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Cloning and Sequencing of Phytoplasma Ribosomal DNA (rDNA) Associated with Kerala Wilt Disease of Coconut Palms

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Using the polymerase chain reaction (PCR) the 16S rRNA gene of phytoplasma associated with Kerala wilt disease of coconut palm (*Cocos nucifera* L) was amplified from infected leaf samples. Within the three universal primer pairs P1/P6, P1/P7 and P4/P7, the primer pair P4/P7 only showed an amplification of 650 bp DNA fragment. Since P4/P7 amplifies the 16S-23S intergenic spacer region of 16S rRNA gene, the PCR product 650 bp of Kerala wilt disease palm indicates the phytoplasma DNA. The amplified fragment was sequenced and deposited in Genbank data library (Accession No. AY158660). The absence of restriction sites for *Bcl*I and *Rsa*I in 650 bp indicates phytoplasmic nature of DNA and its strain difference. A comparison of the 650 bp sequence with other phytoplasmas and its restriction profile indicates Kerala wilt disease phytoplasma as a separate 16S rRNA group in the classification of phytoplasmas. To our knowledge, this report records the first finding of the phytoplasma DNA using universal primers and its sequence analysis in coconut palms of Kerala, South India.

Key words: Kerala wilt disease, phytoplasma, PCR, 650 bp, restriction analysis.

Kerala wilt disease of coconut palms, formerly named as root (wilt) disease is the most important single threat to coconut production in Kerala, South India. The appearance and spread of the disease has immobilized the economy and culture of the state. It is a paradox that with the largest area under coconut cultivation, Kerala has the 9th position in productivity in India as per the coconut statistics (1). Almost all regions under coconut cultivation in Kerala are susceptible to this non-lethal menace. Ribbing, unseasonable discolouration of leaves and generalized decline of palm growth have been considered as the criteria for preliminary diagnosis of disease as phytoplasma. The symptoms of Kerala wilt disease (KWD) showed similarity with coconut lethal yellowing (CLY) and coconut lethal decline (CLD) reported internationally. Association of phytoplasma DNA by PCR based methods in both CLD and CLY confirmed their etiology.

The existing schools of thought on etiology of Kerala wilt disease have twin focus: non-phytoplasmic and phytoplasmic. Researchers on non-phytoplasmic etiology advocates metal toxicity and geochemical factors as the cause of the disease (2,3). Observation of pleomorphic membranous bodies in the phloem of leaf and root tissues

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of infected palms, by electron microscopy was the first evidence to suggest the etiology as phytoplasma by Solomon *et al* (4). Even though electron microscopic method is considerably more sensitive, the small size, low titer and sporadic distribution of phytoplasma in woody plants especially in monocots have made the detection of phytoplasma more cumbersome. A new era in phytoplasma research began when DNA based methods were introduced about a decade ago, following the development of procedures to extract and enrich phytoplasma DNA from infected plants or insects (5-10). Sequence analysis of the conserved regions of 16S rRNA gene became a standard method for the phylogenetic classification of phytoplasma groups (10). The first molecular indication of phytoplasma DNA of Kerala wilt diseased coconut palms by PCR amplification as 558 bp using the primers designed from the conserved regions of *Oenothera lamarkiana* was reported by Sharmila *et al* (11, 12). Since the primer pair designed by Ahrens and Seemuller (11) for the broad phytoplasma detection was not specific for phytoplasmas, the observation of 558 bp DNA fragment in the infected leaf tissue of Kerala wilt diseased coconut palms, arouse disagreement for considering phytoplasma as the cause of Kerala wilt disease. Hence the objective of the present study was to detect and identify phytoplasmas in Kerala

wilt disease coconut palms using universal primers by polymerase chain reaction (PCR) assay, restriction analysis and sequencing of the phytoplasma gene to determine its phylogenetic status.

Materials and Methods

Sources of healthy and diseased palms — Coconut palms from disease prevalent areas of Alappuzha district were used for the experimental study. As control, healthy palms from disease free area of Thiruvananthapuram district were selected. The healthy nature of the palm was confirmed by the assay of the marker enzyme (13). Tender leaf samples of both diseased and healthy palms were used for the DNA preparation.

Preparation of Kerala wilt disease (KWD) phytoplasma DNA — DNA extract was prepared from both healthy and disease leaf samples using the phytoplasma enrichment method of Kirkpatrick *et al* (5) with some modifications. Fresh leaf tissue (25 g) was ground in ice cold extraction buffer with a composition of 125 mM potassium phosphate, 30 mM ascorbic acid, 10% sucrose, 0.15% bovine serum albumin (BSA) and 2% polyvinylpyrrolidone (PVP) at pH 7.6 using 4 ml of buffer per gram tissue. The slurry was strained through cheesecloth and the extract was clarified by centrifugation at 4 °C for 5 min at 1,400 x g. The phytoplasma enriched preparation was pelleted from the supernatant by centrifugation at 4 °C for 30 min at 15,000 x g. DNA was extracted from the phytoplasma enriched pellet and purified following the method of Sambrook *et al* (14).

