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Molecular characterization identifies 16SrXI-B group phytoplasma (‘*Candidatus* Phytoplasma oryzae’-related strain) associated with root wilt disease of coconut in India

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ABSTRACT

The root wilt disease of coconut is a major threat to coconut cultivation in southern India. Here we report the species assignment and 16Sr sub group classification of phytoplasma associated with coconut root wilt disease. Leaf samples were collected from root wilt symptomatic palms in Kayankulam district of Kerala. The phytoplasma 16S rRNA gene was amplified using three sets of primers namely, 1F7/7R3-1F7/7R2, 3Fwd/3Rev-3Fwd/5Rev, and P1/P7-R16F2n/R16R2 producing amplicons of 490, 1300, and 1250 bp respectively. Partial *sec*A gene sequence of 480 bp from root wilt disease phytoplasma was amplified using primers cocsf/cocsr. Sequence characterization and phylogenetic analysis of 16S rRNA and *sec*A genes of root wilt disease phytoplasma grouped it with the rice yellow dwarf group phytoplasmas and identified coconut RWD phytoplasma as ‘*Candidatus* Phytoplasma oryzae’ related strain. Further, in silico restriction digestion study of phytoplasmal 16S rRNA gene region between primers R16F2n/R16R2 was performed for 16Sr group/sub group classification. The root wilt disease phytoplasma grouped with the 16SrXI group members. In the sub group classification it produced identical restriction profile as the sugarcane white leaf phytoplasma and arecanut yellow leaf disease phytoplasma, and hence, it was placed in the 16SrXI-B sub group.

1. Introduction

Coconut palm (*Cocos nucifera* L.) is considered as the most useful tree in the tropical countries of the world. Around the world the coconut production and productivity is very low due to old age of the palms, poor management practices, and pest and diseases (Warokka et al., 2006). Different groups of phytoplasmas are associated with diseases of coconut like lethal yellowing in Jamaica (Myrie et al., 2007), Florida (Harrison et al., 1994), Mexico (Harrison et al., 2002) and Nigeria (Ekpo and Ojomo, 1990), yellow decline in Malaysia (Nejat et al., 2009), Weligama coconut leaf wilt in Sri Lanka (Perera et al., 2012), and Kalimantan wilt in Indonesia (Warokka et al., 2006). Many coconut palms, over the years, have been destroyed by these diseases.

In India, among the diseases, root wilt disease (RWD) is a major threat to coconut plantation in south India and causes a loss of 968 million nuts annually. The disease is non lethal. The most consistent and diagnostic symptom of the disease is the characteristic bending of leaflets (termed flaccidity), foliar yellowing and marginal necrosis of the older leaves (Solomon et al., 1999) (Fig. 1). The symptoms are only obvious in palms that are more than 30 months old (Butler, 1908). The microscopic studies, serological evidences (Sasikala et al., 2005) and vegetative transmission studies (Sasikala et al., 1989) have revealed phytoplasma as the pathogen associated with the RWD. Molecular detection based on nested PCR amplification of 16S rDNA confirmed the association of phytoplasma with coconut RWD (Manimekalai et al., 2010).

Phytoplasmas are unculturable, cell wall-less, phloem limited plant pathogens transmitted through sap sucking insect vectors. The putative vector for coconut RWD phytoplasma is Proutista moesta as evidenced through transmission studies (Rajan et al., 2000). The IRPCM Phytoplasma/Spiroplasma working team has proposed a novel genus ‘*Candidatus* Phytoplasma’ to accommodate phytoplasmas. Within this genus, species are identified if organisms share <97.5% similarity among their 16S rRNA gene sequences (The IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma taxonomy group, 2004). Hodgetts et al. (2008) reported the use of additional genes like *sec*A gene as a phylogenetic parameter to produce an alternative phylogenetic analysis of the phytoplasmas. The RFLP analysis of R16F2n/R16R2 fragment of phytoplasma 16Sr DNA using 17 restriction enzymes viz., - *Alu*I,

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Fig. 1. Coconut palm showing symptoms of root wilt disease.

*Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I is routinely employed for classification and 16Sr group assignment to novel phytoplasma strains (Lee et al., 1998). Alternatively, the computer RFLP analysis of the F2nR2 region can be used for phytoplasma classification (Wei et al., 2007).

