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RESEARCH PAPER

Species-specific identification and detection of *Phytophthora pistaciae*, the causal agent of pistachio gummosis

Reza MOSTOWFIZADEH-GHALAMFARSA and Zahra MIRSOLEIMANI

Department of Plant Protection, College of Agriculture, Shiraz University, Shiraz, Iran, 7144133111

Summary. Gummosis is the most important disease of pistachio trees in Iranian orchards. The principal cause, *Phytophthora pistaciae*, is a recently described plant pathogenic oomycete from pistachio trees. This species is very similar, probably due to convergence, to some other non-papillate high temperature species, especially *P. megasperma*. A PCR based method was developed to provide a reliable molecular tool for the identification of *P. pistaciae*. A collection of isolates from different locations representing a diversity of species was examined for unique coding regions as well as for non-coding gene sequences including the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene of rDNA, the heat shock protein 90 gene and ß-tubulin. Eight PCR primers specific for *P. pistaciae* were designed. Annealing temperatures and extension times were optimized for each set of primers to maximize both specificity and amplification efficiency. Each set was tested against purified DNA from other gummosis-inducing *Phytophthora* species from pistachio trees as well as 28 other *Phytophthora* species from different hosts. In conventional PCR, the limit of detection of different primer sets ranged from 10 ng to 50 pg of purified DNA. The best candidate for identification of *P. pistaciae* isolates was the ITS-S1 primer set which was a combination of ITS-SF1 and ITS-SR1. The ITS-S1 set did not amplify purified DNA from other *Phytophthora* species tested. The combination of ITS-S1 with ITS6 and ITS4 universal primers could detect up to 100 fg DNA in nested PCR and also can amplify the specific band in infected tissues, infested soil and water.

Key words: *Oomycota*, internal transcribed spacer of rDNA (ITS), coding and non-coding loci.

Introduction

Pistachio (*Pistacia vera*) is a major economically important crop in Iran. There are about 250,000 ha of orchards producing 477,000 tonnes of nuts per year, worth almost US $ 1.1 billion. Iran is the most important pistachio nut producer in the world and gummosis is the most important disease of pistachio trees in Iranian pistachio orchards. This soilborne disease affects the crowns and roots of the trees, causing average tree mortality of 10–12% or greater (Mirabolfathy, 1988). Several *Phytophthora* species have been reported to be causal agents, of pistachio gummosis in Iran, including *Phytophthora citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, *Phytophthora cryptogea* Pethybr. & Laff., *Phytophthora drechsleri* Tucker, *Phytophthora megasperma* Drechsler, *Phytophthora nicotianae* Breda de Haan, and *Phytophthora parsiana* Mostowfizadeh-Ghalamfarsa *et al*. (Banihashemi, 1983, 1995; Mirabolfathy and Ershad, 1987; Mirabolfathy *et al*., 1989; Aminaee and Ershad, 1991; Mostowfizadeh-Ghalamfarsa *et al*., 2008).

Mirabolfathy *et al.* (2001) re-examined putative *P. megasperma* and *P. drechsleri* isolates from pistachio using RFLPs and sequence comparisons of internal transcribed spacer (ITS) regions of rDNA. Based on their molecular analysis, these isolates were a member of ITS Clade 7 *sensu* Cooke *et al.* (2000), which were neither *P. megasperma* nor *P. drechsleri*. *Phytophthora megasperma*-like isolates from pistachio differed phylogenetically from all known members of this clade, and were assigned as *Phytophthora pistaciae* Mirabolfathy,

Corresponding author: R. Mostowfizadeh
Fax: +98 0711 2286087
E-mail: rmostofi@shirazu.ac.ir

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whereas *P. drechsleri*-like isolates from *Pistacia* sp. had identical sequences with *Phytophthora melonis* Katsura (Mirabolfathy *et al.*, 2001). A more recent analysis of *P. pistaciae* within a wider selection of *Phytophthora* spp. based on multiple-gene genealogies confirmed its position in Clade 7, far different from *P. megasperma sensu stricto* (Blair *et al*., 2008). Furthermore, analysis of ITS regions of rDNA in a wider range of *P. pistaciae* isolates from different areas revealed a high degree of sequence identity among isolates (Mirsoleimani and Mostowfizadeh-Ghalamfarsa, 2011a).

Phytophthora pistaciae is considered to be one of the principal causes of pistachio gummosis in Iran, and together with *P. melonis* represented 90% of all *Phytophthora* spp. recovered from roots, crowns and surrounding soil from the infected pistachio trees (Mirabolfathy *et al*., 2001). There are no other reports of natural infection of hosts by *P. pistaciae*. However, other investigations have shown that *P. pistaciae* is not only a pistachio tree pathogen but could also affect other species from the host family *Anacardiaceae*, and some members of unrelated families such as *Chenopodiaceae, Fabaceae, Juglandaceae, Rosaceae* and *Vitaceae* (Mirsoleimani and Mostowfizadeh-Ghalamfarsa, 2011b).

Phytophthora pistaciae morphologically resembles many non-papillate *Phytophthora* species, especially *P. megasperma*. In the absence of the clear-cut morphological criteria, isolates can only be identified based on sequencing and phylogenetic analysis of ITS regions of rDNA (Mirabolfathy *et al*., 2001; Mirsoleimani and Mostowfizadeh-Ghalamfarsa, 2011a), which is time-consuming, laborious and requires considerable knowledge of the phylogenetics of *Phytophthora* species. Due to the lack of suitable morphological criteria for identification, developing a simple, fast and accurate method of identification for *P. pistaciae* isolates is a necessity. Furthermore, early detection and diagnosis of this pathogen either in plants, soil or irrigation water is also essential to employ efficient control strategies, and molecular identification may be a suitable tactic. The aim of the present study was to develop a molecular diagnostic tool for identification and detection of *P. pistaciae* isolates, using species-specific PCR primers.