Primers and PCR amplification — Universal primers derived from conserved regions of the 16S ribosomal sequence were used to amplify phytoplasma ribosomal DNA (rDNA) from infected samples (Table 1). Universal

Table 1. Universal primers used for PCR amplification of the 16S rRNA gene of phytoplasmas in Kerala wilt disease of coconut palms

Universal Primers	Sequences	Location	Reference
P1	5' - aagagtttgatcctcgctcaggatt - 3'	1-25	15
P6	5' - cggtagggatacctgttacgactta - 3'	1496 - 1471	15
P4	5' - gaagtctgcaactcgacttc - 3'	1275 - 1294	16
P7 (23 S)	5' - cgctcttcacgctgctctt - 3'	59-40	17

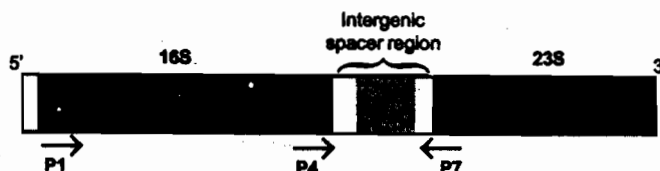


Fig. 1. Diagrammatic representation of phytoplasma rRNA operon, including the 16S and 23S rRNA genes and the intergenic spacer region.

primers (P1/P6, P1/P7 and P4/P7) that prime at the 5' and 3' ends of the 16S rRNA gene and the 16S – 23S intergenic spacer region and the beginning of the 23S rRNA gene were used (Fig. 1). PCR amplification of phytoplasma DNA from Kerala wilt disease leaf samples using the universal primers designed from the conserved regions of 16S rRNA gene was done according to the procedure of Tymon *et al* (9). PCR assay was performed in 50 µl volumes containing 100 ng each primer (Bangalore, Genei), 150 µM dNTPs, 1.25 mM MgCl₂, 0.5 units of Taq DNA polymerase, 10x PCR buffer, and 50 - 100 ng template DNA. Mixtures were subjected to 35 cycles in a PTC-100 thermal cycler (MJ Research) under following conditions: 30s denaturation at 94°C (2 min at 95°C for the first cycle), 60s annealing at 58°C for primer pair P1/P6, 53°C for primer pair P1/P7; P4/P7 and 90s extension at 72°C (10 min in the final cycle). Negative controls contained DNA from healthy palms and water substituted for test DNA. PCR products were analyzed by horizontal electrophoresis, through 1.5% agarose in 1x TAE buffer (40 mM Tris-Acetate, 1 mM EDTA pH 8.0) followed by staining in ethidium bromide and visualization of DNA bands using UV transilluminator. Molecular weight of the bands was determined from the 1 Kb DNA ladder.

Restriction analysis of PCR products — 10 µl of each PCR product was digested with *AluI*, *BclI*, *HindIII* and *RsaI*. Products were separated by electrophoresis through a 15% polyacrylamide gel with 1x TAE as running buffer. Bands were visualized with UV following ethidium bromide staining of gels.

Cloning of PCR products — The PCR products from KWD palms were gel purified using the ultra PCR product clean – up kit (AB gene). The products were ligated into pGEM[®]T vector (Promega) following the instructions given by the manufacturer. 3 µl of the ligation mixture was used to transform high efficiency competent cells of *Escherichia coli* JM109 and plated on Luria – Bertani (LB) medium containing ampicillin, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and isopropyl β-D-thiogalactopyranoside (IPTG). White colonies were

grown in liquid LB containing enough concentration of antibiotic. Plasmid DNA isolated by alkaline lysis method was digested with *EcoRI* to confirm the presence of insert DNA (18).

Sequence analysis — Plasmid DNA for sequencing was prepared with plasmid preparation kit (AB gene). Clone of P4/P7 was sequenced with automated sequencing of Applied Biosystems (ABI prism) following dideoxy chain terminator sequencing protocol (Perkin – Elmer). Sequence analysis was carried out using OMIGA – DNA sequence analysis software (Oxford molecular company). Sequence from other known phytoplasmas obtained from the Blast search of National Centre for Biotechnological Information (NCBI) was compared with the sequence of P4/P7 product and submitted to Genbank.

