Identity of *Phytophthora* associated with arecanut and its relationship with rubber and cardamom isolates based on RFLP of PCR-amplified ITS regions of rDNA and AFLP fingerprints

Arecanut (*Areca catechu* L.) is the most profitable tree crop grown in humid tropics of India, realizing the highest economic returns per unit area. It is vital to small land holders as a source of sustainable income. It is grown over an area of 2,64,000 ha with an annual production of 313,000 t. The fruit rot, commonly known as ‘kole rogi’ or ‘mahali’ in local language, is a major yield-limiting factor, causing yield losses of up to 90% (ref 1).

The fungus causing fruit rot of arecanut was first described as *Phytophthora arecae* (Coleman) Pethybridge. However, the species concept for *P. arecae* has been controversial, since it was merged with *P. palmivora* (Butl.) Butl. and *P. meadii* McRae due to lack of sufficient diagnostic characteristics. Later, *P. arecae* and *P. meadii* were separated from *P. palmivora* based on sporangial morphology and lack of chlamydospore production. The current taxonomic key is also recognized *P. arecae* as a distinct species, which is morphologically close to *P. palmivora*. Serological, isozyme, and mtDNA-RFLP data indicated that *P. arecae* and *P. palmivora* are identical.

Minor differences in the lack of or lesser chlamydospore formation and production of irregular-shaped sporangia and sporangiophores for *P. arecae* as described were found to be within limits of isolate variability. The delimitation of *P. arecae* and *P. palmivora* remains an area of debate, with conflicting conclusions cited in the literature.

Later, *Phytophthora* isolates infecting arecanut were re-classified as *P. meadii* based on morphology and the same fungus has been found to cause abnormal leaf fall of rubber and fruit rot of cardamom. It is not clear whether *P. arecae* or *P. meadii* is the causal agent of fruit rot of arecanut. Further, intra-specific variation within isolates of *P. meadii* causing diseases of arecanut, rubber and cardamom has not been established due to paucity of suitable morphological criteria.

In addition to morphological criteria, molecular techniques have been found to be useful for detailed analysis of genetic variability within and between species. Current approaches have focused on endonuclease restriction digest analysis of the internal transcribed spacer region of ribosomal DNA (ITS-RFLP) and amplified fragment length polymorphism (AFLP) analysis. These methods have resolving powers between and within species. In the present study, ITS-RFLP and AFLP fingerprints were used to characterize *Phytophthora* species associated with fruit rot of arecanut in India. These were compared with isolates of *P. meadii* causing leaf fall of rubber and fruit rot of cardamom and *P. palmivora* isolates from cocoa and coconut.

*P. meadii* isolates from arecanut (40), rubber (5) and cardamom (5), *P. palmivora* from cocoa (30) and coconut (10) derived from different regions of Karnataka and Kerala were used in this study. Reference cultures of *P. arecae* from coconut in Indonesia (IMI 348339, 348342, 348347 and 348348), *P. meadii* from rubber in Sri Lanka (IMI 352862), Malaysia (IMI 330533) and India (IMI 335650) and from arecanut in India (IMI 352313 and 352314) were also examined for comparison. Active growth of each fungal isolate was obtained by culturing on potato carrot agar (PCA) medium at 25°C. After three days, 3 mm diameter mycelial plugs from the actively growing region of PCA were cut and inoculated into 100 ml of sterile V8 juice in a 250 ml flask. Cultures were incubated for 3 days at 25°C on orbital shaker (100 rpm). Mycelium was harvested by vacuum filtration and freeze-dried.

DNA was extracted from the fungal mycelium according to a method described elsewhere. PCR amplification of the ITS region of rDNA was performed using primers ITS1(5′-TCCGTAAGGTG-AACCTGGG-3′) and ITS4(3′-CCTCC-GCTTATGGATATGC-5′) according to the method of White et al. in Hybrid PCR Express for 34 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s and extension at 72°C for 1.5 min, with an initial denaturation of 4 min at 94°C, before cycling and final extension of 5 min at 72°C after cycling. A portion (5 μl) of the amplified product was run on 2% agarose gel in Tris–Borate–EDTA (TBE) buffer, stained with ethidium bromide and visualized under UV illumination. Amplified PCR product was digested with restriction enzymes, namely HinfI, MspI, HaeIII and RsaI. Restriction digestions were performed in 10 μl reaction containing 5 μl PCR product, 1 μl 10X restriction buffer, 3.6 μl PCR-grade water, 0.1 μl bovine serum albumin and restriction enzyme (3 U/reaction) and briefly centrifuged and incubated at 37°C overnight (16 h). Digestion products were electrophoresed in 2.5% LE agarose gels in TBE buffer, stained with ethidium bromide and visualized under UV illumination. The size of the restriction fragments was determined by comparison of the fragment migration distances with those of known marker fragments (100 bp molecular size ladder, Gibco BRL, UK).

