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SPECIES-SPECIFIC DETECTION OF *Phytophthora inundata* BY SIMPLE AND NESTED-PCR *

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Abstract

Phytophthora inundata has been recently described and depicted as a 'difficult' taxon for identification due to its morphology and unusually high upper temperature limit for growth which has been mistaken for other non-papillate and high temperature tolerant plant pathogenic *Phytophthora* species. A simple as well as a nested-PCR based method was developed for the identification of *P. inundata*. A collection of isolates from different hosts representing diversity of species were examined for unique regions of coding as well as non-coding gene sequences. Based on internal transcribed spacers 1, 2 and 5.8S gene of rDNA (ITS), heat shock protein 90 gene (HSP), triosephosphate isomerase/glyceraldehyde-3-phosphate dehydrogenase fusion protein (TIG), and 60S ribosomal protein L10 gene (RPL) ten PCR primers specific for *P. inundata* were designed. Annealing temperatures and extension times were optimized for each set of primers for maximum specificity and efficiency. To evaluate the specificity of the method, 28 morphologically and molecularly characterized *Phytophthora* species were tested. In most cases neither set of primers amplified purified DNA from these non-homologous *Phytophthora* species. Analysis showed that the best candidate for identification of *P. inundata* isolates is ITS-I1 set which is a combination of ITS-IF1 and ITS-IR1. The optimized annealing temperature for this set was 69 °C and the best extension time was 40 sec. It seems that nested-PCR by ITS-I1 set together with universal ITS6 and ITS4 as external primers is at least 50 times more sensitive than conventional PCR.

Keywords: *Phytophthora inundata*, *Oomycota*, internal transcribed spacer of rDNA (ITS), identification, detection.

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Introduction

Phytophthora inundata Brasier, Sanchez-Hernandez & S. A. Kirk is a newly described taxon among plant pathogenic *Oomycota* (Brasier *et al.* 2003b). This species has been isolated from a variety of riparian habitats in Europe, on susceptible hosts such as the ornamental *Aesculus* and *Salix* spp., and also from the commercially cultivated *Olea* and *Prunus* spp. after soil flooding or waterlogging (Sánchez-Hernández *et al*. 2001, Brasier *et al.* 2003a). *P. inundata* is isolated from parsley crops and soil around carrot in Australia (Cunnington *et al.* 2006), diseased alfalfa roots in USA (Ho *et al.* 2006), foot and root rot of citrus trees in Chile (Vial *et al.* 2006) and more recently from native vegetation in Australia (Stukely *et al.* 2007). This species also is isolated from pistachio trees and infected sugar beet and *Solanum dulcamara* roots in Iran (Mostowfizadeh-Ghalamfarsa *el al.* 2006). This shows the world wide distribution of this species.

P. inundata morphologically resembles many *Phytophthora* species, with non-papillate sporangia and high optimum temperature for growth, such as *P. gonapodyides*, *P. cryptogea*, *P. drechsleri, P. melonis, P. parsiana* and *P. cinnamomi*. Therefore, it is sometimes impossible to discriminate *P. inundata* from these morphologically convergent species only based on morphological and/or physiological charactristics. Brasier *et al.* (2003b) in their original description of the species, depicted *P. inundata* as a 'difficult' taxon for diagnosis and recommended an analysis of the internal transcribed spacers (ITS) of rDNA sequence for accurate identification. By searching the literature, it was obvious that in all incidence reports the isolates has been identified only based on sequencing and phylogenetic analysis of ITS regions which is time-consuming, laborious and requires considerable knowledge about phylogenetics of the *Phytophthora* species (*e.g.* Cunnington *et al.* 2006, Ho *et al.* 2006, Vial *et al.* 2006, Stukely *et al.* 2007). Because of the lack of suitable morphological criteria for identification, invention of a simple, fast and accurate method of identification for *P. inundata* isolates seems to be a matter of necessity. In the other hand, in order to employ efficient control strategies, early detection and diagnosis of this pathogen either in plants, soil or irrigation water is also essential and molecular identification could be a suitable tactic. The aim of this study was to develop species-specific primers for PCR identification of *P. inundata*.