Earlier, Sharmila et al. (2004) reported the cloning and sequencing of Kerala wilt disease (or root wilt disease) phytoplasma. Edwin and Mohankumar (2007) did the phylogenetic analysis and grouped the phytoplasma in to 16SrIV group. However, the Kerala wilt disease phytoplasma sequence (GenBank accession no. AY158660) shares no homology with any phytoplasma sequence in the database. This led to further work regarding its correct identification and characterization and with this background, the present study was undertaken to characterize the 16S rRNA and *sec*A genes of coconut RWD phytoplasma for *Candidatus* Phytoplasma species assignment. The RWD phytoplasma was assigned appropriate 16Sr group/sub-group based on virtual RFLP banding pattern.

2. Materials and methods

2.1. Sample collection and nucleic acid preparation

Spindle leaf samples were collected from 12 RWD symptomatic palms from Kayankulam (Kerala state). Sample collected from a disease free area, Kidu (Karnataka state), was used as the negative control. Sugarcane showing the grassy shoot disease symptom, obtained from Sugarcane Breeding Institute, Coimbatore, was used as positive control. The DNA was extracted from 3 g fresh tissue sample using modified phytoplasma enrichment protocol (Ahrens and Seemuller, 1992). 2% PVPP was included in the extraction buffer. The homogenized samples were incubated at 4 °C for 5 min before proceeding for extraction. The DNA was finally dissolved in TE buffer (pH 8) and checked on 0.8% agarose gel. The DNA concentration was measured in spectrophotometer and diluted to 25 ng/μl for further analysis.

2.2. Primers for RWD phytoplasma and PCR amplification, cloning and sequencing

The universal phytoplasma primers P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) and the nested primers R16F2n/R16R2 (Gundersen and Lee, 1996) were initially used to amplify RWD phytoplasma. Two additional sets of semi nested primers 1F7/7R3-1F7/7R2 and 3Fwd/3Rev-3Fwd/5Rev were also used for amplifying phytoplasma 16S rDNA.

The PCR assays were performed in 15 μl volume containing 50 ng of DNA template, 0.2 μM of each primer, 150 μM of each dNTPs, 0.5 U of Taq DNA polymerase (Bangalore Genei) and 1X PCR buffer with 1.5 mM $MgCl_2$. First round amplifications with primers P1/P7, 3Fwd/3Rev, and 1F7/7R3 were performed to 35 cycles in a Mycycler (BIO RAD) thermocycler under following conditions: initial denaturation at 95 °C for 2 min followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C (55 °C for P1/P7), and 1 min and 30 s primer extension at 72 °C followed by a final extension at 72 °C for 10 min. The products of first PCR were diluted 1:4 with sterile water and 2 μl of each dilution was used as template during 35 cycles of PCR with nested primer pairs R16F2n/R16R2, 3Fwd/5Rev, and 1F7/7R2 respectively. The positive control contained DNA from grassy shoot diseased sugarcane and negative controls contained coconut DNA samples from disease free area or sterile water substituted for test DNA. Primers cocsf/cocsr (Manimekalai et al., 2012) were used to amplify partial *sec*A gene of coconut root wilt phytoplasma. PCR conditions followed were as already mentioned, the annealing temperature was 50 °C. The final PCR products were analyzed in 1% agarose in 1X TBE buffer (90 mM Tris borate, 2 mM EDTA, pH.8) containing ethidium bromide. The gels were documented using image analyzer (Gene Genius, Bio imaging system). Amplicons of expected sizes were excised from the gel, purified using QIAGEN gel extraction kit, cloned in to pTZ57R/T vector (M/s MBI Fermentas Inc.), and sequenced with M13 forward and reverse primers.

2.3. Homology and phylogenetic analysis

The nucleotide sequences from the M13 forward and reverse primers were screened for the presence of vector contamination using VecScreen (Altschul et al., 1997) and the vector sequences were removed using the bioedit software (Hall, 1999). The sequences were then subjected to similarity search using a local alignment search algorithm, blastn (Altschul et al., 1997). Representative sequences were deposited in the NCBI GenBank.

