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RESEARCH PAPERS

Characterization of *Phytophthora pistaciae*, the causal agent of pistachio gummosis, based on host range, morphology, and ribosomal genome

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Summary. Gummosis is the most important disease of pistachio trees in Iranian pistachio orchards. *Phytophthora pistaciae*, the main causal agent of this disease, was isolated and described from Kerman province of Iran for the first time. The present study aimed to establish the host range, morphological characteristics and ribosomal genome of *P. pistaciae* isolates. During 2009–2010, isolates of *P. pistaciae* were sampled from 12 infected pistachio plantations in Kerman and Yazd provinces of Iran. Based on phylogenetic analysis of ITS regions, all isolates belonged to a monophyletic clade and clustered with *P. pistaciae* from other studies. The isolates were high temperature tolerant and produced non-papillate, ellipsoid to ovoid sporangia with polar caps, and were non-caducous, sympodial and had internal and external proliferation. The isolates were homothallic and produced spherical oogonia with smooth walls and paragynous, rarely amphigynous, spherical to ovoid antheridia. Oospores were spherical, colorless and aplerotic. Isolates generated chlamydospores in host tissues. The host range of *P. pistaciae* is not limited to pistachio species. Almond, walnut, apricot, grape, mango, sour cherry, among woody plants, and pea, different types of beans, chickpea, faba bean, safflower and okra, among herbaceous plants, were shown to be potential hosts of the pathogen. This study showed that isolates of *P. pistaciae* were homogenous in ITS rDNA genome and their host range, and morphological traits were similar.

Key words: *Oomycota*, root rot, phylogeny.

Introduction

Pistachio is one of the most important fruit trees in Iran. Because of its adaptability to unfavorable conditions such as saline irrigation water, drought and water deficiency this tree crop has been considered the most important economical crop in arid regions (Abrishami, 1995). Pistachio is cultivated in more than 101,000 ha in Iran, and Iran is the greatest pistachio producing country in the world with annual production of 447,000 tons (FAOSTAT, 2012). Root and crown rot or gummosis disease is the most important disease of pistachio trees in Iran (Mehrnejad and Javan Shah, 2010). The disease occasionally causes yield losses of about 10–12% (Mirabolfathy *et al*., 1990). To date, several species of *Phytophthora*, including *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, *P. cryptogea* Pethybr. & Laff., *P. megasperma* Drechsler, *P. drechsleri* Tucker, *P. nicotianae* Breda de Haan, and *P. parsiana* Mostowf., D.E.L. Cooke & Banihash. have been isolated from diseased trees, and their pathogenicity on pistachio trees has been confirmed. *Phytophthora megasperma* and *P. drechsleri* are the most frequent disease pathogens (Ershad, 1971; Banihashemi, 1983b, 1995; Mirabolfathy and Ershad, 1987; Mirabolfathy, 1988; Mirabolfathy *et al.*, 1989; Aminaee and Ershad, 1991; Mostowfizadeh-Ghalamfarsa *et al*., 2008).

Phytophthora megasperma isolates were first reported from Kerman (Mirabolfathy *et al*., 1990) and

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Yazd (Fattahi Ardekani *et al*., 2000) provinces of Iran. Based on morphological, isozymic, and pathological studies and application of molecular markers, *P. megasperma* isolates from pistachio trees were a homogenous population distant from other complex groups of *P. megasperma* isolates, and the pathogen from pistachio was described as a new species named *Phytophthora pistaciae* by Mirabolfathy *et al*. (2001). In that study, *P. pistaciae* was shown to be a phylogenetic species. Because of the convergent morphological and physiological characteristics, its identification was possible only using the sequence of the internal transcribed spacers (ITS) nucleotides.

Phytophthora pistaciae as well as *P. iranica* Ershad (Ershad, 1971) and *P. parsiana* (Mostowfizadeh-Ghalamfarsa, 2008) are *Phytophthora* species which were first reported from Iran. However, there is no report of the distribution of *P. iranica* and *P. pistaciae* from other parts of the world, so these two fungi can be considered as quarantine species for other countries. The studies on *P. pistaciae* isolates are limited to the primary description by Mirabolfathy *et al*. (2001) and as part of multiple-gene genealogies of *Phytophthora* species (Blair *et al*., 2008), in which the position of one isolate has been confirmed among the other species in the second part of the *Phytophthora* Clade 7. Therefore, morphological and molecular information about *P. pistaciae* isolates is limited. Due to structural convergence of this species with other non-papillate and high-temperature tolerant species such as *P. drechsleri, P. cryptogea, P. melonis* Katsura and *P. inundata* Brasier, Sánch. Hern. & S.A. Kirk which have different host ranges, the investigation of host range and comparison among the isolates is required. In the present study valid isolates of *P. pistaciae,* the causal agent of pistachio gummosis, were evaluated in terms of morphological characteristics and host range, and genome comparisons were made based on phylogenetic analysis of ribosomal DNA.

Materials and methods

Pathogen isolation

Plant samples (0.5 cm) were cut from the regions between healthy and infected tissues of infected roots and crowns of pistachio trees and placed on CMA-PARPH medium (CMA 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 and Martin, 1986). To isolate the pathogen from soil, a baiting method using citrus (Banihashemi, 1983a) and pistachio leaves was employed. The isolates obtained were preserved on cornmeal agar

Table 1. Isolates of *Phytophthora pistaciae* recovered from infected tissues of pistachio trees or infested soil around the trees in different parts of the Iran, and their ITS accession numbers from the GenBank database.

Isolate	Matrix[a]	Year of isolation	Location[b]	ITS accession numbers
SURf14	T	1993	Kerman, Kerman	AY659415
SURab6	S	2009	Kerman, Abbasabad	HM585361
SUP.p6	T	2009	Kerman, Azadegan	HM585362
SUP.p7	T	2009	Kerman, Azadegan	HM585363
SUP.p24	T	2009	Kerman, Azadegan	HM585364
SUP.p26	S	2009	Kerman, Azadegan	—
SUKAA	S	2010	Yazd, Yazd	HM585365
SUKAB	S	2010	Yazd, Yazd	HM585360
SURno4	T	2009	Kerman, Noogh	HM585366
SURka7	T	2009	Kerman, Kabootarkhan	HM585367

[a] T = infected trees. S = infested soil around the infected trees.
[b] Province and region.

(CMA; ground corn extract 40 g L^{-1}, agar 15 g L^{-1}) at 15ºC for long-term storage. Details of the *P. pistaciae* isolates examined in this study are listed in Table 1.

Growth rate

CMA plates were inoculated using three replicate plates per isolate, and incubated at 5, 10, 15, 20, 25, 30, 35 or 37°C. Growth rates of colonies was recorded 5 d after the onset of linear growth. The experiments were repeated twice for the temperatures of 30, 35 or 37°C.

