Molecular discrimination of *Phytophthora* isolates on cocoa and their relationship with coconut, black pepper and bell pepper isolates based on rDNA repeat and AFLP fingerprints

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Phytophthora palmivora isolates from cocoa and coconut and *P. capsici* isolates of cocoa, black pepper and bell pepper collected from different localities of India were examined at the molecular level using the ITS1 and ITS2 primers to amplify the internal transcribed spacer (ITS) regions of rDNA gene repeat and by AFLP analysis. An isolate of *P. capsici* (P575) from cocoa in Mexico was also included for comparison. ITS-RFLP revealed consistent polymorphisms that are correlated to morphological description. The AFLP fingerprints of *P. palmivora* from cocoa and coconut showed similar patterns but amongst the isolates of *P. capsici*, four AFLP fingerprint groups were evident that were distinct from the *P. palmivora* type. Thus, ITS regions can be used as taxonomic markers for the identification of *Phytophthora* associated with black pod disease of cocoa and AFLP fingerprints are useful for assessing intra-specific population variation.

COCOA (*Theobrama cacao* L.) is mainly cultivated as an understorey crop in arecanut (*Areca catechu* L.) and coconut (*Cocos nucifera* L.) gardens in India. At present, cocoa is grown over an area of 14,193 ha with an annual production of 5592 tonnes, well short of the domestic requirement of 14,000 tonnes[1]. The projected demand by the processing industries is 30,000 tonnes by 2005, thus necessitating a four-fold increase[1]. In India, black pod disease is a major production constraint, causing crop losses ranging from 20 to 30 per cent[2]. The etiological agent of this disease was identified as a species of *Phytophthora* when it was reported from India[3]. Recent taxonomic studies have indicated that in addition to *P. palmivora*[4], *P. capsici*[5] and *P. citrophthora*[6] also cause this disease in certain localities of Kerala. Further, *P. palmivora* (causal agent of bud rot coconut) and *P. capsici* (foot rot of black pepper and betel vine) are also prevalent in the palm-based cropping systems[7]. These species were mainly identified using morphological criteria[8] but has limitations in understanding genetic diversity and genetic distance between fungal species in the cropping systems.

Electrophoretic protein banding patterns[2,9], isozyme profiles[10] and repetitive DNA polymorphism of total DNA[11,12] and restriction fragment length polymorphism (RFLP) analysis of mitochondrial and nuclear DNA[13] have been used to estimate intra- and inter-specific relatedness in *Phytophthora* of cocoa. Analysis of mtDNA[13] and isozyme patterns distinguished the electrophoretic types within *P. palmivora* isolates from a wide variety of hosts and two distinct subpopulations (Cap A and Cap B) within *P. capsici* isolates of cocoa. Protein electrophoresis is often faced with insufficient resolution because of low variation in allozyme markers. Requirement of large amount of DNA, difficulty in standardization of technique, variation in sensitivity and generation of relatively small number of polymorphisms are the limitations of RFLP. In recent years, several researchers[14–16] have demonstrated the use of ribosomal gene repeat (rDNA) in studying variation in *Phytophthora*. Polymorphisms have been revealed either by restriction digestion of the amplified product or by sequencing a part or this entire region[14]. The ITS1 and ITS2 sequences of the *P. palmivora* and *P. capsici* have been published and detected excellent variation only at the species level[14,16].

A recently developed powerful PCR-based technique, amplified fragment length polymorphism (AFLP), reported to be free of PCR artifacts by retaining time efficiency, has been shown to be more useful in identifying molecular markers in symbiotic fungi cultured by the fungus growing ant (*Cyphomyrex minutus*)[17] and *Colletotrichum* pathogens of alfalfa[18]. AFLP has been found to be more useful for assessing intra-specific population variation and determining race/cultivar specificity and virulence.

The objectives of the present study were to characterize *Phytophthora* species associated with black pod disease in India based on ITS regions of rDNA for rapid identification and to investigate their relationships with *P. palmivora* of coconut and *P. capsici* from black pepper and bell pepper using AFLP fingerprints.

P. palmivora from cocoa (31 isolates) and coconut (10) and *P. capsici* from black pepper (10), bell pepper (05) and cocoa (05) derived from different places of Karnataka and Kerala were used in this study. An isolate of *P. capsici* (P575) from cocoa in Mexico was also included for comparison.