The phylogenetic analysis of RWD phytoplasma 16S rRNA gene between primers R16F2n and R16R2 and the *sec*A gene between primers cocsf and cocsr was employed for *Candidatus* Phytoplasma species assignment. The 16S rDNA sequences from 29 strains of the genus '*Candidatus* Phytoplasma' were retrieved from GenBank and trimmed and aligned using ClustalW. Similarly, the *sec*A gene sequences of 41 other phytoplasma strains were retrieved from GenBank trimmed and aligned. The phylogenetic tree was constructed using neighbor joining method with MEGA software version 4 (Tamura et al., 2007) with 1000 bootstrap replications. *Acholeplasma laidlawii* and *Bacillus subtilis* respectively were taken as the out group to root the phylogenetic trees.

2.4. In silico RFLP analysis.

The RWD phytoplasma sequence corresponding to the F2nR2 region was subjected to in silico RFLP analysis using pDRAW32 program developed by AcaClone Software (http://www.acaclone.com). The in silico RFLP analysis included 15 other phytoplasma 16S rDNA sequences from the database. All the sequences were aligned and trimmed. The sequences were

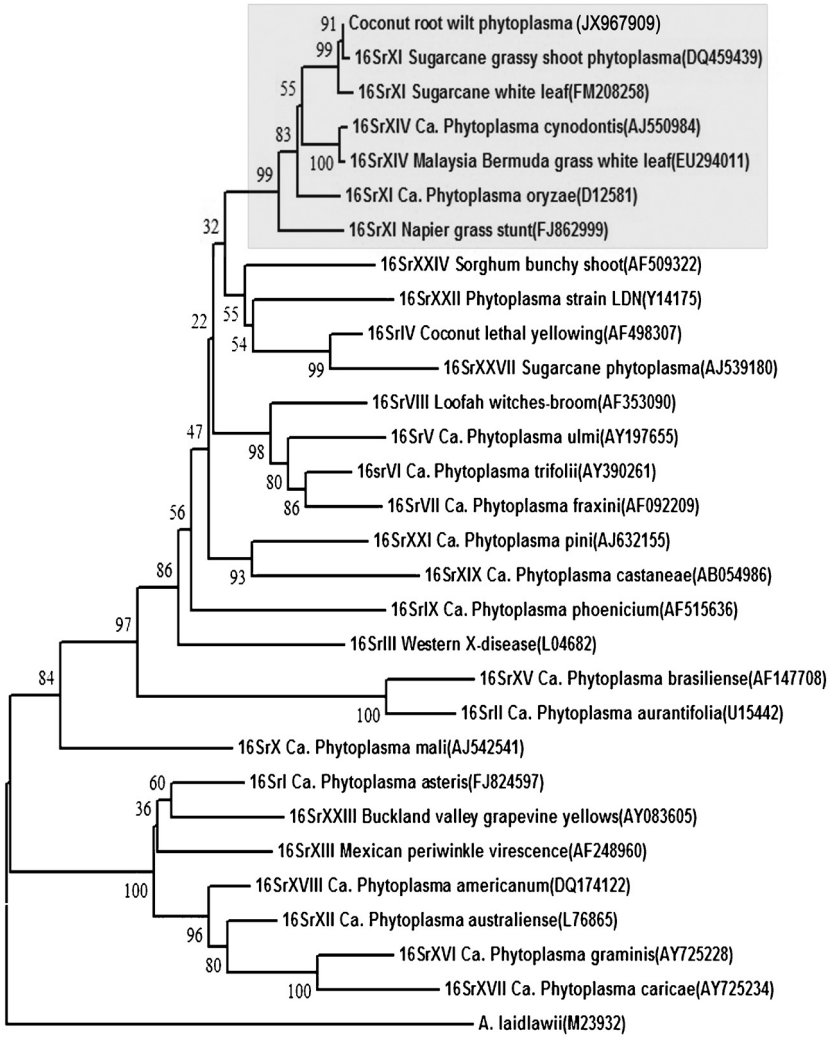

Fig. 2. Phylogram constructed by neighbor-joining method using Mega software based on 16S rRNA gene sequences, showing the phylogenetic relationships between the R16F2n/R16R2 amplified product of coconut root wilt phytoplasma with 29 other known phytoplasmas and *A. laidlawii* as an out group. 16Sr groupings are based on published literature. Genebank accession numbers and sequences are obtained from NCBI. Bootstrap values are expressed as percentage of 1000 replications and branch lengths are proportional to the inferred character state transformation.