Morphological characteristics of sporangia and hyphal swellings

Ten sterile hemp (*Cannabis sativa*) seeds were placed on 7 d-old cultures grown on CMA at 20°C in the dark. After 24 h the seeds were transferred to a 6 cm Petri dish and flooded with sterile soil extract (extract of 1 kg field soil in 1 L of tap water) (Mircetich and Matheron, 1981). The Petri dishes were placed under continual fluorescent light (*ca.* 1000 lx) for 24 h at room temperature. Dimensions and characteristic features of 30 fully mature sporangia, chosen at random, were determined for each isolate using light microscopy (Schmitthenner, 1970). The possibility of production of hyphal swellings was investigated on various solid media including: CMA, hemp seed agar (HAS; ground hemp seed extract 60 g L^{-1}, agar 15 g L^{-1}), potato dextrose agar (PDA; potato extract 300 g L^{-1}, dextrose 20 g L^{-1}, agar 15 g L^{-1}) and water agar (WA; agar 10 g L^{-1}); on liquid media including: corn meal broth (CM; ground corn extract 40 g L^{-1}), hemp seed broth (HS; ground hemp seed extract 60 g L^{-1}) and potato dextrose broth (PD; potato extract 300 g L^{-1}, dextrose 20 g L^{-1}); and on hemp seeds with sterile soil extract or with non-sterile soil extract. The Petri dishes were placed under continual fluorescent light (*ca.* 1000 lx) for 48 h at room temperature production of hyphal swellings production was investigated using light microscopy.

Chlamydospore production and germination

Chlamydospore production was determined on a variety of solid media including: CMA, HSA, PDA, carrot agar (CA; carrot extract 250 g L^{-1}, agar 15 g L^{-1}), French bean agar (FBA; extract of ground French beans 30 g L^{-1}, agar 15 g L^{-1}) and WA; on liquid media including CM, HS, PD, carrot broth (CB; carrot extract 250 g L^{-1}), and French bean broth (FB; extract of ground French beans 30 g L^{-1}); and on hemp seeds in sterile distilled water or sterile soil extract was investigated at 25 or 4°C. In order to stimulate chlamydospore production 30 mg L^{-1} β-sitosterol and 1 g L^{-1} $CaCO_3$ were added to all media. Chlamydospore production was investigated each month for 9 months using light microscopy (Ribeiro, 1978). The possibility of chlamydospore production in infected plant tissues was also tested. Infected crown and roots of French bean, faba bean and pistachio were washed and placed in plastic bags containing sterile moist sand and incubated at 4ºC, and production of chlamydospores was investigated each month for 4 months. In order to examine chlamydospore germination, small parts of crown and roots containing chlamydospore-like structures were mixed with 1 mL of 3% KOH and the extract was centrifuged (Sigma, Taufkirchen, Germany) at 3000 rpm for 10 sec. Then, 0.1 and 0.01 dilutions of the supernatants were placed on WA medium in Petri dishes. The dishes were incubated at 25°C with 16:8 h light:dark photoperiod and were investigated daily to observe colony growth from chlamydospores (Safaie Farahani, 2009).

Characterization of oospores, oogonia and antheridia

Isolates of *P. pistaciae* were placed in 6 cm diam. Petri dishes containing HSA and incubated at 20°C in dark for 1 month. For each isolate 30 oogonia, oospores and antheridia, chosen at random, were measured using light microscopy.

Pathogenicity

Inoculum preparation. A mixture of 200 mL of vermiculite and 120 mL of hemp seed extract (extract of 60 g L^{-1} ground hemp seed) were autoclaved at 121ºC for 20 min (three times, every other day). Twelve 10 mm diam. blocks of CMA containing pathogen were added to plastic bags containing the mixture and incubated at 25°C in the dark for 4 weeks (Banihashemi and Fatehi, 1989).

Host range in annual and perennial plants. The seeds of annual plants from different families were selected (Table 2). The seeds were planted in 1 L pots containing sterile soil mix (soil:sand, 2:1 v/v). Ten seeds from each plant were planted and kept in a

Table 2. Susceptibility of roots of annual and perennial plants to *Phytophthora pistaciae* isolates.

Family	Common name	Scientific name	Re-isolation			Colonization	PCR detection
			SUP.p6	SURf14	SUKAB		
Anacardiaceae	Pistachio	*Pistacia vera*	+	+	+	+	Positive
	Mango	*Mangifera indica*	+	+	+	+	Positive
Juglandaceae	Walnut	*Juglans regia*	+	+	+	+	Positive
Moraceae	Fig	*Ficus carica*	-	-	+	+	Positive
Rosaceae	Sour cherry	*Cerasus vulgaris*	+	+	+	+	Positive
	Apricot	*Prunus armeniaca*	+	+	+	+	Positive
	Almond	*Prunus dulcis*	+	+	+	+	Positive
	Apple	*Malus domestica*	+	+	-	+	Positive
	Peach	*Persica vulgaris*	-	+	-	+	Positive
	Cherry plum	*Prunus divaricata*	-	-	-	-	Negative
	Medlar	*Mespilus germanica*	-	-	-	-	Negative
	Quince	*Cydonia oblonga*	+	-	-	+	Positive
	Hedge thorn	*Crataegus persica*	-	+	-	+	Positive
Rutaceae	Sour orange	*Citrus aurantium*	-	-	-	-	Negative
Vitaceae	Grape	*Vitis silvestris*	+	+	+	+	Positive
Moraceae	Mulberry	*Morus alba*	-	-	-	-	Negative
Punicaceae	Pomegranate	*Punica granatum*	-	-	-	-	Negative
Elaeagnaceae	Oleaster	*Elaeagnus angustifolia*	-	+	-	+	Positive
Asteraceae	Sunflower	*Helianthus annus*	-	-	-	-	Negative
	Safflower	*Carthamus persicus*	+	+	+	+	Positive
Chenopodiaceae	Sugar beet	*Beta vulgaris*	+	+	+	+	Positive
Cucurbitaceae	Watermelon	*Citrullus vulgaris*	-	-	-	-	Negative
	Melon	*Cucumis melo*	-	-	-	-	Negative
	Squash	*Cucurbita pepo*	-	-	-	-	Negative
	Bottle gourd	*Lagenaria vulgaris*	-	-	-	-	Negative
	Cushaw	*Cucurbita moschata*	-	-	-	-	Negative
	Cucumber	*Cucumis sativus*	-	-	-	-	Negative
Cruciferae	Cabbage	*Brassica oleracea*	-	-	-	-	Negative
Fabaceae	Chickpea	*Cicer arietinum*	+	+	+	+	Positive
	Lentil	*Lens esculenta*	-	+	-	+	Positive
	Common bean	*Phaseolus vulgaris*	+	+	+	+	Positive
	Black-eyed bean	*Vigna unguiculata* subsp. *unguiculata*	+	+	+	+	Positive
	Green bean	*Phaseolus*	+	+	+	+	Positive