Phytophthora isolates were grown in 100 ml of sterile V8 juice in a 250 ml flask for 72 h at 25°C on orbital shaker (100 rpm). DNA was extracted from the frozen fungal mycelium by a slightly modified method of Raeder and Broda[19] by the addition of 20 mg/ml RNAase and incubation at 37°C for 10 min after the phenol : chloroform : isoamyl alcohol (25 : 24 : 1) precipitation. This was followed by precipitation with 0.54 volumes of isopropyl alcohol and centrifugation at 10,000 g for 2 min.

The pellet was rinsed with 70% alcohol, dried under vacuum and then the pellet was resuspended in 30 µl of TE buffer (pH 8). DNA was stored at – 20°C.

PCR amplification of the ITS region was performed using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC)[20] in Hybaid PCR Express by 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. Amplified PCR products were separated in 2% agarose gel in Tris-Borate-EDTA (TBE) buffer and visualized under UV after staining with ethidium bromide (0.5 µg/ml).

Amplified PCR product was digested with restriction enzymes namely HinfI, MspI, AluI, HaeIII, RsaI, TaqI and EcoRV. PCR product (5 µl) was digested at 37°C overnight (16 h) with 3 units of enzyme in 10 µl reaction mixture. Restriction products were analysed in 2.5% agarose gels as described above. The size of the restriction fragments was estimated by comparison with 100 bp molecular size ladder.

Amplified fragment length polymorphism (AFLP) was analysed according to the method of Muller et al.[17] Fungal genomic DNA (500 ng) was digested by Pst (20 units), ligated to Pst adaptor at 37°C for 4 h and pre-amplification was carried out at 94°C for 4 min for initial denaturation 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. The pre-amplification PCR products were then diluted 1 : 100 in TE buffer and amplified with AFLP primer E adopting the above thermal cycling parameters. The PCR products were electrophoresed in 2% agarose gels in TBE buffer, and visualized under UV after staining with ethidium bromide.

Primers ITS1 and ITS4 were used to amplify the entire ITS1, 5.8 ribosomal gene and ITS2. PCR products from the isolates contained a single band and size of the amplified product was 900 bp for P. palmivora (Figure 1) and 890 bp for P. capsici.

The amplified products were digested with five restriction enzymes (HinfI, MspI, AluI, HaeIII, RsaI and EcoRV). They detected polymorphisms in the ITS region of both Phytophthora species and resolved individual species in every case (Figure 2). The restriction enzyme EcoRV had no restriction sites in the ITS region of both Phytophthora species. Within the isolates of P. palmivora and P. capsici, no ITS variation was detected by restriction analysis (Figure 2). Restriction fragment sizes of the ITS region of rDNA digested with different enzymes are presented in Table 1. The band sizes were estimated according to the mobility of all standards and total of all bands in each isolate was calculated. The combined sizes of all the fragments were larger than the PCR product in P. capsici isolates when digested with AluI.

The AFLP fingerprints generated with primer E are shown in Figures 3 and 4. All the isolates of P. palmivora from coconut and cocoa showed identical patterns (Figure 3). Among the isolates of P. capsici, four AFLP fingerprint groups were evident that were different from the P. palmivora type (Figure 4). Group I includes Indian isolates from cocoa. Another isolate (Mexican) from cocoa represents group II. Isolates from black pepper fall under group III. The bell pepper isolates formed group IV.

The two species (P. palmivora and P. capsici), segregates of the P. palmivora complex[21] which have caducous sporangia and amphigynous antheridia, have been found to cause black pod disease in India[5,6]. Although these species were distinguishable by characteristic sporangial pedicel length, this character has not been useful to understand genetic diversity and genetic relatedness between them. The present study shows that these species can easily be discriminated by restriction digestion patterns of PCR amplified ITS region of rDNA (Figure 2), providing further support for their separation based on morphological, electrophoretic protein patterns, restric-
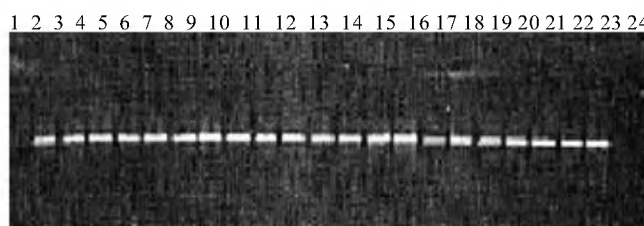