digested with 17 restriction enzymes universally accepted for phytoplasma classification (Lee et al., 1998). The virtual RFLP patterns for 16 sequences produced by the 17 restriction enzymes were compared and analyzed using the software NTSYS-pc version 1.70 (Exeter Software, Setauket, NY, USA). The similarity matrix showing the pairwise similarity coefficients was plotted and clustering was done using the SAHN routine. For assigning 16Sr sub-group to RWD phytoplasma, six representative sequences from the 16SrXI group were selected and in silico digestion was performed using the same 17 enzymes.

3. Results

3.1. Detection of phytoplasma in root wilt affected coconut palms by PCR

In the semi nested/nested PCR, primers 1F7/7R2 (490 bp amplicons), 3fwd/5rev (1200 bp amplicons), and R16F2n/R16R2 (1250 bp amplicons) gave positive results for 11, 6, and 5 samples respectively. The PCR was repeated thrice and consistent results were obtained. Because of low number of amplicons with universal primers, new primers1F7/7R3-1F7/7R2 are found more suitable for detection of RWD phytoplasma as they gave maximum number of positive results. The sequences were deposited in NCBI GenBank Database (Accession No. GU947120, GU947111 and JX273772).

Primers cocsf/cocsr amplified a 480 bp fragment of *sec*A gene from 2 out of the 8 diseased samples checked. The partial *sec*A sequence of coconut root wilt phytoplasma (GenBank Acc. No. JX394030) shared 99% nucleotide identity with SCGS phytoplasma (DQ459440) and arecanut YLD phytoplasma (JX394029), 88% nucleotide identity with NGS phytoplasma (EU168750), and 87% nucleotide identity with Malaysian BGWL phytoplasma (FJ755004) *sec*A genes.

3.2. Homology and phylogeny analysis

In the blastn analysis, the RWD phytoplasma 16Sr gene showed ~99% nucleotide identity with 16Sr gene of SCWL phytoplasma (AB052874), SCGS phytoplasma (DQ459439), arecanut YLD phytoplasma (JN967909), NGS phytoplasma (AY736374), and Iran BGWL phytoplasma (EF444485), and ~98% nucleotide identity with '*Ca*. Phytoplasma oryzae' and '*Ca*. Phytoplasma cynodontis'.

In the phylogenetic analysis based on 16S rDNA (F2nR2 region), the RWD phytoplasma clustered with the RYD and BGWL group phytoplasmas. However, in the sub cluster, the RWD phytoplasma grouped with the SCWL, arecanut YLD and SCGS phytoplasmas,

