بررسی تنوع میزبانی گیاهان علفی و ارتباطات فیلوژنتیکی در میان جدایه‌های *Phytophthora parsiana*

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چکیده

Phytophthora parsiana **به عنوان یک اُمیست مقاوم به دمای بالا با دامنه‌ی میزبانی محدود به گیاهان چوبی، مورد توجه است. در مطالعه‌ی حاضر در یک آزمایش مقدماتی، دامنه میزبانی بیمارگر روی گونه های مختلف گیاهان علفی با استفاده از شش جدایه‌ی *P. parsiana*، شامل جدایه‌های تیپ این گونه از منابع مختلف، در شرایط گل‌خانه بررسی شد. دو جدایه‌ی قارچ، از میان گیاهان علفی شامل کدوییان، فلفل، حبوبات و گیاهان روغنی، تنها روی فلفل بیماریزا بودند. سپس به منظور بررسی واکنش ارقام مختلف فلفل به بیمارگر، بیماریزایی ۱۶ جدایه‌ی *P. parsiana* روی سه رقم فلفل بررسی شد. نتایج نشان داد که *P. parsiana* تنها روی فلفل قرمز و نه فلفل دلمه‌ای بیماریزا بود. این اولین گزارش از میزبان علفی برای *P. parsiana* است. بین جدایه‌های *P. parsiana* بیماریزا روی فلفل قرمز و دو جدایه‌ی دیگر از این گونه که از پسته رفسنجان و یزد جداسازی شده بودند و روی فلفل قرمز بیمارگر نبودند، ارتباطات فیلوژنتیکی مورد بررسی قرار گرفت. بر اساس توالی‌سنجی ناحیه‌ی فاصله‌ی ترانویسی شده‌ی داخلی (ITS)، جدایه‌ی بیمارگر روی فلفل و جدایه‌ی رفسنجان همراه با دیگر جدایه-های *P. parsiana* در تبار ۱۰ از درخت فیلوژنتیکی قرار گرفتند ولی جدایه‌ی یزد هیچ شباهتی با *P. parsiana* نداشت ودر تبار شش درخت فیلوژنتیکی با شباهت بسیار نزدیک (شباهت نوکلئوتیدی ۱۰۰-۹۹ درصد) همراه با *Phytophthora* taxon Walnut گروه‌بندی شد. این اولین گزارش از *Phytophthora* taxon Walnut از پسته در ایران است.**

کلیدواژه: پسته، *Phytophthora* taxon Walnut، ایران، فلفل

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Herbaceous host plants diversity and phylogenetic relationship among isolates of *Phytophthora parsiana*

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Abstract

The high temperature-tolerant oomycete *Phytophthora parsiana* was considered to be limited to woody plants. In the preliminary study, the host range of the pathogen on annual herbaceous plant species using six isolates of *P. parsiana* including type strains from different sources was examined under greenhouse conditions. Among herbaceous species including cucurbits, pepper, pulse and oil crops, two isolates were pathogenic to pepper. Then three cultivars of pepper were used to discriminate pepper cultivars to sixteen isolates of *P. parsiana*. Results showed *P. parsiana* were pathogenic to red pepper ('Anheim' and 'Casabel'cultivars) but not bell pepper. This is the first report of an herbaceous host of *P. parsiana (sensu lato).* Phylogenetic relationships of the pathogenic isolates of *P. parsiana* from pistachio to pepper and two isolates of the species from pistachio from Rafsanjan and Yazd were not pathogenic to pepper were examined. Based on sequences of the internal transcribed spacer (ITS) region of the ribosomal RNA, isolates pathogenic to pepper and one from Rafsanjan were in a group of *P. parsiana* belonging to clade 10 but the Yazd isolate had no genetic similarity to *P. parsiana*, being placed in clade 6 of the ITS tree, closely matching that of *Phytophthora* taxon Walnut (99-100 nucleotide identity). This is the first report of *Phytophthora* taxon Walnut from pistachio in Iran.

