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Rapid and Accurate Species-Specific Detection of *Phytophthora infestans* Through Analysis of ITS Regions in Its rDNA

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Abstract Polymerase chain reaction (PCR) was used to specifically detect *Phytophthora infestans* by analyzing the sequences of the ribosomal internal transcribed spacer regions (ITS) in the rDNA of the *Phytophthora* species. Based on the sequence data, PISP-1 together with the ITS3 primer were used to detect *P. infestans*. A single ca. 450 bp segment was observed in *P. infestans*, but not in the other fungal or bacterial isolates. Two factors, the annealing temperature and template DNA quantity, were investigated to determine the optimal conditions. Using these species-specific primers, a unique band was obtained within annealing temperatures of 55°C–61°C and at template DNA levels of 10 pg–100 ng.

Key words: *Phytophthora infestans*, annealing temperature, ITS, rDNA

Phytophthora infestans (Mont) de Bary, the cause of the late blight disease in the potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill) which are members of the plant family Solanaceae, is an important plant pathogen. The populations of *P. infestans* in Central Mexico are highly diverse, indicating that this area is the center of the origin of the pathogen [7, 8, 11]. It is therefore probable that the late blight pathogen has migrated from this region to other parts of the world via airborne spores or infected potatoes [5, 10].

The identification of most fungi is principally based on their morphological characteristics. For several genera of fungi, including *Phytophthora*, an accurate identification of the species could be very difficult and time consuming, because the morphological features can vary significantly [6]. Major problems in accurately identifying species include the facts that most morphological features are common between species and pure culture conditions for identification are difficult to attain, and that the observation time is often too long. As a result, Clare [4] used electrophoretic pattern of proteins for differentiating *Pythiaceous* fungi, and isozyme and restriction fragment length polymorphism (RFLP) have also been used in identifying most fungi with similar characteristics [24, 26, 27, 29]. In particular, utilization of PCR is very effective, not only because PCR requires a small amount of DNA but also because of savings in time and labor [12, 33]. Using a rapid and convenient RAPD technique, Choi *et al.* [2, 3] and Lee *et al.* [18] analyzed the genetic diversities of the main soil-borne pathogens including *Fusarium* and *Rhizoctonia*. Kim *et al.* [16] also detected a *P. infestans*-specific molecular marker by RAPD fingerprinting. Recently, many researchers have studied the ITS regions to analyze the genetic differences and taxonomical relationship between the species of *Phytophthora* [17], *Pythium* [1], *Peronosclerospora* [34], *Verticillium* [20], and *Fusarium* [22], and mycorrihizae and rust [9], since their ITS regions are of a suitable and available size for PCR amplification, restriction analysis, and sequencing procedures [12, 28, 35].

If *P. infestans*, which is the factor most destructive to potato production, could be rapidly and accurately detected, this would be useful in the prediction and control of late blight, reduction of pesticide costs, harvest of healthy potatoes, and differentiation of *P. infestans* from other *Phytophthora* species. Therefore, this study was carried out to develop a PCR-based method, using the ITS II region, for a more rapid, sensitive, and accurate species-specific detection of *P. infestans* than that of previous works [15, 30, 31].

Fungal Strains and Genomic DNA Isolation

The isolates used in this study including *P. infestans* were either isolated from infected plants or were provided by other researchers (Table 1). The *P. infestans* isolates were cultured in a rye medium (60 g of rye grain and 20 g of dextrose) in 1 l of distilled water for 10 days at 18°C [13],

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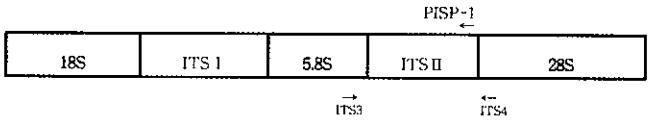
Table 1. Fungal isolates used for species-specific detection of *Phytophthora infestans*.