Material and methods

Origin and maintenance of isolates

Details of the *P. pistaciae* isolates examined in this study are listed in Table 1. The isolates were isolated from host tissue onto 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 (CMA-PARPH) (Jeffers and Martin, 1986). Some isolates were recovered from soil samples by plating 5 mm discs of pistachio leaves used as baits on PARPH medium. 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 also grown on CMA 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 were washed with sterilized distilled water, freeze-dried and stored at -20°C. Freeze-dried mycelia were homogenized using sea sand (Fluka, Darmstadt, Germany) and plastic disposable pestles. Freeze-dried plant materials were also homogenized using mortars and pestles. DNA was extracted from homogenized preparations using a Genomic DNA Purification kit, (Fermentas, Ontario, Canada) according to the manufacturer's instructions. The amount of DNA obtained was estimated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Primer design

Sequenced regions of ten genes from *ca.* 72 *Phytophthora* species, along with a collection of *P. pistaciae* isolates from different hosts and matrices either from our previous studies (Mirsoleimani and Mostowfizadeh-Ghalamfarsa, 2011a) or others (Mirabolfathy *et al.*, 2001; Blair *et al*., 2008) were recovered from GenBank using the Nucleotide Sequence Search Program provided by the National Centre for Biotechnology Information (NCBI, http://www3.ncbi.nlm.nih. Gov/Entrez) (Bethesda, MD, USA). The genes included 28S ribosomal DNA, 60S ribosomal protein L10 (RPL), β-tubulin (TUB), cytochrome c oxidase subunit I (COX), enolase, heat shock protein 90 (HSP), the internal transcribed spacers (ITS1 and ITS2) and 5.8S gene of rDNA (ITS), NADH dehydrogenase subunit I, translation elongation factor 1 α, and triosephosphate isomerase/glyceraldehyde-3-phosphate dehydrogenase fusion protein (TIG). Multiple sequence alignments of each set of

Table 1. Isolates of various *Phytophthora* spp. recovered either from infected tissues of pistachio trees or from infested soil around the trees in different parts of Iran, and species-specific amplification of their DNA by different primer sets developed in this study.

Species	Isolate	Matrix	Year of isolation	Location	Amplification using				
					ITS-S1[a]	ITS-S2[b]	ITS-S3[c]	HSP-S1[d]	TUB-S1[e]
P. pistaciae									
	SUD44	T[f]	1993	Kerman	+	+	+	+	+
	SURf14	T	1993	Kerman	+	+	+	+	+
	SURab6	S[g]	2009	Abbasabad	+	+	+	+	+
	SUP.p6	T	2009	Azadegan	+	+	+	+	+
	SUP.p7	T	2009	Azadegan	+	+	+	+	+
	SUP.p24	T	2009	Azadegan	+	+	+	+	+
	SUP.p26	S	2009	Azadegan	+	+	+	+	+
	SUKAA	S	2010	Yazd	+	+	+	+	+
	SUKAB	S	2010	Yazd	+	+	+	+	+
	SURno4	T	2009	Noogh	+	+	+	+	+
	SURka7	T	2009	Kabootarkhan	+	+	+	+	+
P. citrophthora									
	SURRfc1	T	2010	Rafsanjan	-	-	-	-	-
P. drechsleri									
	SUAbd1	T	2009	Abbasabad	-	-	-	-	-
	SUAzd2	S	2009	Azadegan	-	-	-	-	-
	SUFrd3	T	2009	Fersodieh	-	-	-	-	-
	SUFrd3	S	2009	Kabootarkhan	-	-	-	-	-
P. inundata									
	SUC21	S	1993	Kerman	-	-	-	-	-
	SUAbi1	T	2009	Abbasabad	-	-	-	-	-
	SUNoi2	T	2009	Noogh	-	-	-	-	-
P. melonis									
	SUD43	T	1993	Kerman	-	+	+	+	-
	SURf5	T	1993	Kerman	-	+	+	+	-
	SURf8	T	1993	Kerman	-	+	+	+	-
P. melonis									-
	SURf9	T	1993	Kerman	-	+	+	+	-
	SURf10	T	1993	Kerman	-	+	+	+	-

(*Continued*)

Table 1. *Continues.*

Species	Isolate	Matrix	Year of isolation	Location	Amplification using				
					ITS-S1[a]	ITS-S2[b]	ITS-S3[c]	HSP-S1[d]	TUB-S1[e]
	SURf13	T	1993	Kerman	-	+	+	+	-
	SUAzm1	T	2009	Azadegan	-	+	+	+	-
	SUFrm1	T	2009	Fersodieh	-	+	+	+	-
	SUKbm1	T	2009	Kabootarkhan	-	+	+	+	-
	SUNom1	S	2009	Noogh	-	+	+	+	-
P. parsiana									
	SUC19	T	1992	Kerman	-	-	-	-	-
	SURf6	T	1993	Kerman	-	-	-	-	-
	SURf17	T	1993	Kerman	-	-	-	-	-
	SUAnp1	T	2009	Anar	-	-	-	-	-
	SUFtp1	T	2009	Fathabad	-	-	-	-	-
	SUKbp1	T	2009	Kabootarkhan	-	-	-	-	-
	SUNop1	S	2009	Noogh	-	-	-	-	-

+, Positive PCR product. -, Negative PCR product.
[a] Combination of: ITS-SF1 and ITS-SR1 primers; [b] ITS-SF1 and ITS-SR2 primers; [c] ITS-SF1 and ITS-SR3 primers; [d] HSP-SF1 and HSP-SR1 primers; [e] TUB-SF1 and TUB-SR1 primers.
[f] Infected trees.
[g] Infested soil around the infected trees.

genes were made using ClustalX (Thompson *et al*. 1997) with subsequent visual adjustment. Sequences were examined for conserved regions unique to *P. pistaciae*. Selected primers were analyzed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) against the sequence on which they were originally based. Primers were then evaluated for chemical-physical features such as melting temperature (T_m), self-dimerization, self-annealing, potential hairpin formation and G-C content using Oligo Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) (Kibbe 2007).

Having compared specificity and sensitivity of primer sets, ITS-based and β tubulin-based primers were also selected for nested-PCR. Universal forward ITS6 (Cooke and Duncan, 1997) and reverse ITS4 (White *et al*., 1990) primers 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 *Phytophthora* spp. (Cooke and Duncan, 1997). In the case of β tubulin-based primers, TUBUF2 and TUBUR1 primers (Kroon *et al*., 2004a) were applied as external primers.

