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Molecular identification of *Proutista moesta* as the vector and the phylogenetic analysis of KWD phytoplasma

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The purified genomic DNA from the tissues of *Proutista moesta* and *Stephanitis typica*, considered as possible vectors of Kerala wilt disease (KWD) of coconut palm, was subjected to PCR assay using the primer pairs P1/P6, P1/P7 and P4/P7. In case of *P. moesta*, the amplified products resolved a prominent band of 650 bp for the universal primer pair P4/P7; however, no bands were noticed for the primer pairs P1/P6 and P1/P7. In case of *S. typica* no bands were noticed for all sets of primers. Since P4/P7 amplifies the 16S-23S intergenic spacer region of 16SrRNA gene, the 650 bp product from *P. moesta* indicates the presence of phytoplasma DNA. The restriction enzyme analysis of the 650 bp product, using the enzymes *Alu*I, *Bcl*I, *Hind*III and *Rsa*I, further supports the phytoplasmic nature of DNA. The presence of 650 bp product in the genomic DNA of *P. moesta* shows the insect being a vector of KWD phytoplasma. The sequential similarity of 650 bp of both KWD phytoplasma and the insect phytoplasma again supports the transmission of phytoplasma through the vector *P. moesta*. From the cladogram, it is obvious that the KWD phytoplasma is evolutionarily closest to the phytoplasma causing coconut lethal yellowing of Mexican palms within the group 16SrIV.The present study is the fist confirmed record of *P. moesta* as the vector of KWD by detecting KWD phytoplasma in the insect tissues by PCR based methods.

Keywords: *Proutista moesta*, Kerala wilt disease, phytoplasma, Universal primers, 650bp

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Coconut palm (*Cocos nucifera*) is one of the most significant cash crops grown in Kerala that contributes 35% of the agricultural and 15% of the annual income of the state[1]. Root wilt disease of coconut is the most important single threat of coconut production in Kerala and attributed as the major reason to the low productivity of the crop. For its first appearance and predominance in Kerala, the disease is now renamed internationally as Kerala wilt disease (KWD) of coconut palms. PCR based detection of pathogen by universal primers and RFLP analysis have provided sufficient evidence for confirming the causative organism of KWD as phytoplasma[2]. Plant host range of phytoplasma is determined largely by a number of natural insect vector species that are capable of transmitting them and by the feeding behaviour of these vectors[3]. Previous studies on transmission of KWD disease indicated that *Proutista moesta* and *Stephanitis typica* were the possible vectors of the disease[4,5]. With this background the main objective of the present investigation is to detect the appropriate vector of KWD by PCR based methods using universal primers.

Areas having KWD affected palms were monitored for the presence of insects, *P. moesta* and *S. typical*, in their leaflets. The insects were collected from diseased palms and stored in small cages in live form. Prior to isolation of phytoplasma, both the insects were kept in microfuge tubes at −20°C in a freezer. From such frozen insect samples, phytoplasma enriched pellet (PEP) was prepared and DNA was isolated[2]. For the isolation of DNA, PEP was resuspended in DNA extraction buffer preheated at 60°C with a composition of 2% SDS, 500 m*M* NaCl, 20 m*M* EDTA and 100 m*M* Tris-HCl, *p*H 8.0[6]. The purity of the DNA samples was checked spectrophotometrically by measuring the absorbance at 280 and 260 nm and the concentration of DNA was determined. Universal primers derived from conserved regions of the16S ribosomal sequence were used to amplify phytoplasma DNA (rDNA) from infected samples. Universal primer pairs P1/P6, P1/P7 and P4/P7 (P1-5′-aagagtttgatcctggctcaggatt-3′; P6-5′-cggtagggataccttgttacgactta-3′; P4-5′-gaagtctg-caactcgacttc-3′; P7-5′-cgtccttcatcggctctt-3′) that prime at the 5′ and 3′ ends of the 16S rRNA gene, 16S-23S intergenic spacer region and the beginning of the 23S rRNA gene were used. PCR amplification of phytoplasma DNA from insect tissue was done according to the procedure of Tymon *et al*[7]. PCR products were analyzed by 1.5% horizontal agarose gel electrophoresis. 10 µL of the PCR product was digested with 5 units each of *Alu*I, *Bcl*I, *Hind*III and

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*Rsa*I (Genei, Bangalore) in a final reaction volume of 25 µL with 1 × concentration of appropriate buffers as per the manufacturer's instruction[8]. The PCR product from KWD palms was gel purified using the ultra pure PCR product clean-up kit (AB gene) and ligated into pTZ57R/T vector (Promega).