Results and Discussion

Detection of phytoplasma DNA by universal primers —

The purified DNA fractions from tender leaf samples of both diseased and healthy palms were subjected to polymerase chain reaction (PCR) using universal primers P1/P6, P1/P7 and P4/P7. The PCR assay was done identical to the conditions designed by Tymon *et al* (9) for the three primer pairs. The amplified product of the test DNA from the tender leaf samples of diseased coconut palms primed with P4/P7 resolved a prominent band of 650 bp at annealing temperature 53°C (Fig. 2). The primer pairs P1/P6 and P1/P7 showed no amplification in the test DNA of KWD palms.

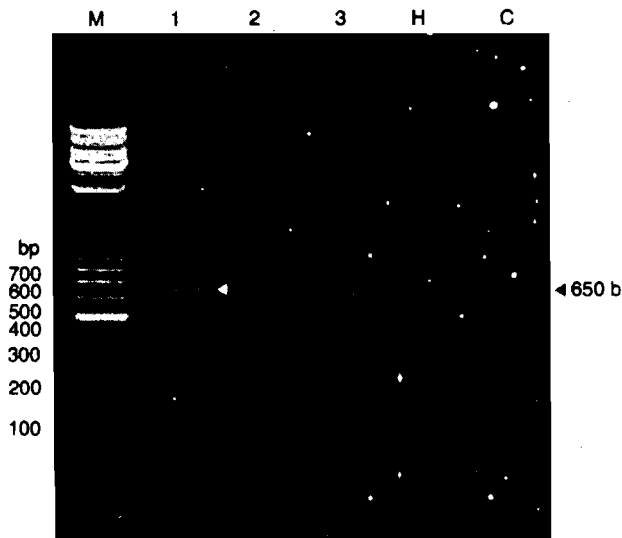


Fig. 2. PCR amplification of 16S rDNA fragment from Kerala wilt disease coconut palms using universal primers. M: marker DNA ladder, lane 1: P4/P7 showing 650 bp, lane 2: P1/P6, lane 3: P1/P7, H: healthy palm and C: negative control.

No product was amplified from the healthy palms as well as in the negative control (i.e., without test DNA). The earlier reports on PCR amplification of phytoplasma DNA using the primer pairs P1/P6, P4/P7 and P1/P7 revealed the amplified products in the range of 1.5 Kb (P1/P6), 0.5 Kb (P4/P7) and 1.8 Kb (P1/P7) in size (9). The DNA fragment 650 bp amplified from KWD palm was different from the expected PCR product as published earlier for coconut phytoplasmas using the same primer pair P4/P7. Since the primer pair P4/P7 is specific to the 3' end of the 16S rRNA gene and the beginning of the 23S rRNA gene the PCR product 650 bp clearly indicates the amplification of 16S-23S intergenic spacer region (Fig. 1).

Restriction analysis of 650 bp — The resulting PCR product was subjected to restriction analysis. The amplified product 650 bp primed by P4/P7 was digested with restriction enzymes *AluI*, *BclI*, *HindIII* and *RsaI*. Based on the nature of the amplified product, the restriction pattern of 650 bp using *AluI* and *HindIII*, the restriction profile showed a pattern of 69, 86 and 407 bp for *AluI* and 67, 114 and 451 bp for *HindIII*. The restriction analysis showed no sign of restriction digestion for *BclI* and *RsaI* (Fig. 3). Since the restriction enzyme *BclI* is site specific to plant chloroplast 16S rDNA, the absence of its cutting site in 650 bp again indicates the phytoplasmic nature of DNA (19-21). The presence or absence of restriction sites of the enzyme *RsaI* in the restriction analysis of phytoplasma 16S rDNA was used as a marker for differentiating the phytoplasma strain (22). Hence the absence of restriction sites for *RsaI* in the 16S-23S rRNA intergenic spacer region of Kerala wilt disease phytoplasma is an indication of its strain difference.

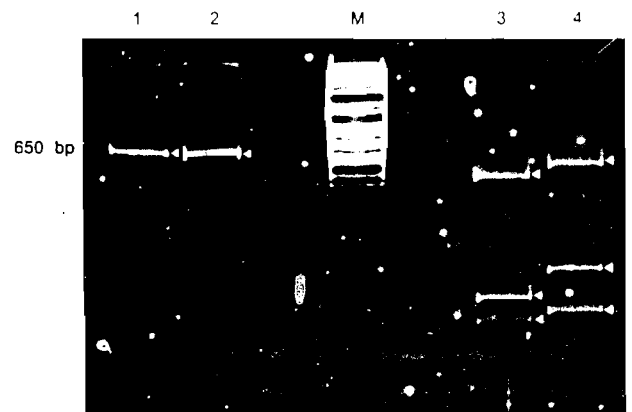


Fig. 3. Restriction analysis of 650 bp fragment amplified in polymerase chain reaction (PCR) primed by the primer pair P4/P7. M: marker DNA ladder, lane 1: *BclI*, lane 2: *RsaI*, lane 3: *AluI* and lane 4: *HindIII*.