(Continued)

Table 2. (Continued)

Family	Common name	Scientific name	Re-isolation			Colonization	PCR detection
			SUP.p6	SURf14	SUKAB		
	Garden pea	*Pisum sativum*	+	+	+	+	Positive
	Soybean	*Glycine max*	-	-	-	-	Negative
	Green gram	*Phaseolus radiatus*	-	-	-	-	Negative
	Faba bean	*Faba vulgaris*	+	+	+	+	Positive
	Persian clover	*Trifolium resupinatum*	-	-	-	-	Negative
Malvaceae	Okra	*Hibiscus esculentus*	+	+	+	+	Positive
	Cotton	*Gossypium herbaceum*	-	-	-	-	Negative

greenhouse at 18–30°C. After 3 weeks the inoculation was conducted using 10 mL of inoculum prepared from isolates SUP.p6, SUKAB and SURf14, and the pots were flooded for 24 h. The sterile mixture made of vermiculite and hemp seed extract was used as a negative control. In the case of perennial plants propagation was by cuttings. The bottom of each pot was sealed using melted paraffin and 50 mL of the inoculum was spread under the soil surface around the crowns of 1–2-year-old seedlings. The pots were then flooded for 24 h. Control plants received 50 mL of sterile vermiculite and hemp seed extract mixture. Plants were evaluated for wilt, defoliation and development of lesions on crowns 3 to 8 weeks (for herbs) and 6 to 12 months (for perennial plants) after inoculation. To confirm the presence of pathogenic *Phytophthora*, re-isolation from plant roots and crowns was carried out (Banihashemi, 2004). The pathogen detected in all infected tissues using PCR by species-specific primers (Mostowfizadeh-Ghalamfarsa and Mirsoleimani, 2013).

Root colonization. Inoculated plant roots (Table 2) were pulled from soil (4–6 weeks after inoculation for annual plants and after 12 months for woody plants), and washed well using distilled water. The roots were cut into 0.5 mm pieces and placed on CMA-PARP medium. The resulting fungal colonies were identified.

Pathogenicity on detached tree twigs. One-year-old detached twigs (*ca.* 9–12 mm diam.) of some species of fruiting and non-fruiting trees (Table 3) were detached from leaves and cut to about 10–15 cm lengths and surface-disinfected using 70% ethanol. The cut ends of each detached twig and any other sites of damage were covered with melted paraffin. A *ca.* 10 mm diam. plug was removed from the bark of each twig and a 5 mm agar disc, cut from the edge of a 5–10-day-old culture, was placed under the bark and the cut was sealed with Nescofilm (Bando Chemical Ind. Ltd., Kobe, Japan) to avoid desiccation. Control plants received a CMA disc only. The twigs were placed in a bowl containing 150 mL sterile distilled water to avoid desiccation and kept at room temperature for 1 month. The progress of the isolates in the twigs was examined by culturing the infected tissue on Petri dishes containing CMA-PARP semi-selective medium. The experiment was performed on twigs in active (summer) and dormant (winter) growth stages. Blocks of CMA containing *Aspergillus* spp. were used as negative controls, and of *P. cactorum* as positive controls (Safaie Farahani, 2009).

Pathogenicity test on fruits, tubers and roots. The fruits, tubers and roots of different plants (Table 4) were washed and disinfected with 70% ethanol. A 7 mm diam. plug was removed from each fruit, tuber or root. A 5 mm disc cut from a CMA culture of a young fungus colony was inserted into the plug hole, and the hole was sealed with transparent adhesive tape. Control plants received a CMA disc. The fruits, tubers or roots were placed in paper packets and kept at room temperature. Two to 7 d after inoculation, the pieces of infected tissue of the fruits, tubers or roots were transferred on CMA-PARP medium to deter-

Table 3. Susceptibility detached tree twigs to *Phytophthora pistaciae* isolates tested in active (Act.) or dormant growth stage (Dor.).

Family	Common name	Scientific name	Re-isolation					
			SUP.p6		SURf14		SCKAB	
			Act.	Dor.	Act.	Dor.	Act.	Dor.
Aceraceae	Plane-leaved maple	*Acer platanoides*	-	-	-	-	-	-
Caesalpiniaceae	Judas tree	*Cercis siliquastrum*	-	-	-	-	-	-
Juglandaceae	Walnut	*Juglans regia*	+	+	+	+	+	+
Moraceae	Fig	*Ficus carica*	-	-	-	-	-	-
	Red mulberry	*Morus rubra*	-	-	-	-	-	-
Oleaceae	Flowering ash	*Fraxinus ornus*	-	-	-	-	-	-
	Olive	*Olea europea*	-	-	-	-	-	-
	Persian jasmine	*Syringe persica*	+	-	+	-	+	-
Papilionaceae	Locust tree	*Robina pseudoacacia*	-	-	-	-	-	-
Plantanaceae	Plane tree	*Plantanus orientalis*	-	-	-	-	-	-
Punicaceae	Pomegranate	*Punica granatum*	-	-	-	-	-	-
Rosaceae	Sweet almond	*Prunus amygdalus* var. *dulcis*	+	+	+	+	+	+
	Quince	*Cydonia oblonga*	+	-	+	-	+	-
	Plum	*Prunus domestica*	+	-	+	-	+	-
	Apple	*Malus domestica*	+	-	+	-	+	-
	Peach	*Persica vulgaris*	-	-	-	-	-	-
	Apricot	*Prunus armeniaca*	+	+	+	+	+	+
Rutaceae	Sour orange	*Citrus aurantium*	-	-	-	-	-	-
Salicaceae	Weeping willow	*Salix babylonica*	-	-	-	-	-	-
Ulmaceae	Common elm	*Ulmus carpinifolia*	-	-	-	-	-	-

Sum. = summer. Win. = winter.

mine the progress of the isolates. The experiment was repeated six times for each treatment.

Molecular assays

DNA extraction. Isolates were grown in 20 mL still culture of potato extract (extract of 300 g L^{-1} potato) at 20 ºC. After vacuum filtration, the mycelia were harvested and freeze-dried. Five hundred μL of CTAB (hexadecyltrimethylammonium bromide) buffer (0.121 g of Tris-HCl, 0.074 g of EDTA, 0.818 g of NaCl, 0.2 g of CTAB, 200 μL of 2-mercaptoethanol, and distilled water up to 10 mL) containing 2% proteinase K (Fermentas, Ontario, Canada**,** UK) was added to 5 mg of freeze-dried mycelium. The mycelia were homogenized using autoclaved sand. The resulting extract was then centrifuged at 13,000 rpm for 10 min and the supernatant was collected. DNA was extracted using a DNG™-PLUS kit (Cinnagen, Tehran, Iran) according to the manufacturer's instructions. The DNA pellet was dissolved in 50 μL of sterile distilled water, and after adding 5 μL of RNase A (Fermentas) solution (5 mg μL^{-1}) was incubated at 37°C for 1 h. The resulting DNA

Table 4. Susceptibility of fruits, tubers and storage roots to *Phytophthora pistaciae* isolates.