Materials and Methods

Origin and Maintenance of Isolates

Details of the *P. inundata* isolates examined in this study are listed in Table 1. The isolates were sourced from the culture collections of the author or in case of Iranian isolates directly isolated from the host tissue on PARPH medium (cornmeal agar amended with 10 µg ml^{-1} pimaricin, 200 µg ml^{-1} ampicillin, 10 µg ml^{-1} rifampicin, 25 µg ml^{-1} PCNB, and 50 µg l^{-1} hymexazol) (Jeffers & Martin 1986). Isolates were stored on cornmeal agar (CMA; ground corn extract 40 g l^{-1}, agar 15 g l^{-1}) slopes at 15 °C. Routine stock cultures for research studies were grown on French bean agar (FBA; ground French bean extract 30 g l^{-1}, agar 15 g l^{-1}) at 20 °C.

DNA Extraction

Isolates were grown in 50 ml still culture of potato broth (potato extract of 300 g l^{-1} boiled potato, pH=6.2) at 25ºC. After vacuum filtration, the mycelia was washed with sterilized distilled water, freeze-dried and stored at -20°C. Freeze-dried mycelia and/or plant material were homogenized using sea sand (Fluka, Germany) and a plastic disposable pestle. DNA was extracted from homogenized preparation using a Genomic DNA Purification kit, (Fermentas, UK) according to the manufacturer's instructions. The amount of DNA obtained was estimated by a NanoDrop spectrophotometer (NanoDrop Technologies, USA).

Primer Design

Sequenced regions of 28S ribosomal DNA gene, 60S ribosomal protein L10 gene (RPL), β-tubulin gene, cytochrome c oxidase gene subunit I, enolase gene, heat shock protein 90 gene (HSP), internal transcribed spacers 1, 2 and 5.8S gene of rDNA (ITS), NADH dehydrogenase gene subunit I, translation elongation factor 1 α gene, and triosephosphate isomerase/glyceraldehyde-3-phosphate dehydrogenase gene fusion protein (TIG) from *ca* 72 *Phytophthora* species along with a collection of *P. inundata* isolates from different

Table 1. Isolates of *Phytophthora inundata* used in this study and their species-specific amplification of DNA sequences by different primer sets.

Isolates	Host (matrix)	Year of isolation	Country of origin	Amplification using					
				ITS-I1[a]	ITS-I2[b]	ITS-I3[c]	HSP-I1[d]	TIG-I1[e]	RPL-I1[f]
SCRP644[g]	*Sali matsudana*	1972	UK	+	+	+	+	+	+
SCRP645	*Olea* sp.	1996	Spain	+	+	+	+	+	+
SUC21[h]	*Pistacia vera*	1993	Iran	+	+	+	+	+	+
SUC22	?	1999	USA	+	+	+	+	+	+
SUC23	?	1999	USA	+	+	+	+	+	+
SUD47	water	1994	Iran	+	+	+	+	+	+
SUKv1	*Beta vulgaris*	2002	Iran	+	+	+	+	+	+
SUMp1	*Beta vulgaris*	2002	Iran	+	+	+	+	+	+
SUMp8	*Beta vulgaris*	2002	Iran	+	+	+	+	+	+
SUSt6	*Solananthus circinnatus*	2002	Iran	+	+	+	+	+	+
SUSt7	*Solananthus circinnatus*	2002	Iran	+	+	+	+	+	+
MSZ2	*Beta vulgaris*	2007	Iran	+	+	+	+	+	+
MSZ3	*Beta vulgaris*	2007	Iran	+	+	+	+	+	+
MST8	*Beta vulgaris*	2007	Iran	+	+	+	+	+	+
MSTK2	soil	2007	Iran	+	+	+	+	+	+
BNS2	*Beta vulgaris*	2007	Iran	+	+	+	+	+	+
BNS3	*Beta vulgaris*	2007	Iran	+	+	+	+	+	+
BBA1	*Beta vulgaris*	2007	Iran	+	+	+	+	+	+
BBA2	*Beta vulgaris*	2007	Iran	+	+	+	+	+	+
BBA4	*Beta vulgaris*	2007	Iran	+	+	+	+	+	+
BBA5	*Beta vulgaris*	2007	Iran	+	+	+	+	+	+
BBAK2	soil	2007	Iran	+	+	+	+	+	+
GK4	*Beta vulgaris*	2007	Iran	+	+	+	+	+	+