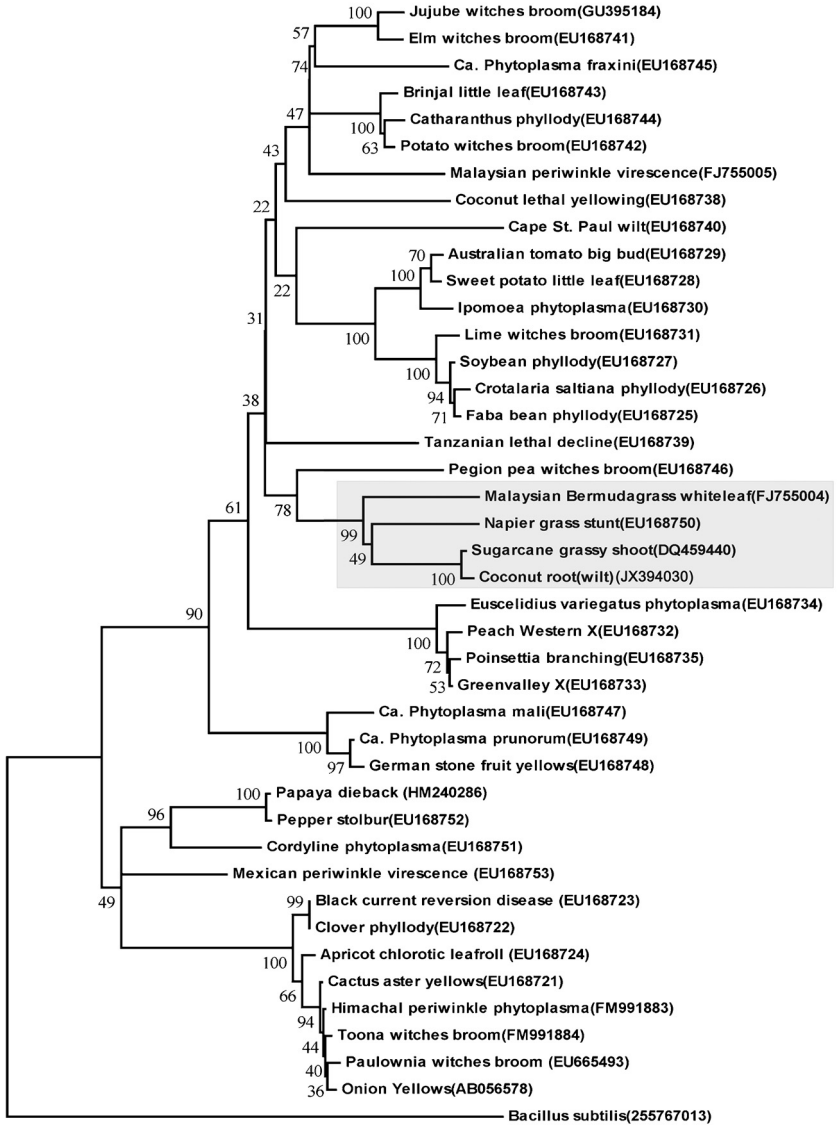

Fig. 3. Phylogram constructed by neighbor-joining method with Mega software showing the phylogenetic relationships of partial *sec*A gene of coconut RWD phytoplasma and 41 other known phytoplasma sequences and *Bacillus subtilis* as the out group. Bootstrap values are expressed as percentage of 1000 replications.

all belonging to the RYD group (Fig. 2). Moreover, the RWD phytoplasma 16S rRNA gene sequence (GenBank Acc. No. JX273772) shares 97.7% nucleotide identity with the '*Ca*. Phytoplasma oryzae' reference strain (GenBank Acc. No. D12581) and 97.9% nucleotide identity with '*Ca*. Phytoplasma cynodontis reference strain' (GenBank Acc. No. AJ550984). For finer differentiation we used the *sec*A gene based phylogeny. Here, the RWD, SCGS, YLD, NGS, and Malaysian BGWL phytoplasmas were found to be diverging from the same parental node. However, the RWD, SCGS, YLD, and NGS, formed separate sub-cluster from the Malaysian BGWL phytoplasma (Fig. 3). The RWD phytoplasma clearly clustered with the YLD, NGS, and SCGS phytoplasma, both belonging to the RYD group. Hence the coconut RWD phytoplasma belongs to the RYD group and we are assigning it as '*Ca*. Phytoplasma oryzae'-related strain.

3.3. *In silico RFLP analysis and phytoplasma classification*

The virtual RFLP analysis of the F2nR2 region of RWD phytoplasma was done for 16Sr group assignment. The similarity coefficient values derived from RFLP analysis placed the RWD phytoplasma with the 16SrXI group members. In the dendrogram also, the RWD phytoplasma clearly clustered with the members of 16SrXI group (Fig. 4). So the in silico RFLP analysis classified the RWD phytoplasma as a member of the 16SrXI group. For sub group classification of RWD phytoplasma, in silico restriction digestion study was performed with representatives of 16SrXI group. Here, the RWD phytoplasma was placed in 16SrXI-B sub group along with SCWL and arecanut YLD phytoplasmas. Comparison of the restriction site maps revealed that the RWD phytoplasma produced virtual RFLP profile identical to SCWL phytoplasma (GenBank Acc. No. X76432) and arecanut YLD phytoplasma (GenBank Acc. No. JN967909) (Fig. 5).