Family	Common name	Scientific name	Re-isolation			PCR detection
			SUP.p6	SURf14	SUKAB	
Actinidiaceae	Kiwifruit	*Actinidia arguta*	+	+	+	Positive
Cucurbitaceae	Cucumber	*Cucumis sativus*	+	+	+	Positive
	Pumpkin	*Cucurbita pepo*	+	+	+	Positive
Rosaceae	Apple	*Malus domestica*	+	+	+	Positive
	Peach	*Persica vulgaris*	-	-	-	Negative
	Nectarine	*Persica* persica var. *nucipersica*	-	-	-	Negative
Rutaceae	Lemon	*Citrus medica* var. *limonum*	-	+	-	Positive
	Orange	*Citrus sinensis*	-	+	-	Positive
	Mandarin	*Citrus nobilis*	-	+	-	Positive
	Sour orange	*Citrus medica* var. *cedrata*	-	+	-	Positive
Solanaceae	Tomato	*Solanum lycopersicum*	+	+	+	Positive
	Egg plant	*Solanum melongena*	-	+	-	Positive
	Potato	*Solanum tuberosum*	-	+	-	Positive
Umbelliferaceae	Carrot	*Daucus carota*	-	-	-	Negative

was stored at -20°C. The quantity and quality of the DNA was determined using a MD-100 Nanodrop machine (NanoDrop Technologies, Wilmington, DE, USA).

DNA amplification. DNA of the internal transcribed spacer regions (ITS) on ribosomal RNA genome was amplified by polymerase chain reaction using the universal primers ITS6 (5′ GAA GGT GAA GTC GTA ACA AGG 3′) and ITS4 (5′- TCC TCC GCT TAT TGA TAT GC -3′) (White *et al.*, 1990; Cooke *et al.*, 2000). Amplifications were performed in a CG1-96 thermocycler (Corbett Research, Melbourne, Australia**,** Australia). The PCR mixture contained: 50 ng of template DNA, 1 μM of each primer, 100 μM of dNTPs, 0.4 U *Taq* DNA polymerase (Cinnagen), 1.5 mM of $MgCl_2$, 2.5 μL of 10× PCR buffer [200 mM Tris-HCl (pH 8) and 100 μM KCl], 100 mM BSA (bovine serum albumen), in a reaction volume of 25 μL. To confirm that the amplifications were not the results of reagent and reaction contaminations, control samples with no DNA were used in each experiment. All reactions were performed with a program of 95°C for 2 min, 30 cycles including 95°C for 20 s, 55°C for 25 s and 72°C for 50 s, and with a final cycle at 72°C for 10 min. 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 and the size of amplified fragments was estimated using 100 bp DNA marker (Gene Ruler, Fermentas).

Sequencing of amplified product. The amplification products of all isolates were purified through purification columns (GeneJet™ PCR Purification Kit; Fermentas) to remove excess primers and nucleotides. PCR products were sequenced in forward and reverse orientation using the primers used for amplification by means of dye terminator cycle sequencing kits (BigDye sequencing kit, Applied Biosystems, Foster City, California, USA) on an ABI377-96 automated sequencer (Applied Biosystems) according to the manufacturer's instructions. The resulting sequences were edited using Bioedit software (Hall, 1999) and submitted to GenBank [Gen-

Bank, NCBI, Bethesda, Maryland, USA; (Online) http://www.ncbi.nlm.nih.gov/] through Internet using BankIit software [GenBank, NCBI, Bethesda, Maryland, USA; (Online) http://www.ncbi.nlm.nih.gov/BankIt/].

Phylogenetic analyses and evaluation of genetic diversity. The phylogenetic study was conducted to find relationships between the resulting isolates and other previously described species, and to provide understanding of the intra-specific genetic diversity for the internal transcribed spacer regions (ITS) on ribosomal DNA. To achieve a clear picture about the amount of interspecific variation and correct comparison between the target species and the others, valid sequenced genomic fragments of other *Phytophthora* species were extracted from GenBank and used in the analysis (Table 5). *Pythium aphanidermatum* was used as the out-group in phylogenetic studies. Primarily alignment of the edited sequences was carried out using ClustalX software (Thompson *et al*., 1997) followed with manual adjustments. The ITS alignment was analyzed by the distance-based method as well as the maximum likelihood method in PHYLIP (Felsenstein, 1993). Model parameters including rate heterogeneity and substitution, and also the expected transition/transversion ratios, γ distribution of α parameters were estimated using the PUZZLE program (Strimmer and von Haeseler, 1996). The maximum likelihood approach using PHYLIP DNAML software (Felsenstein, 1993), and neighbour joining using PHYLIP DNADIST software (Felsenstein and Churchill, 1996), were employed to estimate the topography of the phylogenetic trees. The resulting similarity matrix from PHYLIP DNADIST was used as the input of PHYLIP NEIGHBOR software (Felsenstein, 1993). The robustness of the maximum likelihood trees was tested using 100 bootstrap trials and for neighbour joining with 500 bootstrap trials. The trees were drawn using Treeview (Page, 1996).

Results

Phylogenetic analyses

The studied isolates from pistachio plantations of Kerman and Yazd provinces of Iran (Table 1) were identified as *P. pistaciae* in comparison with reference *P. pistaciae* ITS sequences (Mirabolfathy *et al*., 2001). All of these isolates were also pathogenic on roots of pistachio trees. This is the first report of the pathogen after the original description of the species.

DNA fragments, *ca.* 980 bp, were amplified by PCR reaction using universal ITS4 and ITS6 primers. Neighbour joining (Figure 1) and maximum likelihood (data not shown) phylogenetic analyses of isolates, together with genomic fragments from 80 *Phytophthora* species, generated similar phylogenetic trees. According to the resulting data the similarity of ITS sequences between different isolates of *P. pistaciae* was 99–100%.

Growth rate

The average growth of the isolates is shown in Table 6. Minimum, maximum and optimum growth temperatures were estimated as, respectively, 5, 35 and 30°C (Figure 2).