Figure 1. PCR product amplified with primer pair ITS1 and ITS4. Lanes 1 and 24, size marker (bp ladder); Lanes 2–22, P. palmivora isolates; Lane 23, control.
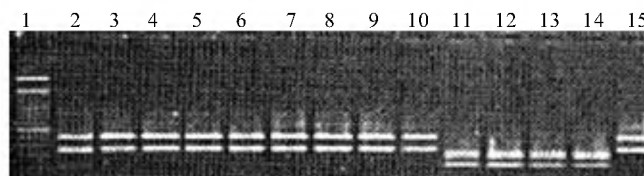

Figure 2. Restriction banding patterns of the ITS rDNA digested with MspI. Lane 1, size marker (bp ladder); Lanes 2–10, P. palmivora (cocoa); Lanes 11–14, P. capsici (cocoa); Lane 15, P. palmivora (coconut).

Table 1. Restriction fragment size (in base pairs) of ITS region digested with restriction enzymes

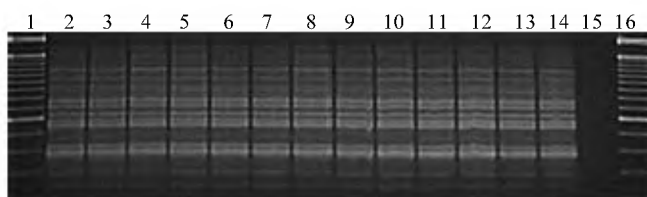|	Restriction fragment size	
Enzyme	P. palmivora	P. capsici
HinfI	310, 240, 180, 170	280, 180, 160, 140, 130
MspI	510, 390	350, 340, 120
HaeIII	900	590, 300
AluI	500, 180, 170, 50	600, 500, 180, 170
RsaI	410, 390, 100	400, 300, 100
TaqI	300, 150, 100, 75, 50	250, 200, 150, 100, 75

Figure 3. AFLP fingerprints of *P. palmivora* from cocoa and coconut. Lanes 1 and 16, size marker (bp ladder); Lanes 2–10, cocoa; Lanes 11–14, coconut isolates; Lane 15, control.
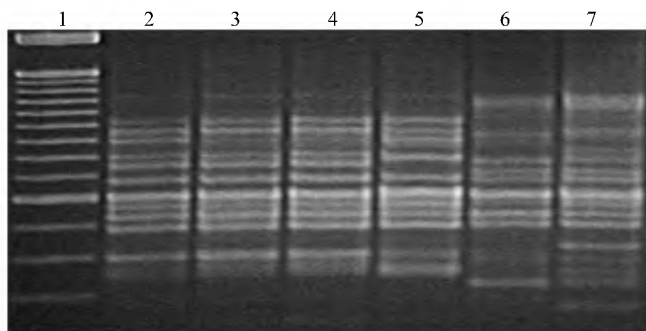

Figure 4. AFLP fingerprints of *P. capsici*. Lane 1, size marker (bp ladder); Lanes 2–5, *P. capsici* (cocoa); Lane 6, *P. capsici* (black pepper); Lane 7, *P. capsici* (bell pepper).

tion digestion patterns of total DNA and serological reactions[11]. Indeed, several workers[14–16] have shown the usefulness of ITS regions for rapid identification of *Phytophthora* isolates by digesting ITS amplification products by restriction enzymes and comparing electrophoretic patterns of the resulting fragments.

A large variation in the sizes and shapes of sporangia between isolates from cocoa and coconut has been reported[21,22]. The isozyme study[10] indicated that cocoa isolates lacked genetic diversity, whereas a limited number of isolates from coconut and one from durian displayed much greater diversity. Further, isozyme studies[23], which included additional isolates from coconut and durian, showed the highest level of genetic diversity amongst the *P. palmivora* isolates from coconut and durian from Indonesia, Malaysia and Thailand. Earlier studies[24] demonstrated the existence of distinct glucose phosphate isomerase isozyme subgroups (cocoa, coconut, fast, intermediate and slow types) within *P. palmivora* causing bud rot of coconut in Indonesia. Our results showed that *P. palmivora* isolates of cocoa and coconut had identical restriction digestion patterns of ITS regions of rDNA and AFLP fingerprints. Pathogenicity studies indicated that *P. palmivora* isolates from cocoa and coconut are cross-inoculable (Chowdappa *et al.*, unpublished). *P. palmivora* has been known as causal agent of bud rot on coconut in India since 1906 (ref. 25). When commercial cultivation of cocoa was started as mixed crop in coconut gardens in the sixties[26], *P. palmivora* isolates might have been

moved from coconut to cocoa and resulted in the appearance of black pod for the first time[3] in 1965. This lack of genetic diversity coupled with similar morphological and pathological features suggests the presence of clonal population of *P. palmivora* pathogenic to coconut and cocoa in India.