Isolate	Pathogen	Group	Host
P-9827	*Phytophthora infestans*	G IV	*Lycopersicon esculentum* MILL.
P-9829	*P. infestans*	G IV	*Lycopersicon esculentum* MILL.
P-9833	*P. infestans*	G IV	*Lycopersicon esculentum* MILL.
BF-8	*P. infestans*	G IV	*Lycopersicon esculentum* MILL.
BN-11	*P. infestans*	G IV	*Lycopersicon esculentum* MILL.
HY-1	*P. infestans*	G IV	*Solanum tuberosum* L.
KD-1	*Phytophthora* sp.	-	-
40401	*P. megasperma*	G V	*Lycopersicon esculentum* MILL.
40412	*P. sojae*	G V	*Glycine max* MERR.
40174	*P. cactorum*	G I	-
40182	*P. cinnamomi*	G VI	-
40184	*P. citricola*	G III	*Zizyphus jujuba* MILL.
40189	*P. cryptogea*	G VI	*Brassica campestris* subsp. *napus* var. *pekinensis* MAKINO
40157	*P. capsici*	G II	*Capsicum annuum* L.
96CC7116	*P. capsici*	G II	*Capsicum annuum* L.
KCT214	*Fusarium oxysporum*	-	*Cucumis sativus* L.
WS5-3	*Fusarium oxysporum*	-	-
R25	*Rhizoctonia solani*	-	*Solanum tuberosum* L.
R28	*Rhizoctonia solani*	-	*Solanum tuberosum* L.
E301629	*Erwinia carotovora* subsp. *atroseptica*	-	*Solanum tuberosum* L.
E301397	*Erwinia carotovora* subsp. *carotovora*	-	*Capsicum annuum* L.
P2108	*Pseudomonas solanacearum*	-	*Solanum tuberosum* L.

whereas the other *Phytophthora* species and fungal pathogen isolates were incubated in V8 juice (200 ml of V8 juice, 5 g of $CaCO_3$) and a PDB (potato dextrose broth, 20 g PDB and dextrose each) medium for 10 days at 24°C in darkness. The bacterial isolates were grown in a Luria-Bertani broth at 27°C overnight [25]. The total genomic DNA of each isolate was extracted by a modification of the method described by Goodwin *et al.* [12], and long-term stocks were maintained as an agar plug in 10% glycerol under liquid nitrogen [28]. The concentration of the RNase-treated DNAs was determined spectrophotometrically, or on 0.8% agarose gels stained with ethidium bromide.

ITS Amplification and Cloning

For the PCR amplification of the ITS II region (Fig. 1) of *P. infestans* and other *Phytophthora* species, 10 ng of each isolate's genomic DNA was added to 24 ml of the reaction



ITS 3: 5′-GCATCGATGAAGAACGCAGC-3′
ITS 4: 5′-TCCTCCGCTTATTGATATGC-3′
PISP-1: 5′-AATGCCAAGCTAAAGAGCCA-3′

Fig. 1. Genetic map of a portion of the rDNA repeat showing the location of the oligonucleotide primer site used to amplify rDNAs from *Phytophthora* species.

mixtures containing 200 μM dNTPs, 0.1 μM of each primer (ITS3 primer: GCATCGATGAAGAACGCAGC, ITS4 primer: TCCTCCGCTTTATTGATATGC), 1 unit of *Taq* polymerase (Dynazyme™, U.S.A.), 1× of the PCR buffer and dH_2O. The reaction conditions consisted of 1 min at 95°C (preheating), 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C, and 8 min at 72°C for 30 cycles followed by storage at 4°C. The reactions were performed on PTC-100™ (MJ Research, Inc., U.S.A.). The amplified PCR products were identified on 3% agarose (Metaphor® agarose, U.S.A.) and purified with Wizard® PCR Preps (DNA Purification System, U.S.A.). The purified DNA fragments were ligated into a pGEM®-T Easy Vector (Promega, U.S.A.) and used to transform the competent cells of the *E. coli* JM109. The inserted plasmids were extracted with the Quanta-Plasmid™ amplification QUKP (Quantum Biotechnologies, Inc., U.S.A.). The ampicillin-resistant colonies were screened using the PUC/M13 forward primer (CGCCAGGGTTTTCCCAGTCACGAC) and PUC/M13 reverse primer (TCACACAGGAAACAGCTATGAC), or by a restriction of the cloned vectors.