4. Discussion

All over the world, coconut palms are affected by a number of phytoplasma diseases. Coconut lethal yellowing, yellow decline, Kalimantan wilt, Cape St. Paul wilt, and Sri Lankan Weligama wilt are a few to name. In south India, the RWD is a major debilitating disease of coconut causing great economic loss. In our earlier studies, we detected the phytoplasma associated with coconut

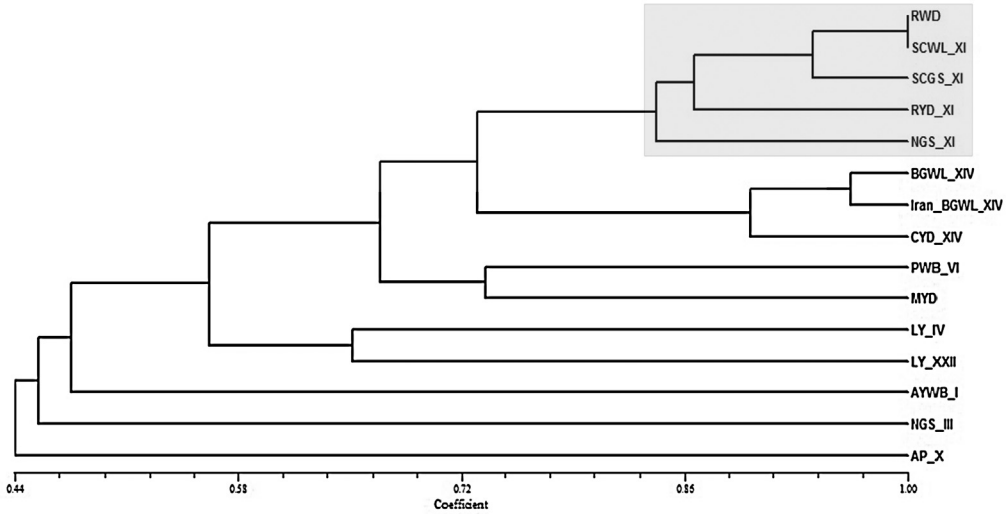

Fig. 4. Dendrogram constructed from similarity coefficient values derived from virtual RFLP analysis through SAHN clustering method with NTSYS pc software. RWD–root wilt disease (JX273772), SCWL_XI–sugarcane white leaf (X76432), YLD_XI- arecanut yellow leaf disease (JN967909), SCGS_XI–sugarcane grassy shoot (DQ459439), RYD_XI–rice yellow dwarf (D12581), NGS_XI - Napier grass stunt (AY736374), BGWL_XIV–Bermuda grass white leaf (GQ403689), Iran BGWL_XIV–Iran Bermuda grass white leaf (EF444485), CYD_XIV–coconut yellow decline (EU636906), PWB_VI - potato witches' broom (AY500818), MYD–Malaysian yellow decline (EU498727), LY_IV–lethal yellowing (DQ631639), LY_XXII–lethal yellowing (EU549768), AYWB_I–aster yellows witches broom (CP000061), NGS_III–Napier grass stunt (DQ305977), and AP_X–apple proliferation (EF392654).

RWD through nested PCR technique (Manimekalai et al., 2010). Here, we report the phylogenetic classification and 16Sr sub group assignment of coconut RWD phytoplasma. The number of positive amplicons with universal primers being low the semi nested primers 1F7/7R3-1F7/7R2 is found ideal for RWD phytoplasma detection since it amplified 11 out of 12 symptomatic samples. The RWD phytoplasma sequence reported here did not show identity with sequence reported earlier for Kerala wilt coconut phytoplasma (GenBank Acc No AY158660), which did not have similarity with any known phytoplasma sequences in the database. Sequence similarity search of 16S rRNA gene of coconut RWD phytoplasma showed that the sequences had similarity with rice yellow dwarf (RYD) and Bermuda grass white leaf (BGWL) group phytoplasmas. In general, the RWD phytoplasma showed similarity with the phytoplasmas infecting the monocots like arecanut, rice, sugarcane, sorghum, bermuda grass, and napier grass. The phylogeny study based on the 16S rRNA gene sequence clustered it with the RYD and BGWL group members but in the sub cluster it grouped with the RYD group members. Further, phylogenetic analysis was performed based on *sec*A gene partial sequence. The *sec*A gene sequences offer an additional approach to phytoplasma diagnostics and strain identification (Hodgetts et al., 2008). In the *sec*A gene based phylogeny, the RWD phytoplasma clustered with SCGS, arecanut YLD and NGS agents, both belonging to the RYD group (Rao et al., 2008; Manimekalai et al., 2012; Jones et al., 2004). Within the sub cluster, the more distantly related one is the Malaysian BGWL phytoplasma. The *sec*A gene sequence of '*Ca*. Phytoplasma oryzae' is not available in the public domain databases and hence could not be included in the phylogeny study. As per IRPCM (2004) rules, a phytoplasma strain can be described as a novel '*Ca*. Phytoplasma' species if its 16S rRNA gene sequence has <97.5% similarity to that of any previously described '*Ca*. Phytoplasma' species. The RWD phytoplasma shares 97.7% nucleotide identity with '*Ca*. Phytoplasma oryzae' which comes under the RYD group and 97.9% nucleotide identity with '*Ca*. Phytoplasma cynodontis' which comes under the BGWL group. However, in the *sec*A gene based phylogeny, the RWD phytoplasma clearly clustered with the NGS and SCGS phytoplasma, both belonging to the RYD group. The phylogenetic characterization hence identifies the RWD phytoplasma as '*Ca*. Phytoplasma oryzae'-related strain.