[a]: Combination of ITS-IF1 & ITS-IR1 primers. [b]: Combination of ITS-IF2 & ITS-IR2 primers. [c]: Combination of ITS-IF1 & ITS-IR2 primers. [d]: Combination of HSP-IF1 & HSP-IR1 primers. [e]: Combination of TIG-IF1 & TIG-IR1 primers. [f]: Combination of RPL-IF1 & RPL–IR1 primers. [g]: SCRP644= IMI389751 and SCRP645= IMI390121 (Braseir *et al.*, 2003b). [h]: Other isolates have been identified in pervious works based on ITS regions of rDNA (Mostowfizadeh-Ghalamfarsa *et al*., 2006; Mostowfizadeh-Ghalamfarsa, 2010) ? = host or matrix not known.

hosts and matrices either from our previous studies (Mostowfizadeh-Ghalamfarsa *et al.* 2006) or others (Blair *et al*. 2008; Cooke *et al*. 2000, Kroon *et al*. 2004a) were recovered from GenBank using the Nucleotide Sequence Search Program provided by the National Center for Biotechnology Information (NCBI, http://www3.ncbi.nlm.nih. gov/Entrez) (Bethesda, MD, USA). Sequences of each set of genes were aligned using the software ClustalX (Thompson *et al*. 1997) and the alignment of all sequences was also checked visually. Sequences were examined for conserved regions unique to *P. inundata*. Selected primers were analyzed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) against the sequence they originally were based. Primers then evaluated for melting temperature (T_m), self-dimerization, self-annealing, potential hairpin formation and G-C content criteria using Oligo Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) (Kibbe 2007). Having compared specificity and sensitivity of primer sets, ITS-based primers were selected for nested-PCR. Universal primers ITS6 and ITS4 (White *et al*., 1990) were applied as external primers. Primer ITS6 is similar to ITS5 (White *et al*.1990), but modified according to the *P. megasperma* 18S rDNA sequence (Förster *et al*. 1990) to allow more efficient amplification in these

taxa (Cooke & Duncan 1997).

PCR Protocol

Each PCR reaction contained 2.5 µl 10× PCR buffer (Fermentas, UK), 2.5 µl 10 mg ml^{-1} BSA, 2.5 µl 1 mM dNTPs, 1.5 µl 25 mM $MgCl_2$ (for ITS based primers) or 3.5 µl 25 mM $MgCl_2$ (for other primers), 0.5 µl 100 µM of each primer, 0.2 µl 5 U/µl *Taq* DNA polymerase (Fermentas, UK) and 100 ng target DNA. The PCR reaction mix was made up to a final volume of 25 µl with water.

PCR amplifications were done on a CG1-96 thermocycler (Corbett Research, Australia). In case of ITS-based primers PCR was originally carried out with a program of 95 °C for 2 min (initial denaturation) followed by 30 cycles of 95°C for 20 sec, a gradient of annealing temperature from 58-70 °C for 25 sec, 72°C for 1 min, and a final extension of 72°C for 10 min for both simple and nested-PCR. In case of other primers PCR was originally carried out with a program of 95°C for 2 min (initial denaturation) followed by 35 cycles of 95°C for 30 sec, a gradient of annealing temperature from 58-70°C for 30 sec, 72°C for 50 sec, and a final extension of 72°C for 10 min for both simple and nested-PCR. The PCR products were visualized in 1.0% agarose gels in 1× TBE buffer (1 h at 80 V). Gels were stained using ethidium bromide and DNA fragments were visualised under UV light.

Specific Identification and Detection of *P. inundata*

Primers specificity. To determine specificity of the primers, PCR was conducted on the high quality genomic DNA of various morphologically and molecularly characterized *Phytophthora* species (Table 4) using the specific primer sets.

Primers sensitivity. To resolve the sensitivity of the primers, spectrophotometrically quantified DNA was serially diluted with HPLC water over 10 orders (100 ng-10 fg) of magnitude (Table 5). Sensitivity of detection was then determined with each specific primer sets for both conventional and nested-PCR.

The sensitivity of the simple and nested-PCR methods were also analyzed by a tenfold serial dilution of DNA in sugar beet root extract (Table 7). Extract of healthy sugar beet prepared by an electric jucer. *Ca* 0.05 g activated charchole added to the extracte, vortexed for 30 sec and cetrifuged at 15,000 rpm (Sigma, Germany) for one min. The supernatant filter sterlized using 0.2 µm polycarbonate membrane and used for serial dilution.