PCR protocol

PCR was carried out in 25 μL reactions containing 2.5 μL of 10× PCR buffer (Fermentas), 100 mM of BSA, 100 mM dNTPs, 1.5 mM of $MgCl_2$ (for ITS based primers) or 2.5 mM of $MgCl_2$ (for other primers), 1 mM of each primer, 0.4 U *Taq* DNA polymerase (Fermentas) and 100 ng target DNA.

Amplifications were performed in a CG1-96 thermocycler (Corbett Research, Melbourne, Australia). In the case of ITS-based primers, PCR was originally carried out with a programme of 95°C for 2 min (initial denaturation) followed by 30 cycles of 95°C for 20 s, a gradient of annealing temperature from 58–70°C for 25 s, 72°C for 1 min, and a final extension of 72°C for 10 min for both direct and nested-PCR. In the case of the other primers, PCR was originally car-

ried 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 direct and nested-PCR. Successful amplification was confirmed by gel electrophoresis (1 h at 80 V) on 1.0% agarose gels in 1 × TBE buffer. Gels were stained using ethidium bromide and DNA fragments were visualised under UV light.

PCR conditions, including annealing temperature and the time of annealing, were optimized to maximize the yield of the desired amplification product while minimizing the levels of non-specific products.

Sequencing of amplified product

The amplification products were purified through GenJET PCR purification kit (Fermentas, Ontario, Canada) to remove excess primers and nucleotides. PCR products were sequenced (Tech Dragon, Hong Kong, China) in forward and reverse orientation using the primers used for amplification and a dye terminator cycle sequencing kit (BigDye® Terminator V 3.1, Applied Biosystems, CA, USA) on a 3730 × l DNA Analyzer (Applied Biosystems) according to the manufacturer's instruction.

Specific identification and detection of *Phytophthora pistaciae*

Primer 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 2) using the specific primer sets.

Primer 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 3). Sensitivity of detection was then determined with each specific primer set both for conventional and nested-PCR.

Detection of *Phytophthora pistaciae* by primers

Detection of isolates in host tissues

In order to detect isolates in infected tissues, DNA from diseased pistachio roots and crowns, as well as host roots (including various cultivars of pistachio) artificially inoculated with *P. pistaciae* isolates was tested (Table 4). Inocula of the isolates were prepared on vermiculite amended with hemp seed extract and inoculated to various plant species (Table 4) according to Mostowfizadeh-Ghalamfarsa *et al.* (2010). DNA was extracted from freeze-dried infected tissues (as above) and amplified using ITS-SF1 and ITS-SR1 primers both directly and as internal primers for nested-PCR with universal ITS4 and ITS6 external primers.

Detection of isolates in infested soil (direct method)

Soil samples were collected from 15-month-old pistachio seedlings with gummosis symptoms after inoculation with different isolates of *P. pistaciae*) SURf14, SUKAB and SUP.p6 (and growth in 3 L capacity pots (see above). Samples from three different pots were mixed thoroughly, dried at room temperature for 3 days and sieved with a sterile 2 mm mesh. Serial dilutions of 1:1, 1:10 and 1:100 infested soils were prepared for each isolate with sterile soil.

The inoculum levels of *P. pistaciae* were assessed using a selective medium CMA-PARPH. Ten g of sieved soil were suspended in 100 mL of sterile water in an Erlenmeyer flask, and shaken with 80 rpm for 40 min. One mL of the suspension was spread on CMA-PARPH, and after 24 h at 20°C, soil was removed from the surface of the plates by washing with water. The plates were incubated again at 20°C for another 3 d (Ippolito *et al*., 2002). The test was performed using three replicates per infested soil. Colonies showing the characteristic morphology of *P. pistaciae* were counted to get the average number of CFU g^{-1} of dry soil. Soil moisture was determined by desiccating 20 g of soil for 24 h at 110°C. DNA was extracted from soil samples (see below) and amplified by ITS-SF1 and ITS-SR1 primers both directly and as internal primers for nested-PCR.

DNA extraction from soil samples

Soil (0.5 g) was suspended in 500 µL of CTAB (hexadecyltrimethylammonium bromide) extraction buffer (0.1 M Tris-Hcl, 0.0002 M EDTA, 0.014 M NaCl, 0.05 M CTAB, and 2% 2-mercaptoethanol) and was homogenized using sea sand (Fluka) and a plastic disposable pestle, and incubated for 1 h at 65°C. The extraction mixture was centrifuged (Sigma, Taufkirchen, Germany) at 13,000 rpm for 10 min at room temperature. The upper phase was extracted

Table 2. Species-specific amplification of DNA sequences from different *Phytophthora* species by designed primer sets for *Phytophthora pistaciae*.

Species	Isolate	Primer sets				
		ITS-S1[a]	ITS-S2[b]	ITS-S3[c]	HSP-S1[d]	TUB-S1[e]
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. irrigata	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 with the expected size; -, The expected size of PCR product did not amplify.
[a] Combination of ITS-SF1 and ITS-SR1 primers which produces a fragment of 395 bp.
[b] Combination of ITS-SF1 and ITS-SR2 primers which produces a fragment of 678 bp.
[c] Combination of ITS-SF1 and ITS-SR3 primers which produces a fragment of 624 bp.
[d] Combination of HSP-SF1 and HSP-SR1 primers which produces a fragment of 207 bp.
[e] Combination of TUB-SF1 and TUB-SR1 primers which produces a fragment of 180 bp.
[*] These gummosis inducing species sourced from other host than pistachio trees.

Table 3. The effect of DNA quantity (per μL sample) on PCR product band density of the putative species-specific primer sets for *Phytophthora pistaciae*.

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-S1 [a]	+++	+++	+	+	+	+	-	-	-	-
ITS-S2 [b]	+++	+++	++	+	+	-	-	-	-	-
ITS-S3 [c]	+++	+++	++	++	+	+	-	-	-	-
HSP-S1 [d]	+++	+	-	-	-	-	-	-	-	-
TUB-S1 [e]	+++	++	+	+	-	-	-	-	-	-

[a–e] See Table 1.
Band density: +++, Very good. ++, Good. +, Reasonable. -, No band.