Keywords: Pistachio, *Phytophthora* taxon walnut, Iran, pepper

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Introduction

Phytophthora parsiana was originally isolated from the crown of a fig tree in southern Iran (Shabankareh in Bushehr province) and reported as a high temperature *P. cryptogea* (Banihashemi and Ghaisi, 1992). Upon phylogenetic studies using different genes it was subsequently described as a new species in clade 10 as *P. parsiana* (Mostowfizadeh-Ghalamfarsa *et al.*, 2008). The host range of this new species was further studied and it was found to be limited to woody hosts (Hajebrahimi and Banihashemi, 2011; Rafiee and Banihashemi, 2013). Among woody plants almond and apricot were the most susceptible hosts (Rafiee and Banihashemi, 2013).

Six isolates of *P. parsiana* from fig and pistachio were inoculated to various hosts by Hajebrahimi and Banihashemi (2011). Fig isolate (PH21-5-90) and an isolate from a pistachio trunk in California, USA (PH21-3-92) were genetically identical but the other isolates from pistachio from Rafsanjan in Kerman province showed genetic variations. The physiological characteristics of identification of *P. parsiana* were high temperature tolerance and formation of botryose hyphal swellings (Banihashemi, unpublished 1992). Further studies then showed that isolates of *P. parsiana* (*sensu lato*) selected according to these criteria were phylogenetically related (Mostowfizadeh-Ghalamfarsa *et al.*, 2008).

Among six original isolates of *P. parsiana* used, almond isolate from Greece was basal to other isolates. Pistachio isolates from Iran including isolates PH21-6-92, PH21-7-92 and PH21-4-92 showed a recent common ancestor, while PH21-3-92 (pistachio trunk isolate from USA) and PH21-5-90 (fig trunk from southern Iran) to be sister taxa and considered to be *P. parsiana* (Mostowfizadeh-Ghalmfarsa *et al.*, 2008) .

The objective of the present study was using more isolates of *P. parsiana* to study their diversity in respect to host range and phylogenetic traits.

Materials and Methods

Sources of isolates

In the preliminary study six isolates tentatively identified as *P. parsiana* (PH21-2-05, PH21-3-92, PH21-4-92, PH21-5-90, PH21-6-92, PH21-7-92) and then sixteen isolates were used (Table 1).

Table1. Isolates of *Phytophthora parsiana* used in this study.

Year	Location	Host	Isolate code
1976	Greece	Almond	PH21.2.05
1992	USA	Pistachio	PH21.3.92
1992	Iran (Kerman)	Pistachio	PH21.4.92
1991	Iran (Bushehr)	Fig	PH21.5.90
1993	Iran (Kerman)	Pistachio	PH21.6.92
1993	Iran (Kerman)	Pistachio	PH21.7.92
2008	Iran (Kerman)	Pistachio	PH21.13.08
2009	Iran (Kerman)	Pistachio	PH21.21.10
2009	Iran (Kerman)	Pistachio	PH21.22.10
2010	Iran (Kerman)	Pistachio	PH21.24.10
2010	Iran (Yazd)	Pistachio	PH21.27.10
2010	Iran (Yazd)	Pistachio	PH21.30.10
2012	Iran (Kerman)	Pistachio	PH21.33.12
2012	Iran (Kerman)	Pistachio	PH21.34.12
2012	Iran (Kerman)	Pistachio	PH21.35.12
2012	Iran (Kerman)	Pistachio	PH21.36.12

Inoculum production

Hyphal tip isolates were grown on corn meal agar (CMA) for 3-4 days at 25°C and few blocks were transferred to vermiculate amended with hemp seed extract (Banihashemi, 2004) and incubated at room temperature for 4-5 weeks.

Herbaceous host range study

Seeds of herbaceous plants from different families including sunflower (*Helianthus annuus*), broad bean (*Faba vulgaris*), chickpea (*Cicer arietinum*), pumpkin (*Cucurbita moschata*), long melon (*Cucumis melo cv. inodorus*) common bean (*Phaseolus vulgaris*), red ('Anheim' and 'Casabel'cultivars) and bell pepper (*Capsicum annuum*), soybean (*Glycine max*) and safflower (*Carthamus tinctorius*) were surface-disinfested in 0.5% sodium hypochlorite for 5 min, washed several times in sterile distilled water and incubated between sterile moist paper towels at 25°C to induce germination.