Detection by Primers. In order to detect isolates in infected tissues, diseased sugar beet roots as well as artificially inoculated sugar beet roots, eggplant tissue and almond roots with *P. inundata* isolates were tested. DNA extracted from freeze-dried infected tissues using above mentioned method and amplified by ITS-IF1 and ITS-IR1 primers both directly and as internal primers for nesting-PCR.

Detection of isolates in infested soil carried out by combination of baiting and PCR methods. One kg of infested soil from sugar beet plantation placed in a plastic container and flooded with distilled water up to 1 cm above the soil. Thirty pieaces of 0.5 cm diamitere disks of four week-old sugar beet leaves put on the surface of water and incubated overnight at 25 ˚C. Disks removed, washed with distilled water, dried on tissue paper and transferred to Petri plates containing PARPH medium. After 3 d at 25 ˚C scraped mycelia used for DNA extraction and PCR detection conducted by specific ITS-based primers. All experiments were repeated twice.

Results

Primer design

Based on ITS, HSP, TIG, and RPL genes, 10 PCR primers specific for *P. inundata* were designed Table 2. No eligable candidate specific for *P. inundata* was found in other genes examined. In ITS-based primers designed, ITS-IF1 located on the ITS1 region of rDNA whereas ITS-IF2, ITS-IR1, and ITS-IR2 sited on the ITS2 region of ribosomal RNA gene. Optimized PCR conditions for each *P. inundata* putative species-specific primer pairs summerized in (Table 3).

Specificity and sensitivity of the designed primers

The expected size of amplification product for each set of species-specific primer has been showed in(Table 3). When each designed primer set was used, an amplicon of the expected size was obtained with DNA from all morphologically and molecularly well-characterized *P. inundata* tested (Table 1). Neither set amplified purified DNA from

Table 4. Species-specific amplification of DNA sequences from different *Phytophthora* species by designed primer sets for *Phytophthora inundata*.

Species	Isolate	Primer sets					
		ITS-I1 [a]	ITS-I2 [b]	ITS-I3 [c]	HSP-I1 [d]	TIG-I1 [e]	RPL-I1 [f]
P. arecae	SCRP18	-	-	-	-	-	-
P. asparagi	SCRP21	-	-	-	-	-	-
P. botryosa	SCRP25	-	-	-	-	-	-
P. cactorum	SCRP27	-	-	-	-	-	-
P. cambivora	SCRP67	-	-	-	-	-	-
P. capsici	2-8-87	-	-	-	-	-	-
P. cinnamomi	SCRP115	-	-	-	-	-	-
P. citricola	SCRP165	-	-	-	-	-	-
P. citrophthora	SCRP179	-	-	-	-	-	-
P. cryptogea	SCRP204	-	-	-	-	-	-
P. drechsleri	SCRP236	-	-	-	-	-	-
P. erythroseptica	SCRP241	-	-	-	-	-	-
P. gonapodyides	SUC6	-	-	-	±	-	±
P. hydropathica	1012	-	-	-	-	-	-
P. inundata	SCRP644	+	+	+	+	+	+
P. irrigate	23J7	-	-	-	-	-	-
P. katsurae	SCRP388	-	-	-	-	-	-
P. meadii	SCRP400	-	-	-	-	-	-
P. medicaginis	10-2-81	-	-	-	-	-	-
P. melonis	SCRP455	-	-	-	-	-	-
P. nicotianae	SCRP468	-	-	-	-	-	-
P. palmivora	SCRP526	-	-	-	-	-	-
P. parsiana	SUC25	-	-	-	-	-	-
P. pistaciae	11-1-05	-	-	-	-	-	-
P. quercina	SCRP541	-	-	-	-	-	-
P. ramorum	Alex1	-	-	-	-	-	-
P. richardiae	SCRP551	-	-	-	-	-	-
P. sojae	SCRP555	-	-	-	-	-	-
P. tropicalis	7Ga	-	-	-	-	-	-

+: Positive PCR product. -: Negative PCR product. ±: Specific product together with non specific bands. [a]: Combination of ITS-IF1 & ITS-IR1 primers which produces a fragment of 439 bp. [b]: Combination of ITS-IF2 & ITS-IR2 primers which produces a fragment of 144 bp. [c]: Combination of ITS-IF1 & ITS-IR2 primers which produces a fragment of 769 bp. [d]: Combination of HSP-IF1 & HSP-IR1 primers which produces a fragment of 619 bp. [e]: Combination of TIG-IF1 & TIG-IR1 primers which produces a fragment of 374 bp. [f]: Combination of RPL-IF1 & RPL-IR1 primers which produces a fragment of 169 bp.