Table 4. Detection of *Phytophthora pistaciae* isolates in inoculated susceptible hosts by direct and nested PCR using species specific ITS-SF1 and ITS-SR1 primers and universal ITS6 (as forward), and ITS4 (as reverse) primers as external set. All pistacio cultivars including Badami, Ghazvini, Sarakhs showed the same results.

Family	Common name	Scientific name	Isolates					
			SURf14		SUP.p6		SUKAB	
			Direct	Nested	Direct	Nested	Direct	Nested
Anacardiaceae	Mango	*Mangifera indica*	-	+	-	+	-	+
	Pistachio	*Pistacia vera*	-	+	-	+	-	+
Asteraceae	Safflower	*Carthamus persicus*	+	+	+	+	+	+
Chenopodiaceae	Sugar beet	*Beta vulgaris*	+	+	+	+	+	+
Fabaceae	Chick pea	*Cicer arietinum*	+	+	+	+	+	+
	Broad bean	*Faba vulgaris*	+	+	+	+	+	+
	Cowpea	*Phaseolus cylindricus*	+	+	+	+	+	+
	French bean	*Phaseolus vulgaris*	+	+	+	+	+	+
	Pinto bean	*Phaseolus* sp.	+	+	+	+	+	+
	Pea	*Pisum sativum*	+	+	+	+	+	+
Juglandaceae	Walnut	*Juglans regia*	+	+	-	+	-	+
Malvaceae	Gumbo	*Hibiscus esculentus*	+	+	+	+	+	+
Rosaceae	Sour cherry	*Cerasus vulgaris*	-	+	-	+	-	+
	Sweet almond	*Prunus amygdalus* var. *dulcis*	+	+	+	+	+	+
	Apricot	*Prunus armeniaca*	-	+	-	+	+	+
Vitaceae	Grape	*Vitis silvestris*	-	+	-	+	-	+

from a homogenized preparation using a Genomic DNA extraction kit DNG™-PLUS, (Cinnagen, Tehran, Iran) according to the manufacturer's instructions. Extracted DNA was resuspended in 50 µL of sterile distilled water. The amount of DNA obtained was estimated by a NanoDrop spectrophotometer (NanoDrop Technologies).

Detection of isolates in infested soil (indirect method)

The detection of isolates in infested soil was carried out by the combination of baiting and PCR. One kg of infested soil from each pistachio orchard was placed in a plastic container and flooded with distilled water up to 1 cm above the soil. Thirty pieces of 0.5 cm diameter disks of young pistachio leaves were put on the surface of the water and incubated overnight at 25°C. Disks were then removed, washed with distilled water, dried on tissue paper and transferred to Petri plates containing CMA-PARPH. After 3 d, scratched mycelia were used for DNA extraction as described above, and PCR detection was conducted using specific ITS-SF1 and ITS-SR1 primers.

Detection of isolates in infested water

Zoospore production

Zoospores were produced by three isolates of *P. pistaciae* (SURf14, SUKAB and SUP.p6) recovered from diseased pistachio trees or infested soil. The isolates were grown on hemp seed agar (HSA; ground hemp seed extract 60 g L^{-1}, and agar 15 g L^{-1} amended with 10 g L^{-1} yeast extract) for 4 d at 25°C. Sporangia were produced by incubating 30 blocks of active margin cultures of *P. pistaciae* isolates for 24–30 h in 20 mL of sterile soil extract (100 g soil suspended in 1 L distilled water for 24 h at room temperature and then filtered) under fluorescent light at room temperature. The zoospore suspensions were filtered through filter paper to remove mycelium and agar plugs. Zoospores were then encysted either by lactophenol cotton blue (LPCB) (Miller and Maxwell, 1984) or vortexing 1 mL of the original suspension in a microtube for 2 min to facilitate counting. The initial concentration of zoospores was determined using a haemocytometer under a light microscope at ×100 magnification. Concentrations of initial suspensions were approximately 1.5×10^3 zoospores mL^{-1}. To obtain higher concentrations of zoospores, 1 to 1.5 mL of each original suspension was centrifuged (Sigma) in a microcentrifuge tube at 10,000 rpm for 1 min, and the pellet was resuspended in 50 µL of sterile HPLC water and diluted with filtered sterile soil extract (Kong *et al*., 2003). We tested a series of 50 mL of artificially contaminated soil extract water containing 1.5×10^4 to 10 zoospores.

DNA extraction from water samples

Propagules were collected from infested water either by filtration or centrifugation. In the filtration method, zoospore suspensions were filtered through 0.45 µm microbiological filters using 10 mL syringes. The filter membranes were removed carefully from the filtering unit and cut into pieces using sterile forceps and scissors. The pieces were each transferred into a sterile 1.5 mL microtube containing 50 µL of sterile soil extract and were ground using some sea sand (Fluka) and a plastic disposable pestle. In the centrifuge-based method, the infested water was centrifuged at 10,000 *g* for 10 min to pellet the propagules and the pellet was resuspended with 50 µL of sterile soil extract. DNA from the water samples was extracted following a protocol derived from Kong *et al.* (2003). The extracted DNA was amplified using ITS-SF1 and ITS-SR1 primers both directly and as internal primers for nested-PCR.

Results

Primer design

Eight PCR primers specific for *P. pistaciae* were designed based on ITS, HSP and TUB genes (Table 5). No eligible candidate specific for *P. pistaciae* was found in any of the other genes examined. In ITS-based designed primers, ITS-SF1 is located on the ITS1 region of rDNA whereas ITS-SR1, ITS-SR2, and ITS-SR3 are situated on the ITS2 region of the ribosomal RNA gene.

Specificity of the designed primers

The expected size of amplification product for each set of species-specific primer is shown in Table 6. 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. pistaciae* isolates tested (Table 1). The ITS-S1 primer set (combination of ITS-SF1 and ITS-SR1) did not amplify purified DNA from 28 other *Phytophthora* species (Tables 1, 2 and Figure 1). This primer set was chosen as the best candidate for additional

Table 5. The putative specific primers designed for detection of *Phytophthora pistaciae*.