Ten to 12 germinated seeds were sown in 15 cm plastic pot containing sterilized soil and sand (2/1 ratio) and incubated in a greenhouse (20–25°C) and irrigated as needed. After two weeks, the number of seedlings in each pot was reduced to 4–5.

Inoculation

In the preliminary study, ten ml of vermiculate inoculum of selected isolates of *P. parsiana* (PH21-2-05, PH21-3-92, PH21-4-92, PH21-5-90, PH21-6-92, PH21-7-92) was placed around seedlings in the pot and flooded overnight with the drainage hole closed. To monitor the activities of the isolates, the drainage holes were opened the next day and drained water was collected and baited with citrus leaf disc (Banihashemi, 2004). Controls received vermiculite hempseed extract. The flooding and drainage baiting were repeated every other week.

Reaction of pepper cultivars to P. parsiana isolates

Based on the preliminary results of herbaceous plants, three cultivars of pepper were used to discriminate pepper cultivars to 16 isolates (Table 1) of *P. parsiana* using red ('Anheim' and 'Casabel') and bell pepper. They were inoculated with 4-week-old vermiculite inoculums of 16 isolates of *P. parsiana* and the activity of the isolates was monitored as described. Isolates PH21-4-92 and PH21-7-92 caused mortality of 33 and 22% in cultivar Anheim and 44 and 22% in cultivar Casabel respectively but could not infect bell pepper. Other isolates of *P. parsiana* failed to infect peppers.

DNA extraction and sequencing

Fungal isolates (PH21-30-10 from Harat in Yazd province and PH21-34-12 from Kerman province) were grown in 50 ml culture of potato broth (extract of 300 gL^{-1} potato) at 20°C for 7 days. After vacuum filtration, the mycelia were harvested and freeze-dried. DNA was extracted from freeze-dried fungal mycelium as described by Mirsoleimani and Mostowfizadeh-Ghalamfarsa (2013). The internal transcribed spacer (ITS) region of the ribosomal RNA genome was amplified by polymerase chain reaction using the universal primers ITS 6 (5′ GAA GGT GAA GTC GTA ACA AGG 3′) and ITS 4 (5′- TCC TCC GCT TAT TGA TAT GC-3′) (White *et al.*, 1990; Cooke *et al.*, 2000). The PCR reaction mixture, PCR conditions and visualization were as described by Mirsoleimani and Mostowfizadeh-Ghalamfarsa (2013). The amplification products of all isolates were purified through purification columns (GeneJet™ PCR Purification Kit; Fermentas) to remove extra 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 AB1377-96 automated sequencer (Applied Biosystems) according to the manufacturer's instructions.

DNA analysis

Sequences were edited using Bioedit software (Hall, 1999) and aligned with ClustaX1.8 (Larkin *et al.*, 2007). Manual adjustment of sequence alignments was performed to accommodate insertions/deletions. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-4439.4317) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4140). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 632 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). The sequence of *Pythium aphanidermatum* AF271227 (obtained from GenBank) was used as outgroup. The GenBank accession numbers of *Phytophthora* species used for phylogenetic studies are listed in Table 2.

Results

Herbaceous plants

From annual herbaceous plants inoculated with isolates of *P. parsiana* only two isolates (PH21-4-92 and PH21-7-92), showed disease symptoms on red peppers after 20 days. Isolate PH21-4-92 was

Table 2. GenBank accession numbers of isolates of *Phytphthora* spp. used for phylogenetic studies.