For sequence analysis, the plasmid DNA was isolated using Nucleospin® plasmid DNA purification column (Macherey-Nagel) as per the manufacturer's instructions. Clone of P4/P7 was subjected to automated sequencing of Applied Biosystems (ABI prism, Model 377, version 3.0, Bangalore Genei). Cladogram was constructed using Bio-edit sequence analysis.

The purified DNA fractions from the insect tissues (*P. moesta* & *S. typica*) were subjected to polymerase chain reaction (PCR) using the universal primers P1/P6, P1/P7 and P4/P7. The electrophoretic profiles of the amplified products of test DNA from the insects, *P. moesta* and *S. typica*, are demonstrated Fig. 1. The PCR product of the test DNA from the tissue of *P. moesta* collected from the KWD palms resolved a prominent band of 650 bp for the primer pair P4/P7 at annealing temperature of 53°C. However, the primer pairs P1/P6, P1/P7 had no amplification in the test DNA. Thus, the presence of 650 bp in the phytoplasma DNA from *P. moesta* is a clear indication of it being an insect vector of KWD phytoplasma. But in the case of lace bug, *S. typica*, no amplification was noticed for all the three sets of primers used, revealing that *S. typica* is a non-vector of KWD phytoplasma. The abundance of the plant hopper, *P. moesta*, on the diseased pockets of coconut palm further supports the affinity of the insect in sucking the cell sap. Also, no product was amplified in the test DNA from the insect tissues collected from healthy palms of the disease free areas, which again supports the role of *P. moesta* as the vector of KWD phytoplasma.

Further, a discrete fragment of 650 bp was amplified in all test DNA from diseased palms and confirms the similarity in amplification pattern (Fig. 2). The DNA fragment 650 bp amplified from KWD palms and from plant hopper, *P. moesta*, very well matches with the expected PCR product published earlier for KWD phytoplasma using the same primer pair P4/P7[2]. Based on the restriction digestion of 650 bp using *Alu*I and *Hind*III, the restriction profile showed a pattern of 69, 86 and 400 bp for *Alu*I, and 69, 121 and 451 bp for *Hind*III (Fig. 3). The restriction analysis showed no sign

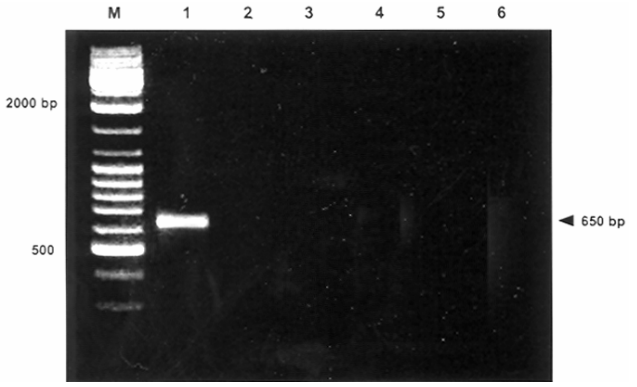

Fig. 1—Agarose gel electrophoresis of the PCR products amplified from the test DNA of *P. moesta* and *S. typica* using universal primers: M, Marker DNA ladder; lane 1, *P. moesta*-P4/P7; lane, -2P1/P6; lane 3, -P1/P7; lane 4, *S. typica*-P4/P7; lane2, -P1/P6; and lane 6, -P1/P7.

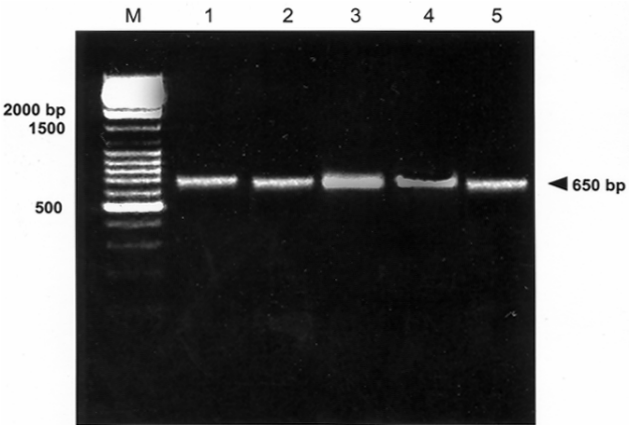

Fig. 2—Agarose gel electrophoresis of 650 bp PCR product amplified from DNA of KWD palms using universal primer pair P4/P7: lanes 1-5, 650 bp amplification; and M, DNA ladder.