Primer	Target DNA	Primer sequence	Accession number[a]	Length	Location[b]
ITS-SF[c]1	ITS[e]	5´ GTC GAT GTC AAA GTC GGC GG 3´	AY659414	20	71-90
ITS-SR[d]1		5´ CGC GCC GCA AGA CAC CC 3´		17	465-449
ITS-SR2		5´ CCA CCC TAC TTC GCA ACA ACA CCG 3´		24	748-725
ITS-SR3		5´ CGG TTC ACC AGC CCA TAC CG 3´		20	694-675
HSP-SF1	HSP[f]	5´ GCG AGA GCA AGA AGG CCG TC 3´	EU080322	20	1235-1254
HSP-SR1		5´ CCT TCT CCA CCT TGT CGT CG 3´		20	1504-1485
TUB-SF1	TUB[g]	5´GGT GCT TGA CGT TGT CCG C 3´	EU080320	19	186-204
TUB-SR1		5´ ACC TTA GGC GAC GGG CAG 3´		18	365-384

[a] Reference to the GenBank accession containing the DNA sequence, on which the primer is based.
[b] Reference to the location of the primer within the original DNA sequence.
[c] Forward. [d] Reverse. [e] Internal transcribed spacers 1, 2 and 5.8S gene of rDNA. [f] Heat shock protein 90. [g] β tubulin.

Table 6. Optimized PCR conditions for *Phytophthora pistaciae* putative species-specific primer pairs and their amplicon length.

Primer sets	Initial denaturation	Number of cycles	Denaturation	Annealing	Extension	Final Extension	Length[f]
ITS-S1[a]	95(120)[g]	30	95(20)	69(25)	72(60)	72(600)	395
ITS-S2[b]	95(120)	30	95(20)	69(25)	72(60)	72(600)	678
ITS-S3[c]	95(120)	30	95(20)	69(25)	72(60)	72(600)	624
HSP-S1[d]	94(120)	35	94(30)	69(30)	72(60)	72(600)	270
TUB-S1[e]	94(120)	35	94(30)	69(30)	72(60)	72(600)	180

[a–e] See Table 1.
[f] Amplicon length (bp). [g] Temperature 'ºC' (time 's').

tests due to its specificity and sensitivity (Tables 1, 2 and 5). Other sets amplified one or two species other than *P. pistaciae* (Tables 1 and 2): the ITS-S2 set amplified a sharp 678 bp band for *P. cinnamomi* and also a faint band for *P. melonis*, whereas the ITS-S3 set only produced a faint 624 bp band for *P. melonis*. The HSP-S1 set amplified a faint 270 bp band for *P. melonis*. The TUB-S1 set not only generated two very faint 180 bp bands for *P. citricola sensu stricto* and *P. richardiae*, but also a number of other non-homologue faint bands for some other species including *P. melonis* (800 bp) and *P. capsici* (500 and 700 bp) (data not shown). In the case of the TUB-S1 primer set the concentration of $MgCl_2$ was reduced to 1.5 M (to reduce non-specific amplifications) instead of the 2.5 M recommended for monocopy genes. Optimized PCR conditions for each *P. pistaciae* putative species-specific primer pairs are summarized in Table 6.

Comparison of the sensitivities of direct and nested PCRs

The sensitivity of direct PCR by various primer pairs ranged from 10 ng to 50 pg purified DNA per µl sample (Table 3), whereas the sensitivity of nested-PCR ranged from 100 pg to 100 fg DNA per µL sample (Table 7).

Comparison of direct and nested-PCR with ITS primer sets showed that nested-PCR was the most sensitive. Nested-PCR was found to be at least 500, 1000, and 500 times more sensitive for ITS-S1, ITS-S2, and ITS-S3, respectively (Tables 3 and 7). However,