Isolate	Species	Accession number
PH21-30-10[a]	*Phytophthora taxon* Walnut	KU577517[1]
PH21-34-12[a]	*Phytophthora parsiana*	KU577518[1]
PIS15	*Phytophthora pistaciae*	AF403506[2]
32F6	*Phytophthora melonis*	EU088256[3]
23J6	*Phytophthora drechsleri*	EU423315[4]
UQ881	*Phytophthora cinnamomi*	AF266764[5]
IMI296829	*Phytophthora syringae*	AF266803[5]
IMI180616	*Phytophthora heveae*	AF266770[5]
MD 9/2	*Phytophthora quercetorum*	DQ313223[6]
CBS678.85	*Phytophthora mirabilis*	AF266777[5]
IMI158964	*Phytophthora iranica*	AJ131987[5]
IMI133317	*Phytophthora megasperma*	AF266794[5]
P246b	*Phytophthora inundata*	AF266791[5]
IMI302303	*Phytophthora humicola*	AF266792[5]
52	*Phytophthora rosacearum*	EU925376[7]
JP-08-328	*Phytophthora taxon* Walnut	KJ405953[8]
P532	*Phytophthora* sp.	AF541910[9]
B164	*Phytophthora taxon* Walnut	KC291550[10]
NZFS310L	*Phytophthora fallax*	DQ297391[11]
NZFS310C	*Phytophthora captiosa*	DQ297402[11]
IMI288805	*Phytophthora insolita*	AF271222[5]
UASWS0198	*Phytophthora polonica*	DQ396410[12]
SUC7 (PH21-3-92 [a])	*Phytophthora parsiana*	AY659737[13]
SUC25 (PH21-5-90 [a])	*Phytophthora parsiana*	AY659739[13]
SUC19 (PH21-4-92 [a])	*Phytophthora parsiana*	AY659738[13]
Rf17 (PH21-7-92 [a])	*Phytophthora parsiana*	AY659741[13]
SURf6 (PH21-6-92 [a])	*Phytophthora parsiana*	AY659740[13]
SCRP237(PH21-2-05 [a])	*Phytophthora parsiana*	AY659736[13]
P21282	*Phytophthora parsiana*	GU594784[14]
1D12	*Phytophthora hydropathica*	EU583797[15]
44A9	*Phytophthora hydropathica*	EU583796[15]
44J1	*Phytophthora hydropathica*	EU583794[15]
GAL	*Phytophthora gallica*	DQ286726[16]
UQ2071	*Pythium aphanidermatum*	AF271227[5]

[1]Submitted in this study. [2]Mirabolfathy *et al.*, 2001. [3]Ho *et al.*, 2007. [4]Gallegly and Hong.,2008. [5]Cooke *et al.*, 2000. [6]Balci *et al.*,2008 [7]Hansen *et al.*,2009. [8]Parke *et al.*, 2014. [9]Brasier *et al.*,2003. [10]Ginetti *et al.*, 2014. [11]Dick *et al.*,2006. [12]Belbahri *et al.*,2006. [13]Mostowfizadeh–Ghalamfarsa *et al.*, 2008. [14]Unpubl. data (Coffey,M.D., Brar,A.K., Xu,E. and Zhang,Y.H.).[15]Hong *et al.*, 2010.[16]Jung *et al.*,2008.[a] Plant Protection Department of Shiraz University code.

more aggressive than the other isolate. Other isolates were not pathogenic to herbaceous plants. The pathogen was re-isolated from symptomatic tissue. All isolates in the pot monitored during the experiment were active with the presence of zoospores. To discriminate isolates of *P. parsiana*, sixteen isolates were inoculated to red and bell peppers cultivars. Isolates PH21-4-92 and PH21-7-92 caused mortality of 33 and 22% in cultivar Anheim and 44 and 22% in cultivar Casabel respectively but could not infect bell pepper. Other isolates of *P. parsiana* failed to infect peppers.

Molecular assays

Two isolates of *Phytophthora* (PH21-30-10 and PH21-34-12) were amplified using the primers ITS6 and ITS4. An amplicon of about 700 bp was obtained for two isolates of *Phytophthora*. BlASTn searches in GenBank showed that ITS sequences of PH21-30-10 and PH21-34-12 isolates had 99-100% identity with isolates of *Phytophthora* taxon Walnut (GenBank KJ405953, AF541910, KC291550) and *Phytophthora parsiana* (GenBank AY659738, GU594784), respectively (Figure 1).

