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 3,13,000 t. The fruit rot, commonly known as 'kole roga' 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[2]. 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 characters[3]. Later, *P. arecae* and *P. meadii* were separated from *P. palmivora* based on sporangial morphology and lack of chlamydospore production[4]. The current taxonomic keys[5,6] also recognized *P. arecae* as a distinct species, which is morphologically close to *P. palmivora*. Serological[7], isozyme[8] and mtDNA–RFLP[9] 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[4] 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[3,4,9–14].

Later, *Phytophthora* isolates infecting arecanut were re-classified as *P. meadii*[15] 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[1].

In addition to morphological criteria, molecular techniques have been found to be useful for detailed analysis of genetic variability within and between species[9,12,13,16–19]. Current approaches have focused on endonuclease restriction digest analysis of the internal transcribed spacer region of ribosomal DNA (ITS–RFLP)[20–24] and amplified fragment length polymorphism (AFLP) analysis[20,25]. 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 325862), 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[26]. PCR amplification of the ITS region of rDNA was performed using primers ITS1(5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4(3′-CCTCCGCTTATTGATATGC-5′) according to the method of White *et al.*[27] in Hybaid 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 *Hin*fI, *Msp*I, *Hae*III and *Rsa*I. 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.*[28]. 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, I 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, repellated 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′-GACTGCGTACATGCAGGT-3′), B (5′-GACTGCGTACATGCAGGA-3′), C (5′-GACTGCGTACATGCAGGC-3′), D (5′-GACTGCGTACATGCAGAC-3′), E (5′-GACTGCGTACATGCAGAG-3′) and F(5′-GACTGCGTACATGCAGCG-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 (bromophenol/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. meadii* (arecanut, rubber and cardamom) and reference cultures of *P. arecae* and *P. meadii*. Digestion of a 900 bp amplicon with *Msp*I, a characteristic profile for *P. palmivora* and *P. meadii*, 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. meadii* isolates. The isolates of *P. meadii* from arecanut, rubber, cardamom and reference *P. meadii* isolates had similar patterns (Figure 1). Within isolates of *P. palmivora* and *P. meadii*, no ITS variation was detected by restriction analysis among the strains studied.

The AFLP fingerprint patterns of *P. palmivora*, *P. arecae* and *P. meadii* 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. meadii* 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 India[2,20,29,30]. 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 oospores[4]. 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. palmivora*[3]. Recent studies indicate that *P. arecae* and *P. palmivora* could be distinguished based on the production of almost spherical to obturbinate, occasionally intercalary sporangia with distorted shapes, forming sympodia only in water[5]. Isozyme[8] and mtDNA–RFLP[9] 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 analysis[31].

P. faberi has been reported as a causal agent on rubber in Sri Lanka[32]. Later, *P. faberi* isolates were merged with

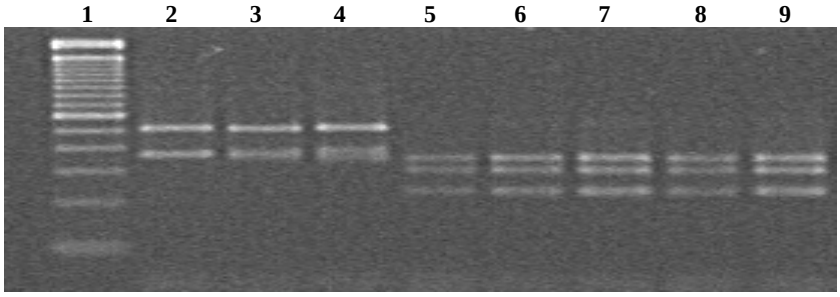

Figure 1. Restriction banding pattern of ITS–rDNA digested with *Msp*I. Lane 1, Size marker (bp ladder); lane 2, *P. arecae* reference isolate (IMI 348342); lanes 3 and 4, *P. palmivora* (cocoa); lane 5, *P. meadii* reference isolate (IMI 330533); lanes 6 and 7, *P. meadii* (arecanut); lane 8, *P. meadii* (rubber); lane 9, *P. meadii* (cardamom).