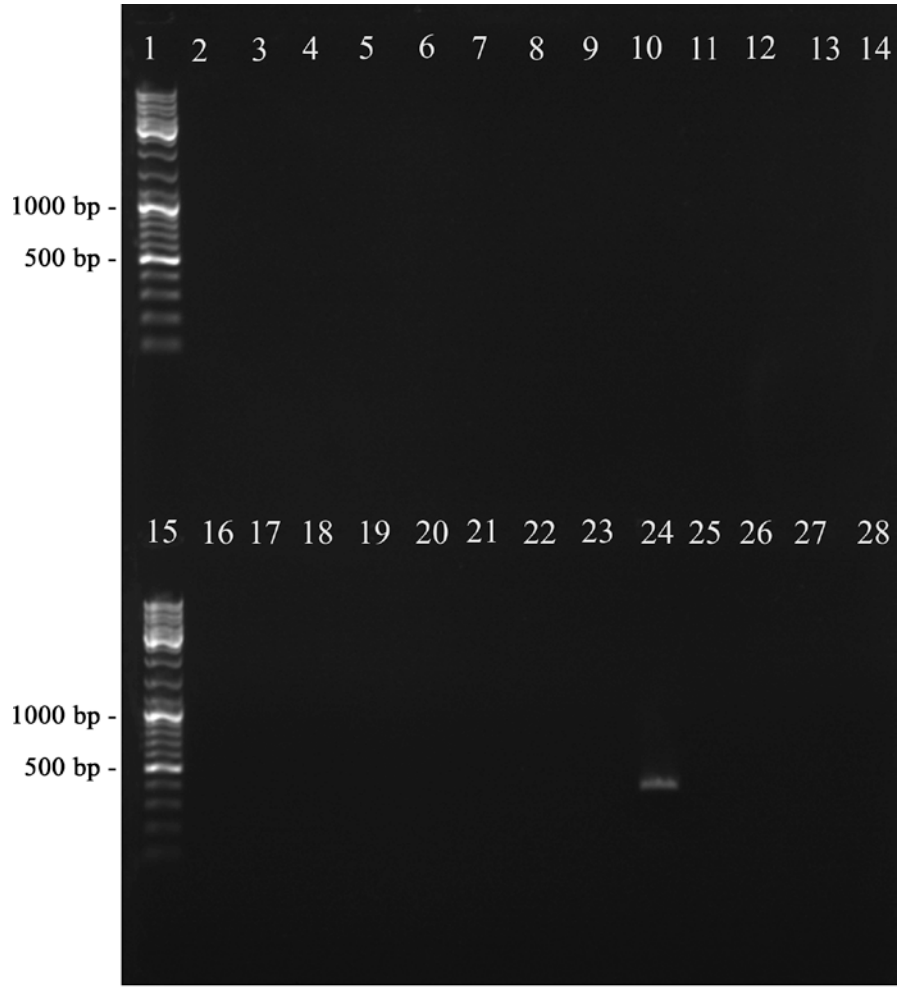

Figure 1. Gel electrophoresis of various *Phytophthora* species after PCR with ITS-IF1 and ITS-IR1 primers. (1) 100 bp DNA ladder, (2) *P. arecae*, (3) *P. asparagi*, (4) *P. botryose*, (5) *P. cactorum*, (6) *P. cambivora*, (7) *P. capsici*, (8) *P. cinnamomi*, (9) *P. citricola*, (10) *P. citrophthora*, (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*.

the quality of bands was much better in nested-PCR with ITS-S1. Nested-PCR for TUB-S1 was at least five times more sensitive than direct-PCR and the generated bands were sharper compared to direct PCR (Tables 3 and 7).

Detection of *Phytophthora pistaciae* in infected plant samples

The ITS-S1 primer set detected *P. pistaciae* in all naturally infected tissues examined. The expected band also amplified in DNA extracted from freeze-dried tissues of inoculated woody and herbaceous hosts (Table 4). The resulting amplicons of both experiments were sequenced and their identity confirmed using a BLAST search (http://blast.ncbi.nlm.nih.gov). All bands were 100% identical to the expected fragment with a full coverage. Single-round amplification could detect target DNA in crowns or roots of all herbaceous plants and also secondary or hairy infected roots in most of the woody plants, but nested-PCR was required for some samples from perennial plants (Table 4). The universal forward ITS6 and reverse ITS4 primers amplified DNA from

Table 7. The effect of DNA quantity (per µL sample) on nested-PCR product band density of the putative species-specific primers for *Phytophthora pistaciae*.

Primer sets	DNA Quantity[f]									
	100 ng	10 ng	1 ng	500 pg	100 pg	50 pg	10 pg	1 pg	100 fg	10 fg
ITS6 & ITS4	+++	+++	++	++	+	-	-	-	-	-
Nested- PCR with ITS-S1 [a]	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
Nested- PCR with ITS- S2 [b]	+++	+++	+++	+++	+++	+++	+++	+++	+	-
Nested- PCR with ITS-S3 [c]	+++	+++	+++	+++	+++	+++	+++	++	+	-
TUBUF2 & TUBUR1	+++	++	+	-	-	-	-	-	-	-
Nested- PCR with TUB-S1 [d]	+++	+++	+++	+++	+++	-	-	-	-	-

[a–e] See Table 1.
[f]Band density: +++, Very good. ++, Good. +, Reasonable. -, No band.

all host tissues and root samples inoculated with the *P. pistaciae* isolates, but no bands were obtained from healthy (uninoculated) controls.

Detection of isolates in infested soil (direct method)

The average inoculum level of *P. pistaciae* in the infested soil with SUP.p6, SUKAB and SURf14 isolates was 610 ±28, 590 ±24, and 540 ±29 CFU g^{-1} of dry soil, respectively. In all tests, no amplified fragments were detected after the single amplification with all species-specific primer pairs. However, specific DNA fragments were detected after nested-PCR from the soils containing *P. pistaciae* propagules and the specific 395 bp band was obtained for all isolates in soil. Pathogen was also detected at a lower dilution of the original infested soil (Figure 2). No unexpected amplifications were obtained with any primer pair in the negative soil controls.

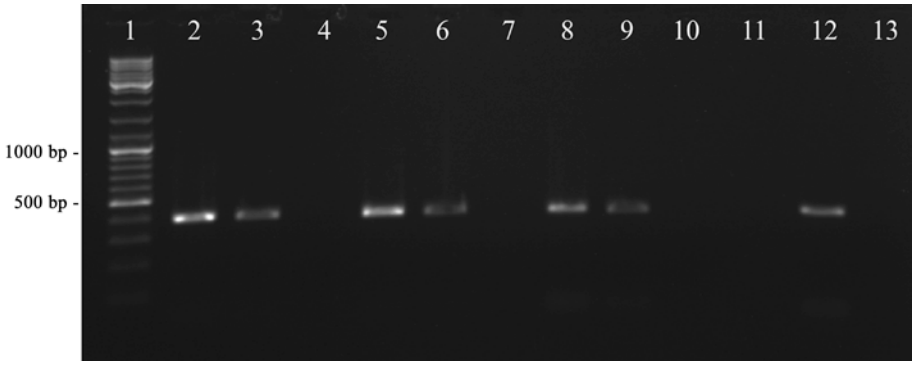

Figure 2. Gel electrophoresis of *Phytophthora pistaciae i*solates detected by nested-PCR with ITS-SF1 and ITS-SR1 primers in diluted (1:1, 1:10 and 1:100) soil samples with sterile soil. Inoculum level of *P. pistaciae* in the initially infested soil samples was 610, 590 and 540 propagules of the pathogen per gram of dry soil for SUP.p6, SUKAB and SURf14 isolates, repectively. (1) 100 bp DNA ladder, (2–4) serial dilutions of soil infested with SURf14, (5–7) serial dilutions of soil infested with SUKAB, (8–10) serial dilutions of soil infested with SUP.p6, (11) negative control with sterile soil, (12) positive control with DNA extract of SURf14 isolate, (13) negative control with HPLC water.

Detection of isolates in infested soil (indirect method)

Phytopthora pistaciae isolates were easily detected from infested soils by the ITS-S1 primer set using a combination of pistachio leaves baits and direct PCR. The bands produced in this test were sequenced and their identity confirmed using a BLAST search (http://blast.ncbi.nlm.nih.gov). All bands were 100% identical to the expected fragment with a full coverage.