Table 5. The effect of DNA quantity (per µl sample) on PCR product band density of the putative species-specific primer sets for *Phytophthora inundata*.

Primer sets	DNA Quantity									
	100 ng	10 ng	1 ng	500 pg	100 pg	50 pg	10 pg	1 pg	100 fg	10 fg
ITS-I1[a]	+++	+++	++	++	-	-	-	-	-	-
ITS-I2[b]	+++	+++	+++	+	+	-	-	-	-	-
ITS-I3[c]	+++	+	-	-	-	-	-	-	-	-
HSP-I1[d]	+++	++	+	-	-	-	-	-	-	-
TIG-I1[e]	++	-	-	-	-	-	-	-	-	-
RPL-I1[f]	+++	+	+	-	-	-	-	-	-	-

[a]: Combination of ITS-IF1 & ITS-IR1 primers. [b]: Combination of ITS-IF2 & ITS-IR2 primers. [c]: Combination of ITS-IF1 & ITS-IR2 primers. [d]: Combination of HSP-IF1 & HSP-IR1 primers. [e]: Combination of TIG-IF1 & TIG-IR1 primers. [f]: Combination of RPL-IF1 & RPL-IR1 primers.
+++: Very good. ++: Good. +: Reasonable. -: No band.
ng = nano (10^{-9}) gram. pg = pico (10^{-12}) gram. fg = femto (10^{-15}) gram.

Table 6. The effect of DNA quantity (per µl sample) on nested-PCR product band density of the putative species-specific primers for *Phytophthora inundata*.

Primer sets	DNA Quantity									
	100 ng	10 ng	1 ng	500 pg	100 pg	50 pg	10 pg	1 pg	100 fg	10 fg
ITS-4 & ITS-6	+++	+++	+	+	-	-	-	-	-	-
Nested- PCR with ITS-I1 [a]	+++	+++	+++	+++	+++	++	++	-	-	-
Nested- PCR with ITS-I2 [b]	+++	+++	+++	+++	+++	-	-	-	-	-
Nested- PCR with ITS-I3 [c]	+++	+++	+++	+++	+++	-	-	-	-	-

[a]: Combination of ITS-IF1 & ITS-IR1 primers. [b]: Combination of ITS-IF2 & ITS-IR2 primers. [c]: Combination of ITS-IF1 & ITS-IR2 primers.
+++: Very good. ++: Good. +: Reasonable. -: No band.
ng = nano (10^{-9}) gram. pg = pico (10^{-12}) gram. fg = femto (10^{-15}) gram.

28 other *Phytophthora* species (Fig. 1). Exceptions were HSP-I1 and RPL-I1 sets (Table 4) which amplified a faint bond for *P. gonapodyides*. The sensitivity of simple PCR by different primer pairs ranged from 100 ng to 100 pg purified DNA per µl sample (Table 5).

Detection of *P. inundata* in infected plant samples and infested soil

Using ITS-IF1 and ITS-IR1 primers as the best candidate for species-specific detection of *P. inundata* in infected plants' tissue, DNA extracted from freeze-dried eggplant infected tissue was directly amplified and produced a sharp band. Whereas, *P. inundata* isolates were detected in sugar beet and almond infected roots only by nested-PCR. In addition to *ca* 800 bp band of ITS region of rDNA from *P. inundata*, some 500-600 bp faint bands were observed in the first round of nested-PCR, in most of the cases (data not shown).

P. inundata isolates also easily detected from infested soils using combination of sugar beet baits and simple PCR by ITS-based primers.

Comparison of the sensitivities of simple and nested-PCRs

Comparison of direct and nested-PCR with ITS species specific primers as internal sets and ITS6 and ITS4 universal primers showed that nested-PCR is more sensitive than simple method in most cases. Nested-PCR was found to be at least 50, 0, and 100 times more sensitive for ITS-I1, ITS-I2, and ITS-I3, respectively (Table 5 & 6). Even in the case of ITS-I2 the amplified band was much sharper than the one generated by simple PCR. The nested-PCR sensitivity was at least 10 ng, 50 pg, and 10 pg of *P. inundata* DNA when diluted in healthy sugar beet root extract and detected with ITS-I1, ITS-I2, and ITS-I3, respectively (Table 7) There were no differences between any replication of entire experiments.