Virtual RFLP patterns of F2nR2 region of RWD phytoplasma placed it in the 16SrXI group. The RFLP profile of F2nR2 region of phytoplasma 16Sr DNA generated using 17 restriction enzymes viz., - *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I is used for classification and 16Sr group/sub group assignment of novel phytoplasma strains (Lee et al., 1998). Alternatively, the computer simulated RFLP can be employed for phytoplasma classification (Wei et al., 2007). The cluster analysis of virtual RFLP bands clearly grouped the RWD phytoplasma with 16SrXI group phytoplasmas. The similarity coefficient values also favored its grouping in the 16SrXI group. These results clearly indicate that in silico RFLP analysis and virtual gel plotting could serve as a convenient and reliable alternative to conventional RFLP analysis. In the 16SrXI group, three sub-groups are reported, viz., 16SrXI-A (RYD strain), 16SrXI-B (SCWL strain), and 16SrXI-C (BVK strain) (Lee et al., 1998). Of the 17 enzymes used in in silico digestion, all the six 16SrXI group sequences studied here lack cutting sites for enzymes *Bam*HI, *Kpn*I, and *Ssp*I. The RWD phytoplasma shows identical banding profile as the SCWL phytoplasma and arecanut YLD phytoplasma and hence we placed it in the 16SrXI-B sub group. The RWD phytoplasma sequence differed from the SCGS sequence (DQ459439) for a single cutting site for the enzyme *Hin*fI. In the phylogenetic analysis, the RWD sequence clustered closely with SCGS sequence while in the RFLP study, the RWD clustered closely with the SCWL phytoplasma, both having identical RFLP profile.

Earlier, the arecanut yellow leaf disease (YLD) phytoplasma from south India was identified as a '*Candidatus* Phytoplasma oryzae' related strain belonging to 16SrXI-B sub group (Manimekalai et al., 2012). In medieval period the movement of people and palms was common between India and Eastern countries and it is probable that the coconut phytoplasma disease reported in Malaysia and Indonesia could spread to India. In Asian countries coconut is grown along with sugarcane. Sugarcane is affected by phytoplasma disease like grassy shoot and white leaf, and these phytoplasma may be transmitted to coconut and arecanut or vice-versa. In the other way, the phytoplasma infecting coconut may be transmitted from infected sugarcane through a common vector. Hence it is not surprising that wherever the coconut is infected by phytoplasma, the sugarcane also shows the occurrence of white leaf and/or