Cloning and sequencing of 650 bp fragment — The amplified product of 650 bp of the primer pair P4/P7 from the DNA of KWD palms were gel purified and cloned using the plasmid pGEM[®]T vector and sequenced. The data showing the partial sequence of the cloned fragment is given in Fig. 4. The sequence of KWD phytoplasma DNA was compared with the GenBank coconut phytoplasma sequences and other 16S rRNA sequences and the cladogram of the phytoplasmas was prepared using BioEdit sequence analysis (Fig. 5). The similarities noticed between the nucleotide of 650 bp with other 16S rRNA gene sequence is considered to be a signature of coconut

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BASE COUNT      100 a      91 c      84 g      121 t
ORIGIN
1  aaagcttgcg actgacgctc tagggaactt gctttggcca ttgaccccca tgcattgtcg
61  ataccagagt ccttcaactc cttcaactcc tctatgagat tggaccgatt attgatgtgg
121  tccatagcac ccttctctatg gacctatcgg agatcagtaa cttcaattgt agcttcagaa
181  agcttcttca aattttgaag cttaccactc gcttctctpa gctttctttt agcttcagaa
241  agctctctct ctttaggtcc ttataaatgg gttttaaagp aaggccctgg ggtgacgatg
301  tggcatcttc agggagtagg ctagtttatg attattcttc gca-gatgag cattotaaaa
361  atgcaaacat gccacagcca aaaaaaaaaa gctttt

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Fig. 4. Nucleotide sequence of Kerala wilt disease phytoplasma gene.

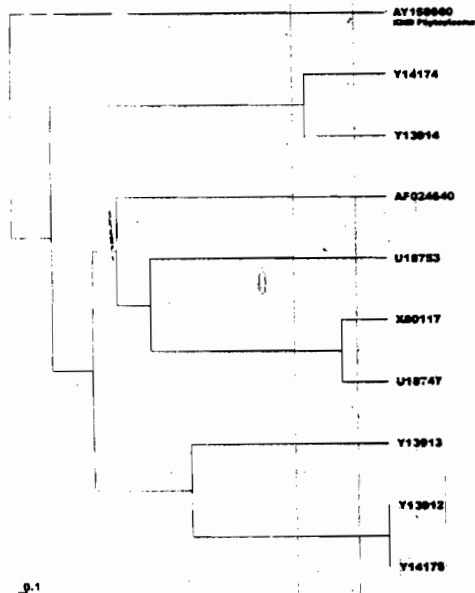


Fig. 5. Cladogram for detecting the phylogenetic status of KWD coconut phytoplasma constructed using Bioedit Sequence Analysis (Fitch - Margoliash Method version 3.573 c). The bar represents 0.1 nucleic acid substitution per site. AY158660: Kerala wilt disease phytoplasma, Y14174: coconut lethal yellowing, sub strain Nigerian Awka disease, Y13914: coconut lethal yellowing, sub strain Ghanaian Cape St. Paul wilt disease, AF024640: Mexican coconut lethal decline, U18753: Yucatan coconut lethal decline, X80117: coconut lethal yellowing, sub strain Tanzanian lethal disease, U18747: coconut lethal yellowing, Florida, Y13913: coconut lethal yellowing, sub strain Tanzanian lethal disease, Y13912: coconut lethal yellowing, sub strain Ghanaian Cape St. Paul wilt disease, Y14175: coconut lethal yellowing, sub strain Nigerian Awka disease.

phytoplasma. Based on high frequency of nucleotide dissimilarity of KWD phytoplasma with other coconut phytoplasmas, KWD phytoplasma may be considered as a separate cluster within the phytoplasma clade. Comparison of 650 bp sequence with other coconut phytoplasma confirms KWD phytoplasma as a different 16S rRNA group phylogenetically. Considering the unique nature of the nucleotide sequence it was deposited in the GenBank data base under accession No. AY158660.

The partial sequence showed similarity with other coconut phytoplasmas by homology search using OMIGA software. The restriction analysis of the fragment is in conformity with putative restriction sites obtained from the DNA sequence analysis. Thus the association of phytoplasma DNA in Kerala wilt diseased coconut palm was confirmed.

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