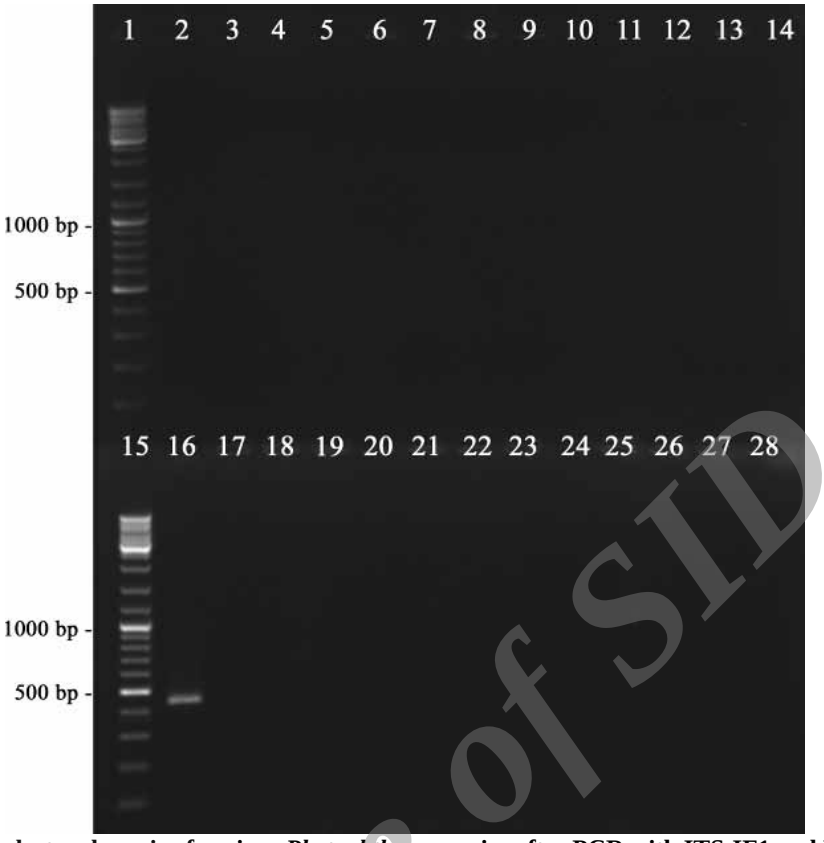

Fig. 1. Agarose gel electrophoresis of various *Phytophthora* species after PCR with ITS-IF1 and ITS-IR1 primers. (1) 100 bp DNA ladder, (2) *P. arecae*, (3) *P. asparagi*, (4) *P. botryosa*, (5) *P. cactorum*, (6) *P. cambivora*, (7) *P. capsici*, (8) *P. cinnamomi*, (9) *P. citricola*, (10) *P. citrophthora*, (11) *P. cryptogea*, (12) *P. drechsleri*, (13) *P. erythroseptica*, (14) *P. gonapodyides*, (15) 100 bp DNA ladder, (16) *P. inundata*, (17) *P. katsurae*, (18) *P. meadii*, (19) *P. medicaginis*, (20) *P. melonis*, (21) *P. nicotianae*, (22) *P. palmivora*, (23) *P. parsiana*, (24) *P. pistaciae*, (25) *P. quercina*, (26) *P. ramorum*, (27) *P. richardiae*, and (28) *P. sojae*.

Table 7. The effect of DNA quantity (per µl sample) in sugar beet filter-sterilized extract on PCR product band density of the putative species-specific primers for *Phytophthora inundata* in direct and nested PCR.