Sporangia, hyphal swellings and chlamydospores

Sporangia were non-papillate, ovoid, ellipsoidal, with polar caps, non-descending, sympodial with internal and external proliferation, mean dimensions of 63.3 (± 4.5) × 37.5 (±2.6) μm (with a range of 38–95 × 29–50 μm), and mean length/width ratio of 1.56 (Table 6). Our observations showed that no hyphal swellings were produced on agar. All isolates produced a few hyphal swellings in all liquid media tested and with hemp seeds and non-sterile soil extract. The hyphal swellings were dark and were peg-like spikes in tandem chains (Table 6).

No chlamydospores were found in monthly investigation on solid and liquid media, but at the end of the fourth month chlamydospores were observed on infected crowns in different treatments of infected tissues (Table 6). The average size of chlamydospores was 11.9 (±1.3) μm (range 8–15 μm) (Table 6). No chlamydospores were found on roots and crowns of control plants. The chlamydospores produced in plant tissues did not germinate even after 2 months on WA.

Morphology of oogonia, oospores and antheridia

Oospore production on HSA medium after 4 weeks showed that all isolates were homothallic. Oogonia were rounded and had smooth walls which

Table 5. GenBank accession numbers of internal transcribed spacer sequences of rDNA (ITS) of *Phytophthora* species used for phylogenetic analyses.

Species	Accession number	Species	Accession number	Species	Accession number
P. andina	AY770741[2]	*P. hedraiandra*	AY707987[24]	*P. niederhauserii*	EU194437[26]
P. arecae	AF266781[1]	*P. heveae*	AF266770[1]	*P. palmivora*	AF266780[1]
P. bisheria	AF408625[21]	*P. hibernalis*	AY369369[20]	*P. parsiana*	AY659739[17]
P. boehmeriae	DQ297406[3]	*P. humicola*	AF266792[1]	*P. phaseoli*	AF266778[1]
P. botryosa	AF266784[1]	*P. idaei*	AF266773[1]	*P. pistaciae*	AY659414[28]
P. brassicae	AF380147[22]	*P. ilicis*	AJ131990[1]	*P. polonica*	DQ396409[13]
P. cactorum	AF266772[1]	*P. infestans*	AF266779[1]	*P. primulae*	AF266802[16]
P. cajani	AF266765[1]	*P. inflate*	AF266789[1]	*P. pseudotsugae*	AF266774[1]
P. cambivora	AF266763[1]	*P. insolita*	AF271222[1]	*P. pseudosyringae*	EU074793[3]
P. captiosa	DQ297405[3]	*P. inundata*	AY659717[16]	*P. psychrophila*	AF449494[23]
P. capsici	AF266787[1]	*P. ipomoeae*	AY770742[2]	*P. quercina*	AJ131986[14]
P. cinnamomi	AF266764[1]	*P. iranica*	AJ131987[1]	*P. quercetorum*	DQ313223[27]
P. citricola	AF266784[1]	*P. katsurae*	AF266771[1]	*P. quininea*	DQ275189[13]
P. citrophthora	AF266785[1]	*P. kelmania*	AY117033[7]	*P. ramorum*	AY540491[15]
P. clandestina	AJ131989[1]	*P. kernoviae*	AY940661[8]	*P. richardiae*	AF271221[1]
P. colocasiae	AF266786[1]	*P. lagoariana*	EF590256[9]	*P. sansomea*	DQ275186[12]
P. cryptogea	AF266796[1]	*P. lateralis*	AF266804[1]	*P. sinensis*	AF266768[1]
P. drechsleri	AF266798[1]	*P. macrochlamydospora*	L41373[10]	*P. siskiyouensis*	EF523387[19]
P. erythroseptica	AF266797[1]	*P. meadii*	AY251649[18]	*P. sojae*	AF266769[1]
P. europaea	AF449491[23]	*P. medicaginis*	AB367511[11]	*P. sulawesiensis*	EF590257[9]
P. fallax	DQ297398[3]	*P. megakarya*	AF266782[1]	*P. syringae*	AF266803[1]
P. fragariae	AF266762[1]	*P. megasperma*	AF266794[1]	*P. tentaculata*	AF266775[1]
P. rubi	AF266761[1]	*P. melonis*	AY659660[16]	*P. trifolii*	AF266800[1]
P. foliorum	EF120469[4]	*P. mirabilis*	AF266777[1]	*P. tropicalis*	AY208125[25]
P. gallica	DQ286726[5]	*P. multivesiculata*	AF266790[1]	*P. uliginosa*	AF449495[23]
P. glovera	AF279128[6]	*P. nemorosa*	DQ275187[12]	*P. vignae*	AF266766[1]
P. gonapodyides	AY659417[16]	*P. nicotianae*	AF266776[1]		

[1] Cooke *et al*., 2000. [2] Unpubl. data (Flier W.G, Adler N, Kroon L.P.N.M and Forbes G.A). [3] Dick *et al*., 2006. [4] Donahoo *et al*., 2006. [5] Unpubl. data (Jung T., Nechwatal J. and Mendgen K.). [6] Unpubl. data (Abad J.A., Abad G.Z. and Shew D.). [7] Unpubl. data (Abad G.Z., Abad J.A., Ivors K.L., Benson M.D. and Creswell T.C.). [8] Brasier *et al*., 2005. [9] Unpubl. data (Coffey M.D. and Peiman M.). [10] Crawford *et. al*., 1996. [11] Unpubl. data (Uddin A.J., Senda M. and Kageyama K.). [12] Unpubl. data (Belbahri L, Calmin G. and Lefort F.). [13] Belbahri *et al*., 2006. [14] Unpubl. data (Cooke D.E.L.). [15] Giltrap *et al*., 2004. [16] Mostowfizadeh-Ghalamfarsa, 2011. [17] Mostowfizadeh-Ghalamfarsa *et al*., 2008. [18] Unpubl. data (Huang L.C. and Liou R.F.). [19] Unpubl. data (Reeser P.W., Hansen E.M. and Sutton W.). [20] Martin and Tooley, 2003. [21] Abad *et al*., 2008. [22] Man in't Veld *et al*., 2002. [23] Jung *et al*., 2002. [24] de Cock and Levesque, 2004. [25] Unpubl. data (Zhang Z., Wang Y. and Zheng X.). [26] Unpubl. data (Perez-Sierra A., Moralejo E. and Belbahri L.). [27] Balci *et al*., 2008. [28] Mirabolfathy *et al*., 2001.