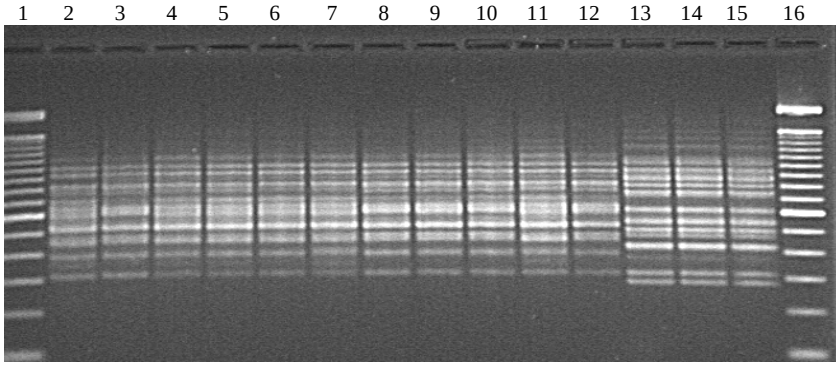

Figure 2. Amplified fragment length polymorphism fingerprint of *P. meadii* and *P. palmivora* isolates after selective amplification with primer E. Lanes 1 and 16, Marker (kb ladder); lanes 2–9, *P. meadii* (arecanut); lane 10, *P. meadii* reference isolate (IMI 352314); lane 11, *P. meadii* (cardamom);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

Table 1. Restriction fragment size (in bp) of *Phytophthora* ITS regions of rDNA digested with restriction enzymes

Phytophthora species	*Hin*fI	*Msp*I	*Hae*III	*Rsa*I
P. arecae	310, 240, 180, 170	510, 390	900	410, 390, 100
P. palmivora	310, 240, 180, 170	510, 390	900	410, 390, 100
P. meadii	350, 200, 180, 170	370, 330, 200	500, 300, 100	410, 110

P. meadii under *P. palmivora*[3]. Subsequent studies separated *P. meadii* isolates from *P. palmivora*[4]. *P. meadii* was first reported from India in 1918 from infected rubber trees[33] and later it was reported as a causal agent on arecanut and cardamom[15]. *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[12,34]. *P. meadii* isolates also differ from *P. palmivora* in isozyme patterns[8]. 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[21]. 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[35]. *P. arecae* and *P. palmivora* have been reported to be con-specific based on morphological, physiological and biochemical[12,13] 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[36]. Using morphological criteria, all the *Phytophthora* isolates infecting arecanut were re-classified as *P. meadii*[15].

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[15]. 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*.