Primer sets	DNA Quantity									
	100 ng	10 ng	1 ng	500 pg	100 pg	50 pg	10 pg	1 pg	100 fg	10 fg
ITS-4 & ITS-6	+++	-	-	-	-	-	-	-	-	-
ITS-I1[a]	+++	+	-	-	-	-	-	-	-	-
Nested- PCR with ITS-I1	+++	+++	+++	+++	++	+	-	-	-	-
ITS-I2[b]	++	-	-	-	-	-	-	-	-	-
Nested- PCR with ITS-I2	+++	+++	+++	+++	+++	+++	-	-	-	-
ITS-I3[c]	+++	-	-	-	-	-	-	-	-	-
Nested- PCR with ITS-I3	+++	+++	+++	+++	+++	++	+	-	-	-

[a]: Combination of ITS-IF1 & ITS-IR1 primers. [b]: Combination of ITS-IF2 & ITS-IR2 primers. [c]: Combination of ITS-IF1 & ITS-IR2 primers.
+++: Very good. ++: Good. +: Reasonable -: No band.
ng = nano (10^{-9}) gram. pg = pico (10^{-12}) gram. fg = femto (10^{-15}) gram.

Discussion

Many diseases caused by *Phytophthora,* especially those cause root and crown rots without any specific foliar symptoms, have not been thoroughly identified, or have been incorrectly attributed to secondary invaders such as *Pythium* or *Fusarium* spp. (Duniway 1977, Tsao 1990). Roots or crowns of some plants can also be simultaneously infected by different *Phytophthora* species (Grato *et al.* 2002). For example sugar beet can be infected by *P. inundata* as well as *P. cryptogea*, and *P. drechsleri* at the same time (Erwin & Ribeiro 1996). Based on classical identification, morphological or physiological characters do not clarify the species boundaries of *P. inundata* isolates and false determinations are frequently made due to convergent evolution of the species (Brasier *et al.* 2003b). Hence, there is a clear rationale for accurate detection and identification of this species. The main goal of this study was to develop a molecular approach for effective identification and sensitive detection of *P. inundata* in pure culture, infected tissues and infested soil.

Isolates from our previous studies (Mostowfizadeh-Ghalamfarsa *et al.*, 2006; Mostowfizadeh-Ghalamfarsa, 2010) and collections has been identified by ITS sequencing followed by phylogenetic analysis together with the set of data from previous works (Cooke *et al.* 2000, Brasier *et al.* 2003b). Ten specific primers were designed based on ITS of ribosomal DNA, heat shock protein 90 gene (HSP), triosephosphate isomerase/ glyceraldehyde-3-phosphate dehydrogenase fusion protein (TIG), and 60S ribosomal protein L10 gene (RPL). Six other examined genes did not have enough polymorphism to be useful for specific primer designing. Although all tested combinations of designed primers could effectively identify the *P. inundata* isolates, it seems that the sensitivity and more importantly the applicability of the primer sets are vary.

For each of HSP, TIG, and RPL genes one forward and one reverse primer designed and tested. These housekeeping genes were previously applied for multi-locus phylogeny of *Phytophthora* species (Blair *et al.* 2008), showing their sufficient polymorphism for discriminating *Phytophthora* isolates at the species level. HSP90 is a member of a class of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures or other stress (De Maio 1999). Amplifying the expected size band in *P. inundata*, HSP-I1 primer set produced a faint band with the same size for *P. gonapodyeides*. Close examination of alignments revealed that although both forward and reveres primers have some differences compared with *P. gonapodyeides* sequence, especially at their 3' ends, they show close match in most of their lengths with HSP of *P. gonapodyeides*. It seems that the members of *Phytophthora* Clade 6 *sensu* Blair *et al.* (2008) (*i.e. P. inundata, P. humicola, P. megasperma, P. humicola,* and *P. gonapodyeides*) are homogenous in their HSP gene. *In silico* analysis of HSP-based primers also revealed that this primers can detect *P. humicola*. Therefore, this set could not be a good choice for discrimination of *P. inundata* isolates among its sister taxa. Such a problem also came about in case of RPL-I1 set. RPL is a highly conserved component of the large ribosomal subunit (60s) that plays a crucial role in protein synthesis (Nguyen *et al.* 1998). Although the high level of conservation makes this gene suitable for phylogenetic inferences at the species level, the lack of runs of multiple nucleotide polymorphisms could be the origin of fake detection of *P. gonapodyeides* isolate by primers based on this gene.