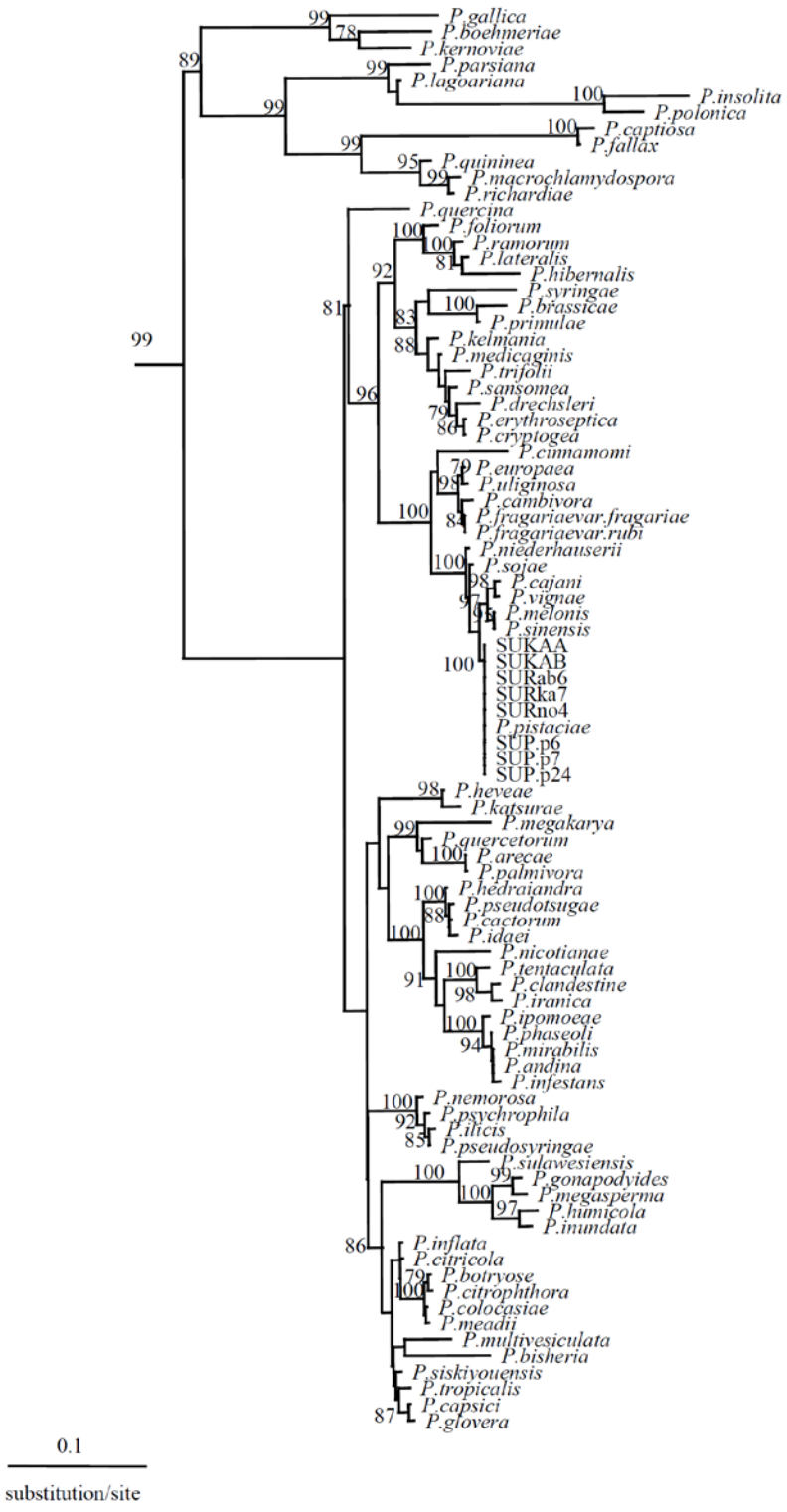

Figure 1. Phylogram of a neighbour-joining analysis of the *Phytophthora pistaciae* isolates examined in this study, together with 80 *Phytophthora* species based on the ITS1, 5.8S subunit, and ITS2 regions of the rDNA. The numbers at the branch points indicate the percentages of bootstrap values ≥50%.

Table 6. Variation in morphological and physiological characteristics of *Phytophthora pistaciae* isolates examined in this study.

Character	Isolate									
	SURf14	**SUP.p6**	**SUP.p7**	**SUP.p24**	**SUP.p27**	**SURab6**	**SURka7**	**SURno4**	**SUKAA**	**SUKAB**
Chlamydospores										
in liquid media	-	-	-	-	-	-	-	-	-	-
in solid media	-	-	-	-	-	-	-	-	-	-
in infected tissues	+	+	+	+	+	+	+	+	+	+
Average diam. (μm)	12. ±1.	11.4±0.6	13±1.3	13±1.3	12.6±0.7	11.2±1.6	11.3±1.2	12.5±1	11.5±1.2	11.1±0.7
Range (μm)	10.3-13. 6	10.8-12.4	10.4-15.3	10.8-15.5	11.3-13.6	9-13.6	8.9-12.8	10.6-13.6	8.9-12.8	10.2-12.1
Sporangia										
Papilla	-	-	-	-	-	-	-	-	-	-
Average length (μm)	51.9±6.7	64.5±1.9	63.6±1.9	64.6±3.1	64.2±2.5	61.7±4.7	69.6±8.1	62.5±3.4	66.3±5.2	64.5±4.3
Range length (μm)	38.1-60.3	62.1-70.5	60.3-67.3	60.6-71.5	61.3-71.5	54.1-70.5	63.3-95.1	55.5-68.5	61.3-84.3	55.3-71.3
Average breadth (μm)	34.1±4.1	42.6±1.9	37.7±1.7	36.6±3.2	34.4±2.2	37.5±4.4	39.3±4.6	38.4±2.1	37.8±3.5	38.6±2.7
Range breadth (μm)	29-43.4	38.3-45.8	35.3-42.6	29.8-44.1	30.4-38.6	30.2-45.1	32.3-5	35.4-42.8	31.3-44.4	32.35-42.4
Length: breadth ratio	1.5	1.5	1.7	1.8	1.9	1.6	1.8	1.6	1.8	1.7
Shape(s)	Ov.	Ellip.	Ov.	Ellip./Ov.	Ellip./ Ov.	Ellip./Ov.	Ellip./Ov.	Ellip./ Ov.	Ellip./ Ov.	Ov.
Tapered base	-	-	-	-	-	-	-	-	-	-
Caducity	-	-	-	-	-	-	-	-	-	-
Proliferation	P/N	P/N	P/N	P/N	P/N	P/N	P/N	P/N	P/N	P/N
Production on solid media	-	-	-	-	-	-	-	-	-	-
Production in liquid media	-	-	-	-	-	-	-	-	-	-
Production on hemp seeds	+	+	+	+	+	+	+	+	+	+
Oogonia										
Average diam. (μm)	32.1±3.8	35.8±4	38.4±4	33.3±2.3	35.4±3.3	33.5±3.1	37.4±3.3	34.6±2.7	38.5±3.1	35.6±2.1
Range (μm)	26-36.1	32.3-43.8	33.4-45	29.7-37.6	30.3-41.3	27.6-36.4	34.1-43.4	29.3-38.8	35.3-44.4	33.3-39.4
Shape(s)	Sph.	Sph.	Sph.	Sph.	Sph.	Sph.	Sph.	Sph.	Sph.	Sph.
Tapered base	-	-	-	-	-	-	-	-	-	-

