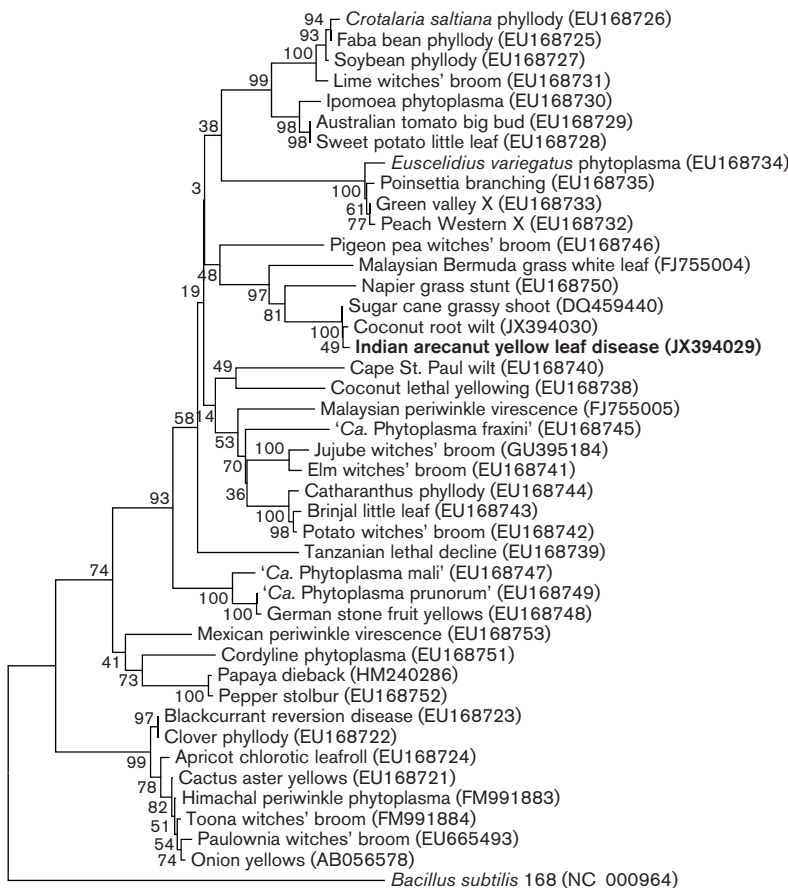
Virtual restriction fragments of 17 phytoplasma 16S rRNA gene sequences generated by 17 selected restriction enzymes



**Fig. 2.** Phylogenetic dendrogram reconstructed by the neighbour-joining method using MEGA software based on 16S rRNA gene sequences showing phylogenetic relationships of the Indian arecanut YLD phytoplasma with 31 other known phytoplasmas, and *Acholeplasma laidlawii* JA1 as an outgroup. 16Sr groupings are based on the published literature. Bootstrap values are expressed as percentages of 1000 replications.

were scored and analysed. The similarity coefficient values showed that the Indian arecanut YLD phytoplasma was most closely related to the SCWL and NGS phytoplasmas,

both belonging to the 16SrXI group. However, in a cladistic analysis, the Indian YLD phytoplasma clustered with members of groups 16SrXI and 16SrXIV, which together



**Fig. 3.** Phylogenetic dendrogram reconstructed by the neighbour-joining method with MEGA software based on partial *secA* gene sequences showing phylogenetic relationships of the Indian arecanut YLD phytoplasma and 42 other known phytoplasmas, with *Bacillus subtilis* 168 as the outgroup. Bootstrap values are expressed as percentages of 1000 replications.

formed a major subcluster (Fig. 4). Here also, the Hainan YLD phytoplasma clearly clustered with group 16SrI. Further *in silico* restriction digestion study including representatives of group 16SrXI placed the Indian YLD phytoplasma in subgroup 16SrXI-B, along with SCWL phytoplasma. Comparison of restriction site maps revealed that the Indian YLD phytoplasma produced a virtual RFLP profile identical to that of the SCWL phytoplasma (X76432), with a similarity coefficient value of 1.00. The SCGS phytoplasma lacks the *Hinf*I site at 766 that is present in SCWL, YLD, NGS, '*Psammotettix cephalotes*' flower stunt phytoplasma BVK and '*Ca. Phytoplasma oryzae*'. All other positions are identical for the YLD and SCGS phytoplasmas. The YLD and NGS sequences differ with respect to enzymes *Hpa*II, *Taq*I and *Bst*UI, while YLD and '*Ca. Phytoplasma oryzae*' differ with respect to *Mse*I (three positions) and *Taq*I. The member of group 16SrXI that is most distantly related to the Indian YLD phytoplasma is BVK, which shows differences in cutting patterns for *Mse*I (three positions), *Taq*I and *Bfa*I (Fig. S2).

