Table 2. Response of ointment A1 and A2 on Tinea infection.

Clinical diagnosis	Fungal species isolated	No. of patients	Worse	No improvement	No. of patients showing improvement: Partial Ist Week	Partial IInd Week	Partial IIIrd Week	Significant Ist Week	Significant IInd Week	Significant IIIrd Week	Completely cured Ist Week	Completely cured IInd Week	Completely cured IIIrd Week
Ointment A1													
Tinea corporis	*Trichophyton rubrum*	7	–	–	1	–	–	4	1	–	2	4	1
	T. simii	5	–	–	2	–	–	3	2	–	–	3	2
Tinea capitis	*T. rubrum*	4	–	–	2	–	–	2	2	–	–	2	2
Tinea manum	*T. rubrum*	4	–	–	2	–	–	–	4	–	–	–	4
Ointment A2													
Tinea corporis	*T. simii*	4	–	–	–	–	–	3	1	–	1	2	1
	T. rubrum	1	–	–	–	–	–	–	–	–	1	–	–
	T. mentagrophytes	1	–	–	–	–	–	1	–	–	–	1	–
Tinea capitis	*T. rubrum*	2	–	–	–	–	–	2	–	–	–	2	–
Tinea manum	*T. rubrum*	2	–	–	–	–	–	2	–	–	–	2	–

Isolation and identification of active ingredient of *T. ammi* oil which is responsible for antidermatophytic activity, is in progress.

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Identification of *Phytophthora* species affecting plantation crops by RFLP of PCR-amplified internal transcribed spacer regions of ribosomal RNA

Arecanut, coconut, cocoa, rubber, black pepper, betel vine and vanilla are the most important plantation crops that have been seriously affected by *Phytophthora* species[1]. Destruction of black pepper by *P. capsici*; heavy loss of cocoa due to black pod and bud rot, and immature nut fall of coconut caused by *P. palmivora*; fruit rot of arecanut and cardamom, and abnormal leaf fall in rubber due to *P. meadii* are some of the destructive diseases of plantation crops caused by *Phytophthora*[1]. Some species cause the same symptoms on a particular host (e.g. *P. palmivora*, *P. megakarya*, *P. capsici* and *P. citrophthora* cause black pod on cocoa)[2], while others have a wide host range (e.g. *P. meadii* infects rubber, cocoa, arecanut and cardamom). As the plantation crops are grown together under high-density, multi-species cropping systems or in the vicinity of each other[1], rapid identification of *Phytophthora* species is of utmost importance.

P. arecae (*P. palmivora*), *P. capsici*, *P. meadii* and *P. nicotianae* are the major species that affect the plantation crops industry[2–5]. These species were mainly identified based on morphological features[6]. It is well recognized that identification of species is a highly specialized, time-consuming and difficult task. The limited number and plasticity of morpho-

logical markers makes species identification difficult. Accurate identification at reasonable time frame is important to offer suitable advice to farmers to adopt plant health legislation and to implement plant quarantine regulations. Ribsomal DNA (rDNA) genes are widely used for molecular taxonomy, as they consist of a mosaic of regions exhibiting various evolutionary rates. As they are highly conserved, the rDNA sequences can discriminate at the levels of orders and kingdoms. At the species level, spacers of the rDNA and mainly the noncoding internal transcribed spacers (ITS), ITS1 and ITS2, are widely used for phylogeny studies, as they usually vary between species within a genus, but show little or no intra-specific variation[7]. Molecular markers based on ITS regions of rDNA have been successfully employed to resolve at an appropriate taxonomic level[8–11]. The objective of the present study is to develop a simple molecular technique based on ITS region of rDNA for rapid identification of *Phytophthora* species affecting plantation crops.

Cultures of *P. capsici* (IMI325900) from cocoa, *P. nicotianae* (IMI 3406160 and IMI 235604), and *P. arecae* (*P. palmivora*) from coconut in Indonesia (IMI 348339, 348342, 348347 and 348348) and *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 examined in the present study. DNA was extracted by placing a small amount of aerial mycelium removed from three-day-old-fungal colonies grown on V8 agar plates at 25 ± 1°C into 100 µl in a 0.5 ml Eppendorf tube, subjected to microwave irradiation for 5 min, and then heating at 95°C for 10 min on a thermal cycler. The ITS region from mycelial extracts was amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (3′-TCCTCCGCTTATTGATATGC -5′). PCR was performed in a 50 µl reaction mixture containing two µl of DNA extract, 50 pmol of each primer, 200 µm each dNTP, 5 µl of 10X PCR buffer and 10 units of Tth enzyme, and the tube was quickly centrifuged. The reaction was performed 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 to determine the number and size of DNA products amplified in the PCR. The 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 gel in TBE buffer, stained with ethidium bromide and visualized under UV illumination. Prior to analysis on agarose gel, the enzyme digests (10 µl) were mixed with 5 µl loading dye (bromophenol blue/sucrose) and loaded on the gel. The size of the restriction fragments was determined by comparison of fragment migration distances with known marker fragments (100 bp molecular size ladder, Gibco BRL, UK).