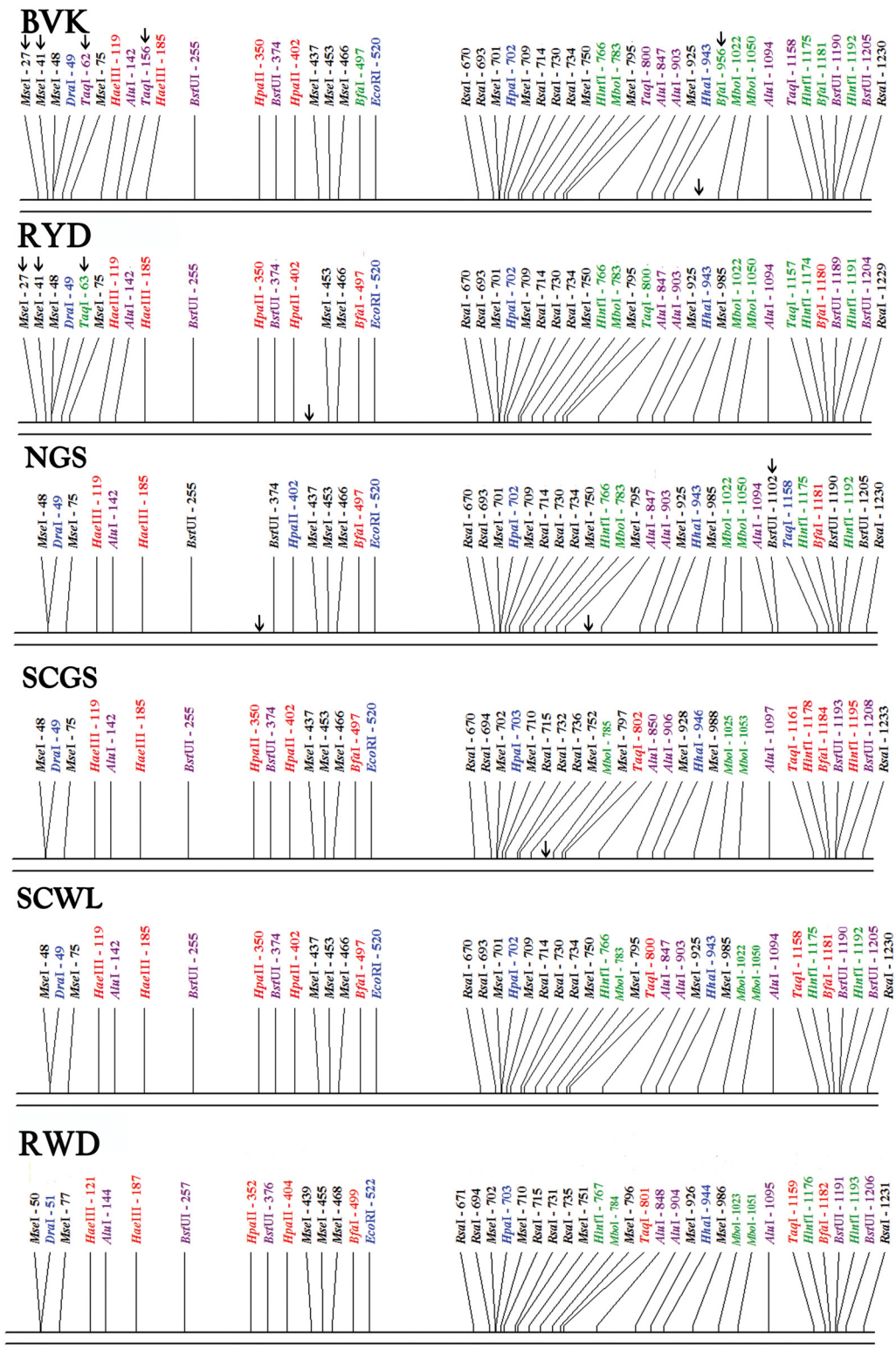

Fig. 5. Restriction site map of F2nR2 region of 16SrXI group phytoplasmas with 17 restriction enzymes (*Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI,*Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I (*Sau*3AI), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I) generated using pDRAW software. The arrows indicate the regions where RWD phytoplasma differs from others. BVK-HQ589192 (*Psammotettix cephalotes* flower stunt phytoplasma), '*Ca*. Phytoplasma oryzae'–D12581, NGS–AY736374, SCGS- DQ459439, SCWL- X76432, YLD- JN967909 and RWD- JX273772.

the grassy shoot diseases (Kumarasinghe and Jones, 2001; Rao et al., 2008). Phytoplasma disease would have spread to coconut even from infected cane samples which were brought to India for cultivation.

5. Conclusion

Our present investigation identified the coconut RWD phytoplasma as '*Candidatus* Phytoplasma oryzae'-related strain and

classified it in to the 16SrXI-B sub group. To our knowledge it is the first report of the association of 16SrXI-B phytoplasma with coconut in the world.

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