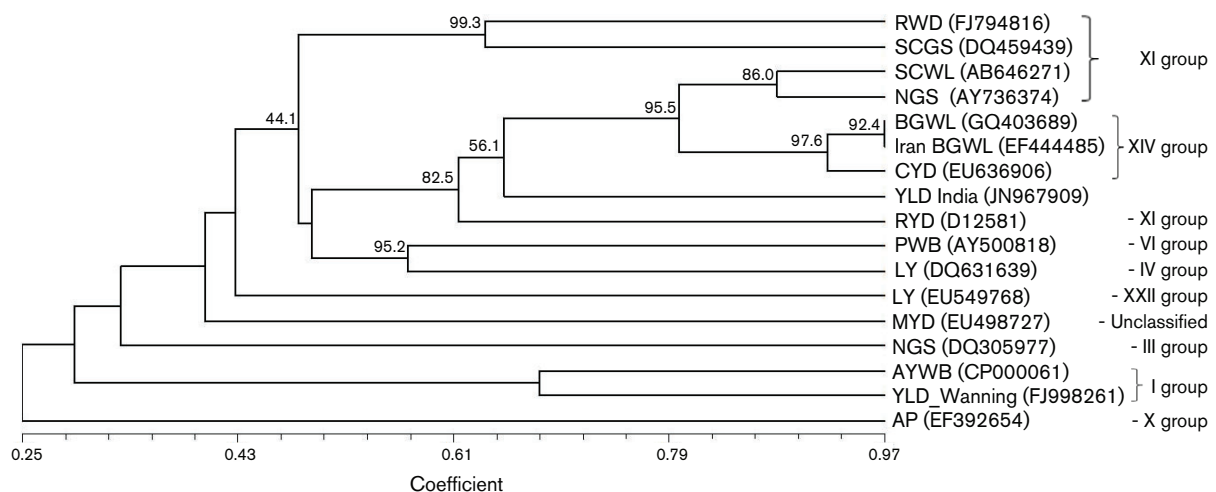
In actual restriction digestion of the Indian YLD phytoplasma R16F2n/R16R2 fragment, the banding pattern obtained was identical to the virtual RFLP pattern except that bands smaller than 100 bp were less clear.

## DISCUSSION

YLD of arecanut is a major production constraint faced by arecanut farmers in south India, especially in the states of Kerala and Karnataka. This disease has also been reported from China, where microscopic and molecular detection confirmed a phytoplasma as the causative agent (Daquan *et al.*, 2001, 2002). Manimekalai *et al.* (2010b) reported the detection of phytoplasma in YLD-symptomatic arecanut samples through a nested PCR assay. In the present study,

we have characterized the 16S rRNA and *secA* gene sequences and assigned the organism to Indian YLD phytoplasma. 16Sr group and subgroup classification of the Indian YLD phytoplasma was done using the computer-simulated RFLP approach.