The ITS regions between 5.8S and 28S for all the *Phytophthora* species were amplified with the ITS3 and ITS4 primers (Fig. 1). A single ca. 650 bp sequence was observed in all the *Phytophthora* species (data not shown). Among the *Phytophthora* species, the isolates P-9827, P-9829, P-9833, BF-8, BN-11, 40401, 40157, and 97CC7112 (Table 1) were used for the insertion of ITS regions into the vectors.

Sequence Analysis and Primer Selection

The thermal cycle sequencing was performed on sequence-grade plasmid DNA with the silver Sequence™ DNA sequencing system (Promega, U.S.A.). The sequence data were compared with the DNA sequence data in the GenBank at the National Center for Biotechnology Institute (NCBI). Subsequently, the identified sequences were then aligned using the DNASTAR Computer software program (DNASTAR Inc., U.S.A.). After screening with the software program, the primers were reacted with various fungal and bacterial genomic DNAs.

All the cloned isolates, P-9827, P-9829, P-9833, BF-8, BN-11, 40401, 40157, and 97CC7112, were analyzed for the nucleotide sequences through manual sequencing (Table 1). As shown in Fig. 2, the ITS of P-9827 consisted of 556 nucleotides. The sequences were then compared with those available in the GenBank sequence database (accession number: PINITS2), and it was found that the ribosomal internal transcribed spacer of *P. infestans* showed a 95% homology (data not shown). The ITS II sequences were aligned using the DNASTAR program (DNASTAR Inc., U.S.A.) to design proper primers that could specifically amplify *P. infestans*. One of these primers, designated as PISP-1 (5'-AATGCCAAGCTAAAGAGCCA-3'), was then selected. The pair primers, ITS 3 and PISP-1, were then amplified in the PCR mixture. Consequently, a 453 bp amplified fragment was achieved only in *P. infestans*, thereby confirming the specific identification and detection of *P. infestans* by the ITS3 and PISP-1 (5'-AATGCCAAGCTA-AAGAGCCA-3') primers. Furthermore, the pair primers were not amplified in other fungal and bacterial DNAs (Fig. 3A).

```
        10         20         30         40
TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAATCT 40
TGCCTGATATCAGGTCAATTGAGATCATCVGgTTACCACA 80
ACATTTCCCAAATGGATCGACCCTCGGCAGCCGAAGCCGC 120
CACTCTACTTCGCAACAGCAAAGCCGATTCAAATGCCAAG 160
CTAAAGAGCCATGGTTCACCAGTCCATCACGsCACAGCAG 200
       210        220        230        240
GAAAATCACCCAATAAGCGCATTGTTCAGCCGAAGCCAAC 240
CATACCACGAATCGAGCATTTCTCCATTAACGCCGCAGCA 280
GACAAACGGGTCGCCAACTCGCTACAATAGCAGCGTCCAC 320
AACCAGCAATGCCACCACTTTTGGAGCAAAGAGAAGTACA 360
GTTTAGTACATTTAAAAGGACTCGCAGTSGGTCCGAAAAC 400
       410        420        430        440
CAACCGCAAGACACTTCACATCTGGCATCTCCTCCACCGA 440
CTACACGGAAGGAAGAAAGCCAAGTTTGTTGTACGGACAC 480
TGATACAGGCATACTTCCAGGACTAACCCGGAAGTGCAAT 520
ATGCGTTCAAAATTTCGATGACTCACTGATCCTGCAATTC 560
GGCATTBWCGCAGTTC 576
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Fig. 2. Complete sequences of the ITS region of *Phytophthora infestans*, P-9827.
The PISP-1 primer is underlined.