Detection of isolates in infested water

Phytophthora pistaciae isolates were detected from infested water using either the filtration or centrifugation methods for collecting zoospores in the sterile soil water extract. No positives PCR reactions were detected by a single round of amplification with species-specific primers. However, specific DNA fragments were detected after nested-PCR was employed for water samples. The centrifugation method was more effective than filtration for collecting zoospores. In the artificially contaminated sterile soil water extract, PCR with the ITS-S1 primer set yielded positive results for up to 50 zoospores per mL soil extract when the centrifugation method was used, whereas in the filtration method the limit of detection was only 100 zoospores per mL soil extract (Figure 3).

There were no obvious differences between any of the replications in any of the experiments.

Discussion

Roots or crowns of some plants can be simultaneously infected by different *Phytophthora* species (Er-

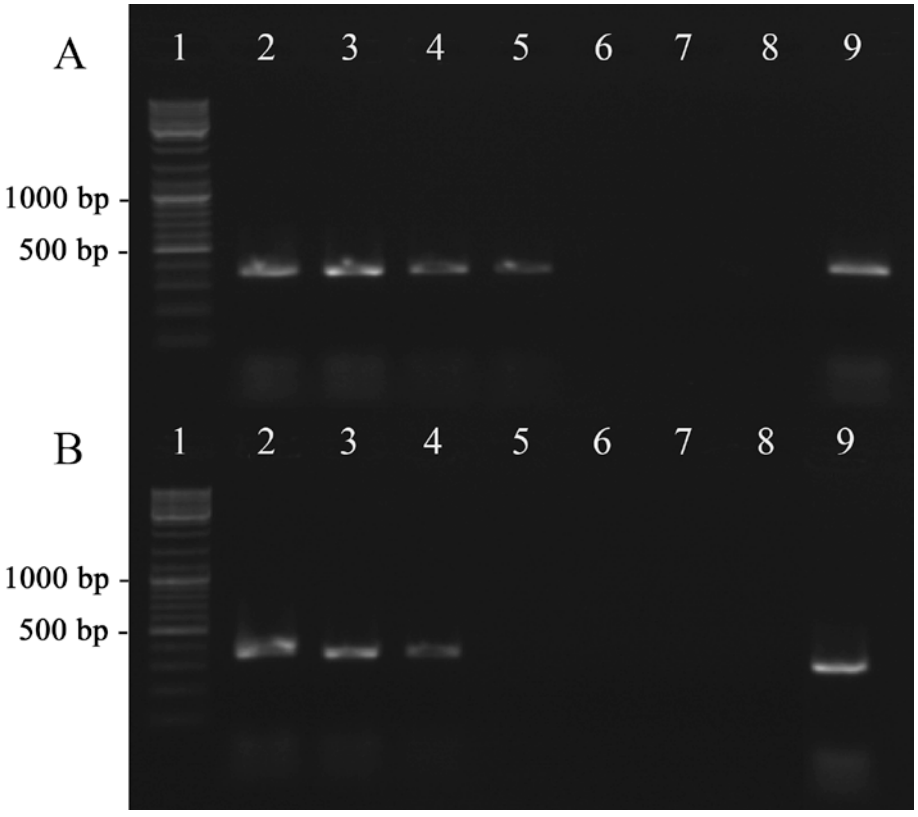

Figure 3. Gel electrophoresis of nested-PCR products obtained with *Phytophthora* pistaciae specific primers ITS-SF1 and ITS-SR1 using centrifugation (A) or filtration (B) methods for collecting zoospores of *P. pistaciae* in soil extract. (1) 100 bp DNA ladder; DNA extracted from a suspension contained 1.5×10^3 (2), 500 (3), 100 (4), 50 (5), 10 (6) zoospores in mL soil extract, negative control with sterile soil extract (7), negative control with HPLC water (8), positive control with DNA extract of SURf14 isolate (9).

win and Ribeiro, 1996; Grote *et al.*, 2002). Moreover, the foliar symptoms of some *Phytophthora* diseases have been incorrectly attributed to other soil invaders such as *Pythium* or *Fusarium* spp. (Duniway, 1977; Tsao, 1990). Pistachio trees are no exception and can be infected by a range of soilborne fungi as well as various *Phytophthora* species, each of which has a different pathogenicity potential and host range (Mirsoleimani and Mostowfizadeh-Ghalamfarsa, 2011b). However, all of them produce characteristic gummosis: gummy exudates from the crowns and lower parts of the trunks of affected plants, with underlying rotted brown tissues or severe root rot (Duncan *et al.*, 2001). Classical identification of the causal agents of pistachio gummosis based on morphological or physiological characters does not clarify the species boundaries, and false determinations are frequently made due to convergent evolution of some species (Mirsoleimani and Mostowfizadeh-Ghalamfarsa, 2011a). Hence, there is a clear rationale for accurate detection and identification of these species, especially one of the major causes of the gummosis, *P. pistaciae,* a recently described phylogenetic species.

Isolates of *P. pistaciae* used in this study were from our previous works (Mirsoleimani and Mostowfizadeh-Ghalamfarsa, 2011a; b) and collections which have been identified confidently by ITS sequencing followed by phylogenetic analysis together with a set of data from earlier studies on *Phytophthora* spp. (Cooke *et al.* 2000; Mirabolfathy *et al.*, 2001). Eight specific primers were designed based on the internal transcribed spacers of ribosomal DNA, the heat shock protein 90 gene (HSP), and β tubulin (TUB). Seven other examined genes did not have enough concentrated polymorphism to be useful for specific primer design. Although all tested combinations of designed primers could effectively identify the *P. pistaciae* isolates, there is variability in the specificity, sensitivity and, more importantly, the applicability of the different primer sets.