Sequence similarity searches with the 16S rRNA gene sequence of Indian arecanut YLD phytoplasma showed that the sequences were similar to those of RYD- and BGWL-group phytoplasmas. Phylogenetic study based on the 16S rRNA gene sequence clustered it with members of the RYD group. Further phylogenetic analysis was performed based on partial *secA* gene sequences. The *secA* gene sequence offers an additional approach to phytoplasma diagnostics and strain identification (Hodgetts *et al.*, 2008). A pair of semi-nested primers was designed based on available sequences of RYD- and BGWL-group phytoplasmas, to which the Indian YLD phytoplasma is more closely related, because the universal phytoplasma *secA* primers (Hodgetts *et al.*, 2008) didn't amplify the Indian YLD phytoplasma *secA* gene. In the *secA* gene-based phylogeny, the Indian YLD phytoplasma clustered with SCGS and NGS agents, both belonging to the RYD group (Rao *et al.*, 2008; Jones *et al.*, 2004). Within the subcluster, the more distantly related strain was the Malaysian BGWL phytoplasma. The *secA* gene sequence of '*Ca. Phytoplasma oryzae*' is not available in public databases, and hence could not be included in the phylogenetic study. According to the recommendations of the IRPCM (2004), a phytoplasma strain can be described as representing a novel candidate species if its 16S rRNA gene sequence has <97.5% similarity to that of any previously described '*Ca. Phytoplasma*' species. The Indian YLD phytoplasma shares 97.5% nucleotide identity with '*Ca. Phytoplasma oryzae*', which comes under the RYD group, and 97.8% nucleotide identity with '*Ca. Phytoplasma cynodontis*', which comes under the BGWL group. However, in the *secA* gene-based



**Fig. 4.** Dendrogram constructed from similarity coefficient values derived from virtual RFLP analysis through the SAHN clustering method with NTSYS pc software.

phylogeny, the Indian YLD phytoplasma clearly clustered with the NGS and SCGS phytoplasmas, both belonging to the RYD group. The phylogenetic characterization hence identifies the Indian YLD phytoplasma as a 'Ca. Phytoplasma oryzae'-related strain.

RFLP analysis of the R16F2n/R16R2-amplified 16S rRNA gene fragment of phytoplasmas with 17 distinct restriction enzymes has classified phytoplasmas into different 16Sr groups and subgroups (Lee *et al.*, 1998). Alternatively, computer-simulated RFLP analysis of the F2nR2 region can be used for rapid differentiation and classification of phytoplasmas (Wei *et al.*, 2007). Our study clustered the Indian YLD phytoplasma with the 16SrXI group, the members of which affect monocots such as coconut (Manimekalai *et al.*, 2010a), rice (Jung *et al.*, 2003) and sugar cane (Rao *et al.*, 2008). Within the 16SrXI group, the Indian YLD phytoplasma shows a banding profile identical to that of the SCWL phytoplasma, and differs in a single position for *HinfI* from the banding profile of the SCGS agent. This is in accordance with a previous report that stated that the SCGS and SCWL phytoplasmas differ only with respect to a single cutting site for *HinfI* (Rao *et al.*, 2008). Another important observation is that all six 16SrXI-group sequences studied here lack cutting sites for *BamHI*, *KpnI* and *SspI*. This was confirmed for *BamHI* and *KpnI* in actual restriction digestion of the Indian YLD phytoplasma 16S rRNA gene. In the 16SrXI group, three subgroups are reported, 16SrXI-A (RYD strain), 16SrXI-B (SCWL strain) and 16SrXI-C (BVK strain) (Lee *et al.*, 1998). With an RFLP profile identical to that of the SCWL phytoplasma reference strain (GenBank accession no. X76432), the Indian YLD phytoplasma is placed in subgroup 16SrXI-B.

The arecanut YLD phytoplasma reported from China (GenBank accession nos FJ998269 and FJ694685) belongs to group 16SrI, and its 16S rRNA gene sequence shares only 91 % nucleotide identity with the sequence of the YLD phytoplasma from India. Our virtual RFLP study of the F2nR2 fragment clearly separated the YLD phytoplasma sequences from India and China into different clusters, where the Hainan sequence (GenBank accession no. FJ998269) fell into group 16SrI. In the 16S rRNA gene-based phylogenetic analysis, the Hainan sequence clustered with 'Ca. Phytoplasma asteris'. These findings support the conclusion that the YLD phytoplasma in India is different from the Hainan YLD phytoplasma reported previously, and hence we name it Indian YLD phytoplasma. To our knowledge, this is the first report of a 16SrXI-B phytoplasma associated with arecanut. 'Ca. Phytoplasma' species assignment based on phylogenetic analysis of 16S rRNA and *secA* gene sequences identified the Indian YLD phytoplasma as a 'Ca. Phytoplasma oryzae'-related strain.

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