When impure DNA extract from mycelial fragments was amplified with primers ITS1 and ITS4, it yielded a PCR product of 860 bp for *P. capsici*, 900 bp for *P. arecae* (*P. palmivora*) and 920 bp for *P. nicotianae* (Figure 1). The ITS–RFLP patterns of *P. arecae*, *P. capsici*, *P. meadii* and *P. nicotianae* generated with restriction enzyme *Rsa*I differed considerably from each other (Figure 2). Isolates of same species, however, showed identical banding patterns. The results are almost similar irrespective of the enzyme used (Table 1). Such profiles are

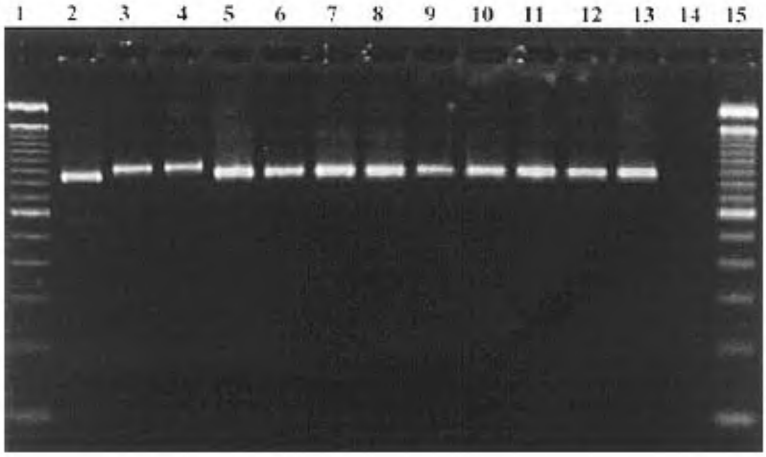

Figure 1. PCR amplification of ITS regions of rDNA. Lanes 1 and 15, Marker (100 bp ladder); lane 2, *P. capsici*; lanes 3–4, *P. nicotianae*; lanes 5–8, *P. arecae* (*P. palmivora*); lanes 9–13, *P. meadii*, lane 14, Control.

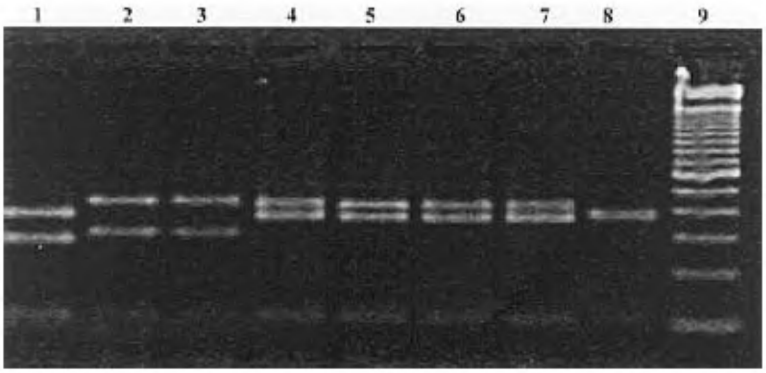

Figure 2. ITS–RFLP patterns generated with restriction enzyme *Rsa*I. Lane 1, *P. capsici* (325900); lanes 2–3, *P. nicotianae* (3406160 and 235604); lanes 4–7, *P. arecae* (348339, 348342, 348347 and 348348); lane 8, *P. meadii* (330533); lane 9, marker (100 bp ladder).

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

Species	*Hin*fI	*Msp*I	*Hae*III	*Rsa*I
P. arecae (*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. capsici	280, 180, 160, 140, 130	350, 340, 200	590, 300	400, 300, 100, 90
P. nicotianae	320, 240, 180, 170	510, 400	800, 110	410, 310, 100, 90

almost invariably species-specific and a comparison against a reference culture offers rapid identification.

There are few reports that PCR can be performed on fungal spores or mycelium following either freeze and thaw protocol[9,12], microwave irradiation[13] or using intact conidiophores as target DNA following initial denaturation step at 94°C[14,15]. Extraction procedures that depend on either toxic chemicals or costly DNA extraction kits can be avoided using this simplified procedure in *Phytophthora* identification. Although sequencing of ITS regions has been found to be useful in differentiation of various *Phytophthora* species[8–11,16], they are time-consuming and cost-prohibitive, and not possible in small laboratories. By using this method, a large number of isolates can be screened routinely and ITS profiles can be obtained within 24 h. Identification of *Phytophthora* based on morphology also requires considerable amount of time and microbiological expertise.

Several workers[5,12,16] 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. The simplified method proposed here allows direct identification of *Phytophthora* from agar plates and can be further adapted for diseased plant samples. Thus, this method, can be used as a taxonomic marker for rapid identification of *Phytophthora* species associated with diseases of plantation crops and also useful for disease diagnosis in plant health clinics and plant quarantine laboratories.

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