AFLP analysis was carried out according to the method of Mueller et al. The restriction and ligation was carried out in a 20 μl reaction containing 500 ng genomic DNA, 0.2 μg adapter, 0.5 mM ATP, 2 μl 10X buffer, 20 units of Pst, 1 unit of T4 DNA ligase. The reaction mixture was incubated at 37°C for 4 h. The restricted/ligated DNA was precipitated by adding 80 μl of water, 50 μl 7.5 M ammonium acetate and 2 volumes of 300 μl 100% ice-cold ethanol followed by centrifugation at 13,000 g for 10 min. The pellet was rinsed with 70% ethanol, repelled by centrifugation at 13,000 g for 5 min, dried under vacuum and re-suspended in TE buffer (pH 8).

PCR amplification of digested/ligated DNA, using adaptor oligo ADA as primer, was carried out in 25 μl reaction mixture containing 50 ng DNA, 50 pmol primer, 0.2 mM dNTP, 25 μl 10X PCR buffer and 6 units of Tth enzyme. The reaction mixture was subjected to thermal cycling in Hybaid PCR Express. An initial denaturation for 4 min at 94°C was followed by 34 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, extension for 1.5 min at 72°C and a final extension step of 5 min at 72°C. A portion (5 μl) of the amplified product was electrophoresed on 2% agarose gel (Seakem, LE, FMC Bioproducts, UK) in TBE buffer, stained with ethidium bro-
mide and visualized under illumination. The presence of a smear of each sample was a check for successful amplification. The ADA pre-amplification PCR products were then diluted 1:100 in TE buffer and used as the DNA templates for amplification with AFLP primers. Six primers, AFLP-A (5’-GACTGGGTACATGCAAGGGTGA-3’), AFLP-B (5’-GACTGGGTACATGCAAGAGGA-3’), AFLP-C (5’-GACTGGGTACATGCAAGGCGTGA-3’), AFLP-D (5’-GACTGGGTACATGCAAGCTGCGTGA-3’), AFLP-E (5’-GACTGGGTACATGCAAGATGCGTGA-3’) and AFLP-F (5’-GACTGGGTACATGCAAGATGCGTGA-3’) were used, each with two selective bases at its 3’ end (underlined). Primers were used at 50 pmol final concentration, using the reaction mixture as for the ADA pre-amplification as given above. The thermal cycling programme was the same as that for the ADA pre-amplification. A sample (10 µl) PCR product was added to 5 µl gel-loading dye (bromphenol/sucrose) and electrophoresed in 2% agarose LE gel in TBE buffer.

PCR products from the isolates contained a single band and size of the amplified product was ca. 900 bp for P. palmivora (coconut and cocoa) and P. meadi (arecanut, rubber and cardamom) and reference cultures of P. arecae and P. meadi. Digestion of a 900 bp amplicon with MspI, a characteristic profile for P. palmivora and P. meadi, was generated (Figure 1). The results were similar irrespective of the enzyme used (Table 1). P. palmivora isolates from coconut and cocoa, and P. arecae (reference) had identical ITS–RFLP patterns which were different from P. meadi isolates. The isolates of P. meadi from arecanut, rubber, cardamom and reference P. meadi isolates had similar patterns (Figure 1). Within isolates of P. palmivora and P. meadi, no ITS variation was detected by restriction analysis among the strains studied.

The AFLP fingerprint patterns of P. palmivora, P. arecae and P. meadi isolates generated with primer E are shown in Figure 2. All isolates of P. palmivora from cocoa and coconut and isolates assigned to P. arecae showed identical patterns. The same was found to be true for isolates of P. meadi from arecanut, rubber and cardamom. Similar groupings were obtained, irrespective of the AFLP primer used.