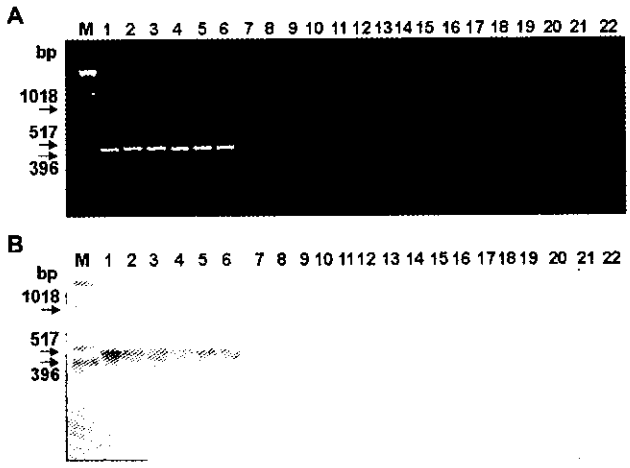

Fig. 3. A, Amplification of 450 bp product using pair primer, PISP-1, and ITS 3, to specifically detect *P. infestans*. B, Result of Southern hybridization with a labeled plasmid pGEM®T-easy vector containing the cloned ITS region from *P. infestans* isolate P9829. Lanes M: 1 kb DNA ladder; 16: P-9827, P-9829, P-9833, BF-8, BN-11, and HY-1; 7: KD-1; 8: 40401; 9: 40412; 10: 40174; 11: 40182; 12: 40184; 13: 40189; 14: 40157; 15: 96CC7116; 16: KCT214; 17: WS5-3; 18: R25; 19: R28; 20: E301629; 21: E301397; and 22: P2108.

Southern Blot Hybridization

The PCR products amplified by the ITS and PISP-1 primers were electrophoretically separated on a 1% agarose TBE gel (0.089 M Tris, 0.089 M Boric acid, 0.02 M EDTA). After the gels were stained with ethidium bromide, they were transferred to a nylon membrane (Hybond™-N⁺, Amersham, England). This membrane was prehybridized and hybridized in a hybridizaion incubator (Robbins Scientific®, U.S.A.). The plasmid inserted with the ITS II region of *P. infestans*, P-9829, was prepared as a probe for a Southern blot analysis by random-primed labeling. A total of 100 µl of the PCR labeling reaction mixture containing 10 ng of template DNA, 0.5 µM of each primers (ITS3 and ITS4), 1x the PCR buffer, 5 units of *Taq* polymerase (Dynazyme, U.S.A.), 0.1 mM of dGTP, dCTP, dATP, 0.09 mM dTTP, and 1 mM digoxigenin-dUTP (Boehringer Mannhein, Germany) was prepared and reacted in a program consisting of 90 sec at 94°C, 90 sec at 50°C, 2 min at 72°C for 24 cycles, and 4 min at 72°C. The color detection was processed using a DIG luminescent detection kit and all procedures were carried out using the supplied instruction manual (Boehringer Mannhein, Germany).

A probe encoding the ITS region of the isolate P-9829 was hybridized with the PCR products made by the ITS II and PISP-1 primers. In a Southern hybridization, the same results as with the pair primers were observed (Fig. 3B).

Investigation of PCR Sensitivity

In order to assess the sensitivity of the PCR amplification with the primer set for molecular detection, the reactions

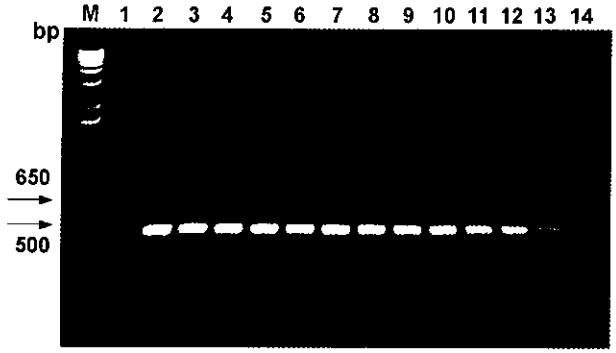

Fig. 4. Primer sensitivity according to template DNA quantities of *P. infestans* by PCR using PISP-1 and ITS3 primers.
The PCR products were resolved by 1% agarose gel electrophoresis. Lane M: 1 kb plus ladder (Gibco, U.S.A.); 1: control (no DNA); 2: 100 ng; 3: 10 ng; 4: 1 ng; 5: 800 pg; 6: 600 pg; 7: 400 pg; 8: 200 pg; 9: 100 pg; 10: 80 pg; 11: 60 pg; 12: 40 pg; 13: 20 pg; and 14: 10 pg.

were performed under annealing temperatures ranging from 55°C to 67°C. Different amounts of genomic DNA ranging from 100 ng to 10 pg were then prepared by continual dilutions. The PCR products were electrophoretically separated on a 1.0% agarose gel, stained with ethidium bromide (10 mg/ml) for 30 min, and visualized with UV light.