Genes encoding triose-phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase are fused and form a single transcriptional unit (TIG) in *Phytophthora* species (Unkles *et al.* 1997) which is phylogenetically informative. Despite the high specificity of the TIG-I1 primer set for identification of *P. inundata*, the quality of amplified band was low on agarose gel and it was not able to detect less 100 ng purified DNA. Structure analysis of both primers displayed tow potential self-annealing sites in TIG-I1 set which could be the source of the low efficiency and the high amount of primer dimers observed on the gel. TIG-I1 set, however, can still be applied as a collaborator tool for identification of *P. inundata* isolates along with other highly efficient primers.

Methods based on ITS of the rRNA repeat unit were widely used for identification of the *Phytophthora* species (*e.g.* Bonants *et al.* 1997, Drenth *et al.* 1999, Ippolito *et al* 2002, Kroon *et al.* 2004b). Within a species ITS tend to be distinct and monomorphic. In addition to a suitable level of sequence variation between different species, since the rDNA gene exists in a high copy number

(*ca* 100-200 copies), the application of rDNA-based methods can increase the sensitivity of a diagnostic test (Drenth *et al*. 2006). Based on ITS region of rDNA two forward and two reverse primers designed and their combinations were tested for species-specific identification of all *P. inundata* isolates. In some cases ITS-I2 primer set produced a fade amplification product of approximately 130 bp together with the expected 144 bp band. Decreasing of the extension time to 30 sec eliminated this non-specific ghost band. Although all ITS-based primer sets were highly specific and sensitive, it seems that ITS-I1 is the best candidate for identification and detection of *P. inundata* isolates according to its specificity, product length (439 bp), fragment quality and consistency, and sensitivity in both simple and nested-PCR.

To promote the sensitivity of the reactions nested-PCR developed using ITS6 and ITS4 universal primers as externals to the fragments of the ITS-based primer sets. ITS6 and ITS4 product has been described by Cooke and Duncan (1997) as being specific for the genus *Phytophthora*. Nested-PCR gave more sensitivity almost in all cases. Although the level of detection did not change in ITS-I2 primer set after nested-PCR, the quality of bands were significantly promoted. These results are in agreement with the results of other authors who were able to detect minor amount of pathogen DNA in host tissues and soil by nested-PCR method (*e.g.* Bonants *et al.* 1997, Grato *et al.* 2002, Ippolito *et al.* 2002). As sugar beet filter-sterilized extract has been used as a carrier for purified target DNA, the level of sensitivity was dramatically changed in all ITS-based primer sets. It appears that simple PCR cannot detect more than 100 ng DNA in the extract of host tissue, whereas nested-PCR could be up to 10^4 times more sensitive and can detect pathogen presence even in low concentration of the target organism. Consistent with this was the detection of *P. inundata* isolates in infected sugar beet and almond roots by nested-PCR as the only applicable approach. In the author's opinion the false negative results obtained by simple PCR could be due to the relatively higher amount of plant DNA, and the presence of plant inhibitors (Wilson 1997). Simple PCR with ITS-I1 primer set, however, easily detect DNA extracted from freeze-dried eggplant infected tissue. In general, simple as well as nested-PCR methods were able to detect almost a higher concentration of *P. inundata* purified DNA when diluted in healthy root extract of sugar beet compared with control DNA diluted in water, which could be due to inhibitory effects of plants materials (Wilson 1997, Grato *et al.* 2002).

Having recovered from infested soil by sugar beet baits, the *P. inundata* isolates could be easily detected by ITS-based primers. The PCR reaction produced the expected band size for each primer set. Sequencing the amplified fragments showed the exact match with their corresponding ITS regions.

Based on our previous studies (Mostowfizadeh-Ghalamfarsa *et al.* 2006) *P. inundata* isolates from different origins have a very high level of genetic similarity. Therefore, the designed primers could be effective tools for identification and detection of *P. inundata* isolates from different hosts and geographical locations. According to the results due to a higher lever of specificity and sensitivity, the nested-PCR by ITS-I1 primer set could be employed as diagnostic tool to identify the pathogen, and it is also a reliable and effective way of *P. inundata* detection in infected host plant parts and infested soil in practice. While all primer sets were not equally specific, it is possible to use the fragments generated by primer sets as a diagnostic character for more confident identification in case of doubt. It seems that the approach described here could be adjusted to be a part of a multiplex PCR system for simultaneous identification of economically important pathogens. With this molecular method there is no need for time consuming and costly sequencing and phylogenetic analysis which is essential for accurate identification of the isolates of *P. inundata*.

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