The taxonomic position of P. arecae has been controversial since it was reported on arecanut in India20,29,30. This species has been separated from P. palmivora based on the presence of irregular sporangiophores, the absence or rare production of chlamydospores and larger oogonia and oospores2. Later, it was found that chlamydospores of P. arecae and P. palmivora were identical, indicating chlamydospore character cannot be used as a criterion for separating P. arecae and P. palmivora3. Recent studies indicate that P. arecae and P. palmivora could be distinguished based on the production of almost spherical to oblong, occasionally intercalary sporangia with distorted shapes, forming sympodia only in water5. Isozyme6 and mtDNA–RFLP8 analyses indicated that isolates of P. arecae and P. palmivora were indistinguishable. The ITS–RFLP and AFLP patterns obtained in this study clearly showed that P. palmivora (from cocoa and coconut in India) and P. arecae (derived from coconut in Indonesia) were identical. Phytophthora isolates from coconut in Indonesia have been assigned to P. arecae based on variable production of chlamydospores and growth at 35°C, but there was caution that the status of these isolates should be decided after subjecting them to molecular analysis11.

P. faberi has been reported as a causal agent on rubber in Sri Lanka22. Later, P. faberi isolates were merged with

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![Figure 1](image1) Restriction banding pattern of ITS--rDNA digested with MspI. Lane 1, Size marker (bp ladder); lane 2, P. arecae reference isolate (IMI 348342); lanes 3 and 4, P. palmivora (cocoa); lane 5, P. meadi reference isolate (IMI 330533); lanes 6 and 7, P. meadi (arecanut); lane 8, P. meadi (rubber); lane 9, P. meadi (cardamom).

![Figure 2](image2) Amplified fragment length polymorphism fingerprint of P. meadi and P. palmivora isolates after selective amplification with primer E. Lanes 1 and 16, Marker (kb ladder); lanes 2–9, P. meadi (arecanut); lane 10, P. meadi reference isolate (IMI 352314); lane 11, P. meadi (cardamom); lane 12, P. meadi (rubber); lanes 13, 14, P. palmivora (cocoa); lane 15, P. arecae reference isolate (IMI 348342).

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<th>Table 1. Restriction fragment size (in bp) of Phytophthora ITS regions of rDNA digested with restriction enzymes</th>
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P. meadii under P. palmivora. Subsequent studies separated P. meadii isolates from P. palmivora. P. meadii was first reported from India in 1918 from infected rubber trees and later it was reported as a causal agent on arecanut and cardamom. P. meadii was distinguished from P. palmivora by production of spherical-to-ovoid, caducous sporangia with medium length pedicels (P. palmivora sporangia have short pedicels), formation of sporangia on sympodium, rare production of chlamydospores and formation of aplerotic oospores. P. meadii isolates also differ from P. palmivora in isozyme patterns. The present study indicates that arecanut (P. meadii) isolates differed from those assigned to P. arecae and P. palmivora in ITS–RFLP and AFLP patterns. PCR-amplified ITS regions of a large number of isolates belonging to P. arecae, P. palmivora and P. meadii have been sequenced, and variations were comparable to RFLP of PCR-amplified ITS regions. Pathological tests showed that P. palmivora isolates from cocoa and coconut did not infect arecanut; P. meadii isolates from arecanut did not infect coconut and cocoa, but caused infection on rubber and cardamom. P. arecae and P. palmivora have been reported to be con-specific based on morphological, physiological and biochemical criteria. Earlier, many isolations from arecanut in India were erroneously assigned to P. arecae (syn P. palmivora) because P. meadii was not considered by some researchers to be distinct. Using morphological criteria, all the Phytophthora isolates infecting arecanut were re-classified as P. meadii.

In this study, P. meadii isolates from arecanut, rubber and cardamom were found to be uniform based on ITS–RFLP and AFLP patterns. Earlier studies based on morphology also showed that P. meadii isolates from these crops were identical. From these results, it is derived that P. meadii may originally have been a causal agent of fruit rot of arecanut in India since 1918 (refs 2, 29), as arecanut is a traditional crop. Later, this pathogen might have moved from arecanut to rubber and cardamom, when these crops were introduced subsequently into India. Thus, P. meadii is the main pathogen causing fruit rot of arecanut in India and there is no evidence of occurrence of P. arecae.

35. Sarsawathy, N., Ph D thesis, Mangalore University, 1993, p. 120.

Received 7 May 2003; revised accepted 8 July 2003

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