For each of the HSP and TUB genes one forward and one reverse primer was designed and tested. These housekeeping genes were previously applied for construction of multi-locus phylogeny of *Phytophthora* species (Kroon *et al.*, 2004a; Blair *et al.*, 2008), showing adequate polymorphism for discriminating *Phytophthora* isolates at the species level. The heat shock protein 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. pistaciae*, HSP-S1 primer set produced a faint band with the same size for *P. melonis*. Close examination of alignments revealed that although forward primers had few differences at its 3´ end compared with the *P. melonis* sequence, both primers showed a close match in most of their lengths with HSP of *P. melonis*. Most of the members *of Phytophthora* Clade 7b *sensu* Blair *et al.* (2008) (*i.e. P. pistaciae, P. melonis, P. sinensis, P. cajani, P. sojae,* and *P. vignae*) are homogenous in their HSP gene to some extent. The lack of sensitivity was the other problem of the HSP-S1 set which could only detect up to 10 ng purified DNA. Therefore, this set cannot be a good choice for discrimination of *P. pistaciae* isolates among its sister taxa, especially *P. melonis*, another major cause of pistachio gummosis (Mirabolfathy *et al.*, 2001).

β-tubulin is a highly conserved gene coding for one of the two microtubules' 50 kD proteins (Ludueňa, 1993). The high level of conservation makes this gene suitable for phylogenetic inferences at the species level (Kroon *et al.*, 2004a). Although the TUB-S1 set did not amplify *P. melonis*, it seems that most parts of the primers' sequences are shared by the β-tubulin sequences of some phylogenetically unrelated species including *P. citricola* and *P. richardiae* and also by some unknown parts of the *P. inundata* genome (it amplified two faint 1200 and 1220 bp bands, data not shown). However, none of the species with false positive 180 bp bands have been reported to induce gummosis in pistachio trees, and this set could be applicable for identification of the disease. While bands were sharper in nested-PCR using TUBUF2 and TUBUR1 external primers, the combination of primer sets did not significantly increase the sensitivity of detection compared with ITS-based primer sets (see below).

ITS-based methods have been widely employed for identification of the *Phytophthora* species (*e.g.* Bonants *et al.*, 1997; Drenth *et al.*, 1999; Ippolito *et al.*, 2002; Kroon *et al.* 2004b). The internal transcribed spacers of rDNA (ITS) contain mosaics of highly conserved and variable regions. Therefore, this region is suitable for species discrimination across a wide range of organisms, including the *Oomycota*. 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, one forward and three reverse primers were designed and their combinations were tested for species-specific identification of all *P. pistaciae* isolates. Although all ITS-based primer sets were highly specific and sensitive, ITS-S2 and ITS-S3 sets amplified a faint band with *P. melonis* of the expected size. The ITS-S2 set also generated a 678 bp band with *P. cinnamomi.* Further examinations on alignments did not reveal any exact match for each of the primers on the ITS sequence of either *P. melonis* or *P. cinnamomi*. This indicates that ITS-S1 is the best candidate for identification and detection of *P. pistaciae* isolates according to its specificity, product length (395 bp), fragment quality and consistency, and sensitivity in both direct and nested-PCR. The reverse ITS-SR1 primer from the ITS-S1 set partly resembles the PIS2rev primer developed for *P. megasperma* isolates from pistachio trees (Mirabolfathy *et al*., 2002). However, there are few nucleotide differences and unlike the PIS2rev combination with PIS1fwd, the ITS-S1 set does not amplify *P. melonis* or other sister taxa of *P. pistaciae*. In addition, the upper limit of ITS-S1 detection is much greater than those reported by Mirabolfathy *et al.* (2002).

To promote the sensitivity of the reactions, nested-PCR was developed using ITS6 and ITS4 universal primers as externals to the fragments of the ITS-based primer sets. ITS6 and ITS4 products have been described by Cooke and Duncan (1997) as being specific for the genus *Phytophthora*. The nested-PCR was at least 500 times more sensitive in all cases. The nested-PCR with the ITS-S1 primer set very adequately amplified the expected bands from the infected pistachio cultivars as well as from other inoculated host tissues. It can also detect a reasonable amount of the pathogen in infested water and soil. These results are in agreement with those of other authors who were able to detect minor amounts of pathogen DNA in host tissues and soil by the nested-PCR method (e.g. Bonants *et al*., 1997; Grote *et al*., 2002; Ippolito *et al*., 2002). In order to ascertain whether the observed negative is valid, or is the result of a failed extraction, multiplex PCR with universal primers must be used as an internal positive control for plant DNA (Garbelotto et al., 1996; Hayden et al., 2004). Hence, to avoid the NanoDrop analysis, this testing is required before making the assay fully operative.

Based on our previous studies (Misoleimani and Mostowfizadeh-Ghalamfarsa, 2011b), *P. pistaciae* isolates from different origins have are genetically very similar. Therefore, the designed primers could be effective tools for identification and detection of *P. pistaciae* isolates from various geographical locations. Our pathogenicity test studies (Misoleimani and Mostowfizadeh-Ghalamfarsa, 2011a) also showed that there could be other potential hosts for *P. pistaciae* and the specific primer sets could be used to detect the pathogen in these hosts.

Our results demonstrate that the nested-PCR with the ITS-S1 primer set can be employed as a diagnostic tool to identify the pathogen, due to a higher level of specificity and sensitivity achieved with this protocol. Furthermore, the method in practice is also reliable and effective for detection of *P. pistaciae* in infected host plant tissues and infested soil and water.

While all primer sets were not equally specific, it is possible to use the fragments generated by primer sets as diagnostic characters for more confident identification in cases of doubt. The approach described here could be adjusted to be a part of a multiplex PCR system for simultaneous identification of economically important *Phytophthora* species. With this molecular method, there is no need for the time-consuming and costly sequencing and phylogenetic analysis which is otherwise essential for accurate identification of the isolates of *P. pistaciae*, especially in affected pistachio tree orchards.

Unlike other newly described *Phytophthora* species from infected pistachio trees (e.g. *P. parsiana*), the isolates of *P. pistaciae* were recovered only from orchards of Kerman and Yazd Provinces of Iran. Therefore, the primers could be a useful tool for domestic and international quarantine purposes, especially for other pistachio producing countries such as the USA, Turkey, Syria and China. However, assessing more environmental samples using these specific primers should improve the reliability of the designed sets. The importance of the pistachio industry, especially in Iran, emphasizes the need for a comprehensive study of the geographic distribution of *P. pistaciae* using the species-specific primer sets.

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