(Continued)

Table 6. (Continued)

Character	Isolate									
	SURf14	SUP.p6	SUP.p7	SUP.p24	SUP.p27	SURab6	SURka7	SURno4	SUKAA	SUKAB
Oospores										
Average diam. (μm)	24.3±3.9	26.4±3.7	30.3±2.8	24.4±2.3	27.3±3.2	24.4±3.4	27.5±2.6	25.5±3.4	31.3±3.5	26.4±3.6
Range (μm)	20.3-32.3	20.1-31.3	26-33.2	20-28.2	22.2-33.3	21.1-31.7	23.4-32.3	20.3-29.6	22.1-34	22.1-31.3
Aplerotic	+	+	+	+	+	+	-	+	+	-
Antheridia										
Average length (μm)	15±1.6	14.3±0.9	15.1±2.1	12.9±2.1	15.3±0.9	14.4±1.2	14.6±1.4	13.4±1.1	16.3±2.1	13.7±0.1
Range length (μm)	13.1-17.7	12.3-15.4	13.1-19.7	8.1-15.2	13.7-16.5	12.3-15.9	12.3-16.7	12.1-15.3	14.2-21	12.3-15.3
Average breadth (μm)	13.9±1.4	13.2±0.9	14.2±1.7	11.6±1.7	14.5±0.8	13.5±1.2	13.2±1.1	12.4±1.1	15.1±1.8	12.7±0.75
Range breadth (μm)	12-16.4	11.1-14.3	12.3-18.5	9-14.3	12.6-15.5	11.5-14.5	11.2-15.4	11.1-14.4	13.1-19	11.1-14.1
Shape(s)	Sph./Ov.	Sph./Ov.	Sph./Ov.	Sph./Ov.	Sph./Ov.	Sph./Ov.	Sph./Ov.	Sph./Ov.	Sph./Ov.	Sph./Ov.
Inception	Par./Amph.	Par.	Par./Amph.	Par.	Par.	Par.	Par./Amph.	Par.	Par.	Par.
Hyphae										
Average diam. (μm)	5.5	5.4	5.9	5.3	4.9	5.3	5.1	5.4	5.7	5.5
Hyphal swellings										
Production on solid media	-	-	-	-	-	-	-	-	-	-
Production in liquid media	-	-	-	-	-	-	-	-	-	-
Production on hemp seeds	+	+	+	+	+	+	+	+	+	+
Average growth rate (mm d^{-1}) on CMA at (°C)										
5	1.8	2.5	1.5	3.3	2.4	2.3	2.2	2.7	1.2	1.8
10	4.3	4.6	3.9	4.5	4.1	4.6	4.7	5.4	4.9	4.3
15	5.5	6.4	5.6	4.8	4.7	5.5	5.6	4.8	4.7	5.1
20	7.2	8.1	7.5	7.6	6.9	7.4	7.5	8.2	7.7	7.5
25	9.1	9.2	9.4	10.3	9.2	9.5	9.4	10.1	9.4	9.1
30	10.3	11.9	10.6	10.7	10.3	10.3	10.9	11.1	11.3	10.1
35	4.1	4.4	3.8	4.6	4.6	4.4	4.4	4.4	4.7	3.8
37	0	0	0	0	0	0	0	0	0	0

+, Feature occurring frequently; -, Feature not observed; Amph., Amphigynous; Ellip., Ellipsoid; .N, Nesting proliferation; Ov., Ovoid; Par., Paragynous, P, Proliferating isolate; Sph., Spherical.

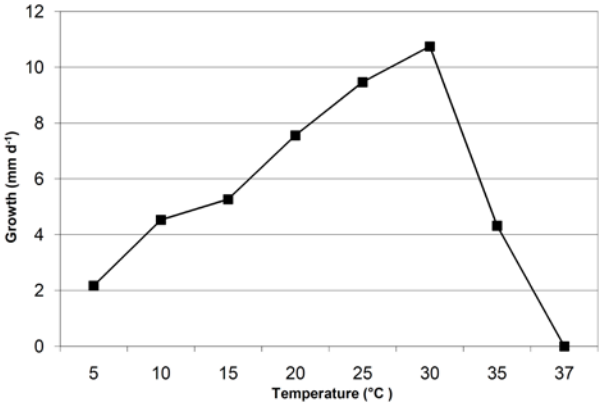

Figure 2. Average radial growth rate of *Phytophthora pistaciae* isolates on cornmeal agar at different temperatures.

were 1–4 μm thick. The average size of oogonia was 35.4 (±2.2 μm; range of 26–45 μm). Antheridia were mostly paragynous, rarely amphigynous and rounded to ellipsoid with average size of 14.2 × 12.9 μm (range 8–21 × 9–19 μm). Oospores were rounded and aplerotic, colourless, with average size of 26.8 (±2.41) μm (range 20–34 μm) with walls that were 1.6–2.4 μm thick (Table 6).

Host range

The results from inoculations of different plants are shown in Table 2. Disease symptoms on plant shoots included wilt, defoliation and decline. In some cases root rots and colour changes in the centres of plant crowns occurred together. *Phytophthora pistaciae* isolates had the ability to infect roots and crowns of annual and perennial plants (Table 2) without inducing any visible symptoms (wilt, decline or defoliation) on aerial plant parts. In all non-infected and non-colonized plants, re-isolation of pathogen from soil in pots by pistachio leaf baiting showed that the isolates were alive and active. All tested isolates infected almond, apricot and walnut detached twigs in both dormant and active growth stages, and infected only dormant stage detached twigs of quince, plum, apple and Persian jasmine (Table 3). All of the isolates caused spreading lesions on some species of fruits (Table 4). None of the isolates produced any symptoms in potato tubers or carrot roots. The identity of re-isolated samples from the infected tissues was confirmed by PCR using species-specific primers (Tables 2 and 4).

Discussion

This is the first report of *P. pistaciae* after the original description of the species. However, after more than 10 years from the primary report (Mirabolfathy *et al*., 2001) there has been no report on the occurrence of the pathogen outside of Iran, This suggests that *P. pistaciae* is a native pathogen in Iran.