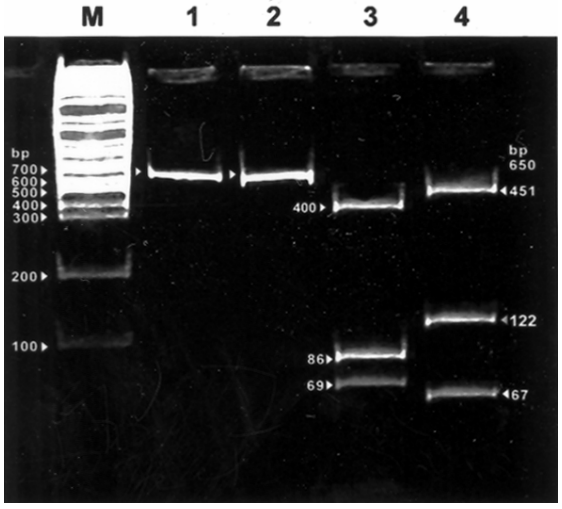

Fig. 3—Polyacrylamide gel electrophoresis of 650 bp digested with restriction enzymes: M, Marker DNA ladder; lane 1, *Bcl*I, lane 2, *Rsa*I; lane 3, *Alu*I; and lane 4, *Hind*III.

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   1 gaagtctgca actcgacttc tagggaactt gcgttggcca ttagcaccca tgccttgtcg
  61 ctaccaagct tcttcactca ctccagctcc tctatgagat ggcactgatt attgatgtgg
 121 tccatagcac tctttctatg gacctatcgg agatgagtaa cttcaattgt agcttcagaa
 181 agcttcttca aattttgaag cttaccctct gctttcttca gcttcttttt agcctgatga
 241 agctctctct ctttaggtcc ttaataatgg gttttaaagg aaggccctgg ggtagcaatg
 301 tggcatcgtc agggagtagg cgagtttatg attattcctc gcacgatgag cattctaaaa
 361 atgcaaacat gccacaggca aaaaaaaaga aaaaatatca tgtccattaa tagaaaaaat
 421 ttcattagaa taaaaaaaaa tgagaacaaa aatataaaaa acttcggact ctgatcacac
 481 gtgcagagac tacagatgcc ggtactcatc tccggctagg tccatacaaa gagagtgatg
 541 gaccacatca aggtcagagg tggggtaaaa aggagctgga gtggaagaac aatctagtta
a 601 tcgacaggcc tgcgcgctat agacacgctt gttctataaa ggcggttgga aacctcaga

   1 gaagtctgca actcgacttc tagggaactt gcgttggcca ttagcaccca tgccttgtcg
  61 ctaccaagct tcttcactca ctccagctcc tctatgagat ggcactgatt attgatgtgg
 121 tccatagcac tctttctatg gacctatcgg agatgagtaa cttcaattgt agcttcagaa
 181 agcttcttca aattttgaag cttaccctct gctttcttca gcttcttttt agcctgatga
 241 agctctctct ctttaggtcc ttaataatgg gttttaaagg aaggccctgg ggtagcaatg
 301 tggcatcgtc agggagtagg cgagtttatg attattcctc gcacgatgag cattctaaaa
 361 atgcaaacat gccacaggca aaaaaaaaga aaaaatatca tgtccattaa tagaaaaaat
 421 ttcattagaa taaaaaaaaa tgagaacaaa aatataaaaa acttcggact ctgatcacac
 481 gtgcagagac tacagatgcc ggtactcatc tccggctagg tccatacaaa gagagtgatg
 541 gaccacatca aggtcagagg tggggtaaaa aggagctgga gtggaagaac aatctagtta
b 601 tcgacaggcc tgcgcgctat agacacgctt gttctataaa ggcggttgga aacctcaga
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Fig. 4—Sequence of the PCR product of 650 bp: a. KWD phytoplasma, b. *P. moesta.*