The two 20 bp oligonucleotide primers for *P. infestans* were used to investigate the range of annealing temperature and detectable concentration of genomic DNA. The annealing temperature was found to be a crucial factor in optimizing the product formation. Although the yields were different, they were detectable on stained agarose gels within a range of 55°C to 61°C (data not shown). In additon, these primer sets amplifed a visible segment at template DNA levels of 10 pg–100 ng (Fig. 4).

Various DNA-based diagnostic methods have been proved to be highly sensitive and species-specific tools. These techniques are very powerful in detecting and determining the taxonomy of fungi. PCR-based techniques are particularly valuable when compared with conventional methods, because they require only small quantities of DNA and little handling time. They can usually be carried out together with positive and negative controls. Previously, Tooley *et al.* [30] developed the specific primer, PINF, for the effective detection of *P. infestans*. Trout *et al.* [31] evaluated the accuracy of the PINF primer using many *Phytophthora* species. *Phytophthora cactorum* and *P. mirabilis* were also detected using the PINF primer, and these two species were further differentiated by digesting the PCR products with certain restriction enzymes [15, 31]. In the present work, we developed another species-specific primer set, ITS3 and PISP-1: The PISP-1 primer was developed according to the stringent standards [21] for designating effectual primers and through sequence alignment, and it could amplify only the specific PCR-band for *P. infestans*. Even though *P. infestans* included sexually compatible types and different metalaxyl resistance types, they were all detectable (data not shown). This was particularly the case with *P. cactorum*, which was directly differentiated from *P. infestans* by restriction enzymes without any additional procedures (Fig. 3A). Further work also ascertained that PISP-1 could not amplify any of the bands of *P. nicotianae* and *P. erythorseptica* among the potato pathogens.

The 453 bp specific fragment, except the primer oligonucleotides, was within the suggested optimal range for the amplification by PCR [14, 23]. Annealing temperature and template DNA quantity were also investigated, since they could influence the rate and specificity of the amplification [14, 19, 23]. The theoretical annealing temperature calculated from the primers was 68°C. However, temperatures between 55°C–61°C were found to be suitable for the observation of the PCR products of *P. infestans* on stained agaroses. These values were far below the theoretical optimal temperature of 68°C. Furthermore, 10 pg–100 ng of *P. infestans* genomic DNA was established to be sufficient for a detectable amplification by PCR (Fig. 4).

The application of PCR technology is effective in detecting a certain fungus both *in vitro* and *in planta,* and can help us to study the taxonomy of an organism. Therefore, further study on the *in planta* detection of *P. infestans* in infected tissues or tubers and at different developmental stages of the host *in vivo* and *in vitro* is in need.

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