Sporangia of the isolates studied here were produced after 1 day in Petri dishes, and their size was not significantly different from that outlined in the first description of the species. However, the size of sporangia (Table 6) was different from those indicated in the identification key Gallegly and Hong (2008), in which the mean size of primary sporangia was 77 × 40 μm and of secondary was 54 × 37 μm. The isolates used by Gallegly and Hong (2008) were the same as those of Mirabolfathy *et al*., (2001) in the original description. Based on the Mirabolfathy *et al*., (2001) measurements, the mean size of oogonia was 35 μm (range 25–44 μm), close to the dimensions of oogonia measured in the present study (35.4±2.2 μm). The antheridia of the species had a mean size of 15 × 14 μm in the first description by Mirabolfathy *et al*., (2001), which was slightly larger than values determined in our study (14.2 × 12.9 μm). The other morphological characteristics of antheridia were consistent with the primary description. However, in the Gallegly and Hong (2008) identification key the antheridia were reported mostly as paragynous and round with mean size of 12–15 μm. There were no differences between the oospore morphological and morphometric characteristics of the present study compared with those described by Mirabolfathy *et al*., (2001) and Gallegly and Hong (2008). The minimum, maximum and optimum growth temperatures of 5, 35 and 30ºC, respectively, were consistent with other studies (Mirabolfathy *et al*., 2001; Gallegly and Hong, 2008).

Production of chlamydospres occurs only in some *Phytophthora* species (Stamps *et al*., 1990). The media compounds, optimum temperature and humidity, and other environmental factors such as pH can affect chlamydospore production. In the primary description of *P. pistaciae*, no chlamydospores were found in the different media tested, and no chlamydospores were observed in the present study. We have demonstrated, however, the isolates we studied are capable of chlamydospore production on the crowns of host plants. This indicates that this species can produce chlamydospores only in plant tissues and in unfavourable conditions, such as low temperature (e.g. 4ºC), in order

to survive. Nevertheless, the lack of chlamydospore germination did not confirm their role in the life cycle of the pathogen. Some *Phytophthora* species, such as *P. drechsleri*, do not normally produce chlamydospores in soil or on agar (Tucker, 1931; Waterhouse, 1963; Ershad, 1971). However *P. drechsleri* can produce chlamydospores after a week in artificially infected Egyptian bean (*Lupinus angustifolius*) maintained in humid soil (Cother and Griffin, 1973).

Generally, in the investigation of species that are not distinguishable using morphological characteristics, or in the investigation of complex species such as *P. megasperma, P. cryptogea* and *P. drechsleri*, it is better to use a combination of both morphological and molecular characteristics (Hansen and Maxwell, 1991). Analysis of ITS regions of rDNA was recognized as the main criterion for *P. pistaciae* identification (Mirabolfathy *et al.,* 2001). The results of ITS analyses showed that the isolates of *P. pistaciae* examined in the present study and those from other studies were in a homogeneic group. This group was clustered in a monophyletic clade in which the closest species were *P. sinensis* Y.N. Yu & W.Y. Zhuang, *P. melonis, P. vignae* Purss and *P. cajani* K.S. Amin, Baldev & F.J. Williams, consisting Clade 7 of *Phytophthora* species (Blair *et al*., 2008).

Although there were high similarities in ITS regions of the samples, the comparison of nucleotide sequences among the studied species showed that single nucleotide polymorphisms (SNPs) occurred in some regions of sequences (e.g. 443, 275, 229, and 208 positions from rDNA of the isolates from the Azadegan region). This confirms that there could be some lines in the population. However, further investigations and sampling are required to attribute the SNPs to specific regions. The SNPs described above are probably a heteroplasmy type, not homozygote, and their divergence rate is not great enough to conclude with certainty that there are distinct lines in the *P. pistaciae* population.

Results of the present study indicate that the host range of *P. pistaciae* is not limited to pistachio. In addition to other closely related plants such as mango, the pathogen can also infect plants in other families including *Juglandaceae, Rosaceae, Vitaceae, Asteraceae, Chenopodiaceae, Fabaceae* and *Malvaceae.* The severe disease symptoms observed on most of the plants in *Rosaceae*, as well as the ability to infect some rosaceous plants without elicitation of symptoms suggests the high susceptibility of this family to the pathogen.

No *P. pistaciae* isolates could infect the hosts affected by morphologically convergent and host specific species such as *P. sojae* Kaufm. & Gerd. (soybean pathogen), *P. trifolii* E.M. Hansen & D.P. Maxwell (clover pathogen) and *P. medicaginis* E.M. Hansen & D.P. Maxwell (alfalfa pathogen), all of which have been previously included in the *P. megasperma* complex. Information from host range investigations can be used to discriminate *P. pistaciae* from other non-papillate *Phytophthora* species, especially those which could be pathogenic on pistachio trees. Isolates of *P. pistaciae* did not cause disease or even colonize the roots of cucurbits. Therefore, these characteristics can be used to distinguish this species from non-papillate *P. melonis* which causes gummosis on pistachio (Mirabolfathy *et al*., 2001). In addition to convergent morphological characteristics, *P. melonis* is a closely related sister group of *P. pistaciae* (Blair *et al*., 2008), and its annual plant host range includes cucurbits (Katsura, 1968). It seems that we could use fababean to discriminate *P. pistaciae* from other pathogenic, non-papillate *Phytophthora* species on pistachio trees. Severe symptoms, including root and crown rot, appeared on faba bean plants after inoculation with all the isolates tested and in all replicates, while faba bean is not the host of other non-papillate morphologically convergent species isolated from pistachio including *P. melonis, P. drechsleri, P. parsiana* and *P. inundata* (Katsura, 1968; Erwin and Riberio, 1996; Safaie-Farahani and Mostowfizadeh-Ghalamfarsa, 2010; Hajebrahimi and Banihashemi, 2011).

The results of inoculation of detached twigs in warm season (active) and cold season (dormant) twigs showed that all the isolates infected almond, apricot and walnut in both stages. However, the infection by isolates on active stage twigs was only observed in twig which had been severely infected in the host range test. All isolates used in this test developed infections and disease on ten different kinds of fruits (Table 4), while none of the isolates infected carrot roots or caused pink rot in potato tubers. Therefore, these species can be easily distinguished from some convergent species like *P. drechsleri, P. cryptogea* and *P. erythroseptica* Pethybr. which can cause pink rot in potato tubers (Mostowfizadeh-Ghalamfarsa *et al*., 2006).

In general, this study has shown that *P. pistaciae* isolates are uniform in terms of morphological, molecular and host range characteristics. Although a few lines can be distinguished using molecular analysis

of rDNA, no significant difference was found among other genotypic characteristics of isolates from various regions. However, there is a possibility that pistachio cultivars react differently to different isolates. According to our observations this species can be distinguished from other sister species using differential hosts. However, exact identification of this species can be achieved only by phylogenetic analysis of the ITS region of the ribosomal genome or using specific primers based on this region (Mostowfizadeh-Ghalamfarsa and Mirsoleimani, 2013). The study of the reaction of pistachio rootstocks to *P. pistaciae* isolates from various geographical locations is under investigation, and this could provide a clearer picture about the pathogenic potential of this species.

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