of digestion for *Bcl*I, and *Rsa*I. Since the restriction enzyme *Bcl*I is site specific to chloroplast 16SrDNA, the absence of its cutting in 650 bp again indicates the phytoplasmic nature of DNA[9-11]. The presence or absence of the restriction sites of the enzyme *Rsa*I in the restriction analysis of phytoplasma 16SrRNA was used as a marker for differentiating the phytoplasma strain[2]. Hence, the absence of restriction sites for *Rsa*I in the 16S-23SrRNA, spacer region of KWD phytoplasma, is an indication of its strain difference. Fig. 4 (a & b) represents the sequential similarity of the PCR product of 650 bp. The sequence was further compared with 26 other coconut phytoplasmas so far sequenced and a cladogram was prepared (Fig. 5). The bootstrap value strongly supports the branches, indicating a robust tree whose branching order is in general agreement with previous branching[12-14]. From the cladogram, it is obvious that the phylogenetic position of KWD phytoplasma (AY158660) was in between the Mexican coconut lethal yellow group (gene accession no. AF024639) and Tanzanian coconut lethal decline (gene accession no. Y13913). Moreover, the tree branching pattern indicates that the KWD phytoplasma was evolutionarily closest to Mexican coconut lethal yellowing palms.

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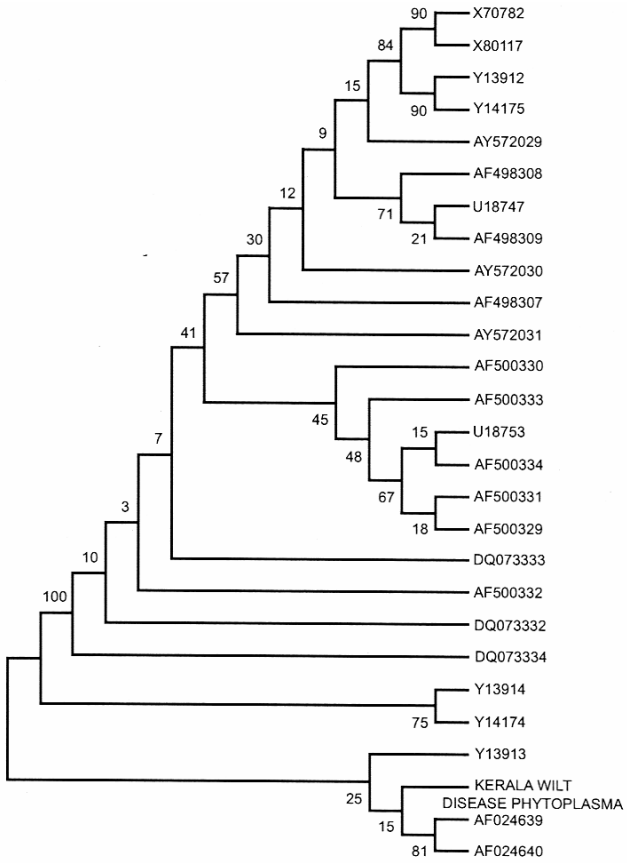

Fig. 5—Cladogram of 16SrRNA gene sequences of coconut phytoplasma and KWD phytoplasma. X70782: Lethal disease of coconut; X80117: Lethal yellowing-Tanzania; Y13912: Lethal yellowing-Ghnanian; Y14175: Lethal yellowing-Nigerian; AY472029: Lethal yellowing-Jamaica; AF498308: Lethal yellowing-Florida; U18747: Lethal yellowing-Florida; AF498309: Lethal yellowing- Florida; AY572030: Lethal yellowing-Jamaica; AF498307: Lethal yellowing-Jamaica; AY572031: Lethal yellowing-Jamaica; AF500330: Lethal yellowing-Mexico; AF500333: Lethal yellowing-Mexico; U18753: Lethal yellowing-Yucatan; AF500334: Lethal yellowing-Mexico; AF500331: Lethal yellowing-Mexico; AF500329: Lethal yellowing-Mexico; DQ073333: Lethal yellowing-Mexico; AF500332: Lethal yellowing-Mexico; DQ073332: Lethal yellowing-Mexico; DQ073334: Lethal yellowing-Mexico; Y13914: Lethal yellowing-Ghnanian; Y14174: Lethal yellowing-Nigeria; Y13913: Lethal yellowing-Tanzania; AF024639: Lethal yellowing: Florida; AF024640: Lethal yellowing-Mexico. The numbers on the branches are bootstrap values (confidence values)

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