1. Sarma, Y. R., Chowdappa, P. and Anandaraj, M., in *IPM System in Agriculture: Key Pathogens and Diseases* (eds Upadhyay, R. K. *et al.*), Aditya Books Pvt Ltd, New Delhi, 2002, vol. 8, pp. 149–187.
2. Butler, F. J., *Mem. Dept. Agric. India, Bot. Ser.*, 1910, 3, 221–280.
3. Tucker, C. M., *Univ. Mo., Agric. Exp. Stn. Res. Bull.*, 1931, **153**, 1–207.
4. Waterhouse, G. M., *Mycol. Pap.* (*CMI*), 1963, **92**, 1–22.
5. Newhook, F. J., Waterhouse, G. M. and Stamps, D. J., Mycological paper No. 143, 1978, London.
6. Stamps, D. J., Waterhouse, G. M., Newhook, F. J. and Hall, G. S., Mycological Papers 162, CAB International Mycological Institute, Kew, London, 1990.
7. Merz, W. G., Burell, R. G. and Gallegly, M. E., *Phytopathology*, 1969, **59**, 367–370.
8. Mchau, G. R. A. and Coffey, M. D., *Mycol. Res.*, 1994, **98**, 1035–1043.
9. Forster, H., Oudemans, P. and Coffey, M. D., *Exp. Mycol.*, 1990, **14**, 18–31.
10. Leonian, L. H., *W. Va. Univ. Agric. Exp. Stn. Bull.*, 1934, **262**, 36.
11. Ploetz, R. C. and Mitchell, M. J., *Plant Dis.*, 1999, **73**, 266–269.
12. Oudemans, P. and Coffey, M. D., *Mycol. Res.*, 1991, **95**, 1025–1046.
13. Oudemans, P. and Coffey, M. D., *Mycol. Res.*, 1991, **95**, 9–30.
14. Thomas, K. M., Ramakrishnan, T. S., Soumini, C. K. and Balakrishnan, M. S., *Proc. Indian Acad. Sci.*, 1947, **B26**, 147–163.
15. Sastry, M. N. L. and Hegde, R. K., *Curr. Sci.*, 1987, **56**, 367–368.
16. Mchau, G. R. A. and Coffey, M. D., *Mycol. Res.*, 1995, **99**, 89–102.
17. Mills, S. D., Forster, H. and Coffey, M. D., *Mycol. Res.*, 1991, **95**, 31–48.
18. Nygaard, S. L., Elliot, C. K., Cannon, S. J. and Maxwell, D. P., *Phytopathology*, 1989, **79**, 773–780.
19. Old, K. H., Maran, G. F. and Bell, J. S., *Can. J. Bot.*, 1984, **62**, 2016–2022.
20. Chowdappa, P., Brayford, D., Smith, J. J. and Flood, J., *Curr. Sci.*, 2003, **84**, 101–104.
21. Cooke, D. E. L. and Duncan, J. M., *Mycol. Res.*, 1997, **101**, 667–677.
22. Cooke, D. E. L., Kennedy, D. M., Guy, D. C., Russell, J., Unkles, S. E. and Duncan, J. M., *Mycol. Res.*, 1996, **100**, 297–303.
23. Crowford, A. R., Bassam, B. J., Drenth, A., Maclean, D. J. and Irwin, J. A. G., *Mycol. Res.*, 1996, **100**, 437–443.
24. Lee, S. B. and Taylor, J. W., *Mol. Biol. Evol.*, 1992, **9**, 636–653.
25. Neill, N. R., van berkum, P., Lin, J. J., Kuo, J., Ude G. N., Kenworthy, W. and Saunders, J. A., *Phytopathology*, 1997, **87**, 745–750.
26. Raeder, U. and Broda, P., *Lett. Appl. Microbiol.*, 1985, **1**, 17–20.
27. White, T. J., Bruns, T., Lee, S. and Taylor, J., in *PCR Protocols – A Guide to Methods and Applications* (eds Inns, M. A. *et al.*), Academic Press, San Diego, 1990, pp. 315–322.
28. Mueller, U. G., Lipari, S. E. and Milgroom, M. G., *Mol. Ecol.*, 1996, **5**, 119–122.
29. Butler, F. J., *Mem. Dept. Agric. India, Bot. Ser.*, 1907, **1**, 82–84.
30. Coleman, L. C., *Bull. Dept. Agric. Mysore, Mycol. Ser.*, 1910, **2**, 1–92.
31. Hall, G. and Warokka, J. S., in *Coconut Phytophthora*, CIRD-CP, Montpellier, 1992, pp. 69–70.
32. Gadd, C. H., *Ann. R. Bot. Gard. Peradeniya*, 1924, **9**, 47–84.
33. McRae, H., *Agric. J. India*, 1918, **14**, 566–577.
34. Dantanarayana, D. M., Peries, O. S. and Liyanage, A de S., *Trans. Br. Mycol. Soc.*, 1984, **82**, 113–126.
35. Sarsawathy, N., Ph D thesis, Mangalore University, 1993, p. 120.
36. Holiday, P., *Fungus Diseases of Tropical Crops*, Cambridge Univ. Press, Cambridge, UK, 1980, p. 607.

Received 7 May 2003; revised accepted 8 July 2003

P. CHOWDAPPA[†,*]
D. BRAYFORD[#]
J. SMITH[#]
J. FLOOD[#]

[†]*Central Horticultural Experiment Station, Hirehalli 572 168, India*
[#]*CABI Bioscience UK Centre, Bakeham Lane, Egham, Surrey, TW20 9TY, UK*
**For correspondence.*
e-mail: pallem@rediffmail.com