P. capsici has been described[27] as having generally ovoid sporangia with extremely variable sizes and brown coloured zoospores, lacking chlamydospores being pathogenic to pepper plants (*Capsicum annum* L). Later, many isolates of *Phytophthora* from other hosts, which exhibited similar morphological features, have been included under the species *P. capsici*. *Phytophthora* isolates from tropical plantation crops such as black pepper and cocoa, previously described as *P. palmivora* MF4 and some of which formed chlamydosopres, were re-designated as an amended description of this species[28]. In contrast, a new species, *P. tropicalis* (previously identified as *P. capsici*) for isolates of tropical origin on morphological grounds has been proposed[29]. Later, it was found that three isozyme subgroups[10] within *P. capsici* CAP1, CAP2 and CAP3 and isolates described as *P. tropicalis*[29] fell within both CAP2 and CAP3. Further studies on isozyme variability[23,30] have shown the presence of only two subgroups CAP A and CAP B within *P. capsici* from a wide array of hosts. They noted the occurrence of both CAP A and CAP B on black pepper and *Piper betle* and only CAP B on cocoa from India. This situation might have arisen from multiple introductions either on different hosts or at different time intervals. In the present study, AFLP fingerprints clearly showed the existence of two genetic groups on cocoa and they are genetically distinct from isolates of black pepper (Figure 3), supporting the existence of two subgroups within *P. capsici* from cocoa based on colony morphology, size and shape of sporangia, response to antibiotics and fungicides, serological reactions and pathogenicity[11].

Inoculation tests suggest that cocoa isolates infect the black pepper and vice versa (Chowdappa *et al.*, unpublished). Since *P. capsici* infect a wide number of crops grown near or under coconut or arecanut cropping systems in India (e.g. cocoa, black pepper, betelvine, bell pepper, brinjal, bauhinia, *Dolichos lablab* and *Ailanthus excelsa*), it is clearly important to examine the host specificity further and can correlate with AFLP fingerprints by examining a large number of isolates from these hosts to derive definite conclusions. Thus, the present study suggests that ITS regions of rDNA can be used as a taxonomic tool for rapid identification of *Phytophthora* causing black pod disease of cocoa in India and AFLP fingerprints are useful for assessing intra-specific population variation only in *P. capsici*.

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A case study of twilight probing of the atmosphere during Leonid meteor shower 2001

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Twilight sounding method is used to retrieve the vertical distribution of the dust particles throughout the middle atmosphere. The photometry observations of the twilight sky brightness were carried out during the Great Leonid meteor storm of November 2001 at a tropical inland station Pune (18°32′N, 73°51′E), India. This experiment gave an opportunity to monitor height distribution of meteoric dust between 16 and 150 km. Normalization of the current set of data with a reference data set shows the evidence of influx of the fine meteor dust with a broad peak at around 80 km in the evening twilight of November 18, the day of meteor storm peak, and its subsequent descent to the lower stratosphere. The magnitude of enhancement of dust due to this meteor shower at the peak of distribution is about seven times the normal.

MANY observers, ever since the discovery of the twilight phenomenon, have shown that the characteristics of the light scattered downward by the atmosphere during the twilight period are sensitive indicators of the aerosol component of the atmosphere. The twilight variations that occur as the solar depression angle changes can be used to derive information on the vertical distribution of atmospheric aerosols[1].

The twilight sounding method, involving ground-based photometry of the twilight zenith sky brightness, can be used to derive the vertical distribution of dust particles intruding the Earth's atmosphere during the active meteor showers[2], volcanic eruptions[3,4] and also aerosols forming within the atmosphere. This method is based on the fact that the luminosity of the twilight sky at a given moment depends on the momentary height of the Earth's shadow. Bulk of the scattered light comes to an observer from a narrow atmospheric layer. The lower cut-off of this layer is determined by the shadow of the solid Earth. The contribution of the rest of the atmosphere above this layer may be neglected due to an exponential decrease of air density.

Bartusek *et al.*[5], in Australia, carried out simultaneous twilight and Lidar observations of the atmospheric aerosols. The results of his comparative study showed a good confirmation of the validity of twilight data. The earlier results of twilight sounding method showed that some