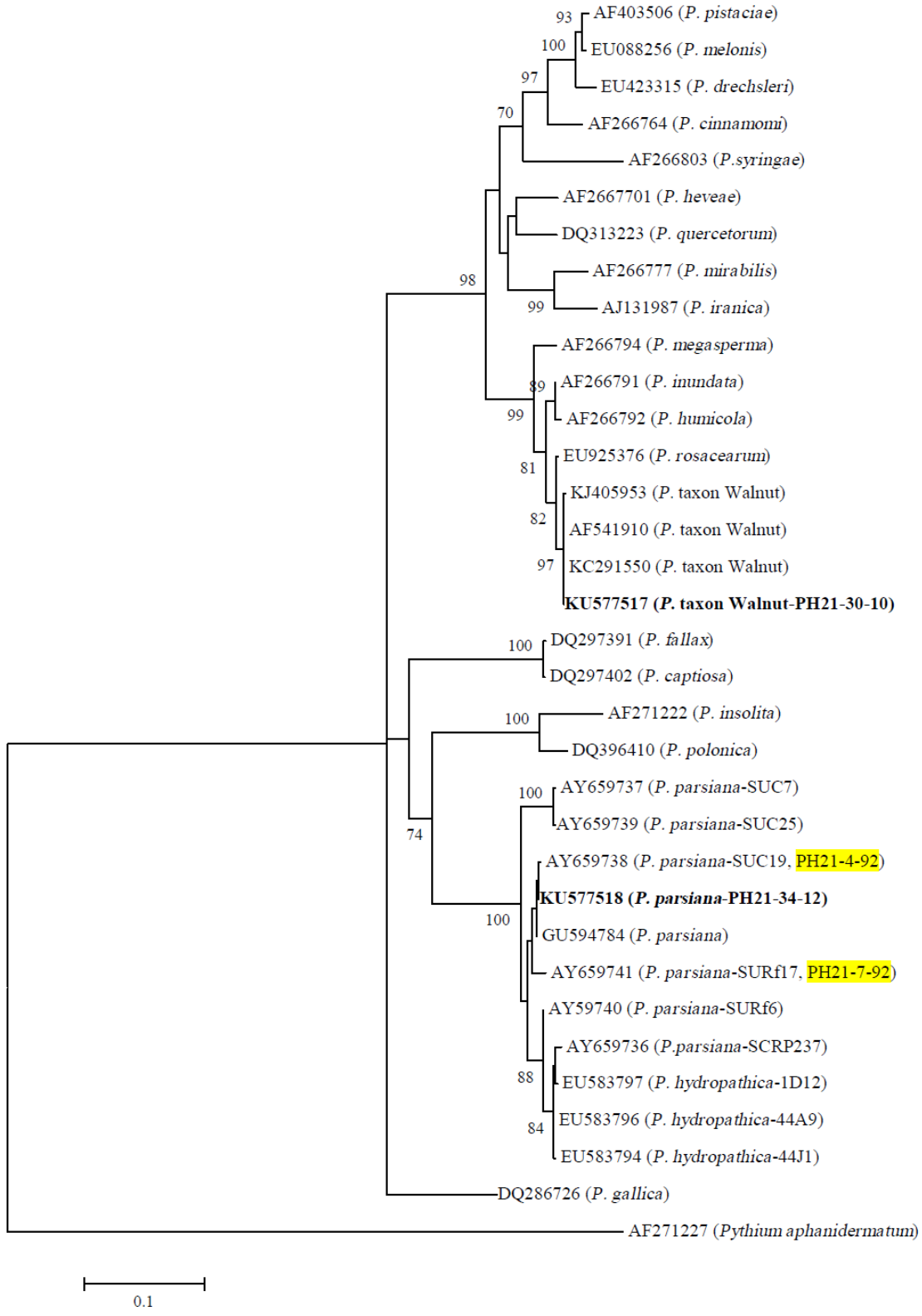

Figure1. Phylogram of maximum likelihood analysis of the *Phytophthora parsiana* and *Phytophthora* taxon Walnut isolates examined in this study (indicated in bold), together with 20 *Phytophthora* species based on the rDNA ITS region. The numbers at the branch points indicate the percentages of the bootstrap values (≥70%).

Discussion

Among annual herbaceous plants only two isolates of *P. parsiana* from pistachio in Rafsanjan (PH21-4-92 and PH21-7-92) infected red pepper plants and PH21-4-92 was more aggressive. This is the first indication of an herbaceous plant as host for *P. parsiana*.

The two type cultures of *P. parsiana* from Bushehr in fig and stem canker of pistachio in USA along with the rest of the isolates used failed to infect red pepper and were limited to woody plants. An isolate of the pathogen from pistachio recovered from Kerman province (PH21-34-12), although related to PH21-4-92 and PH21-7-92 based on ITS sequencing (Figure 1), was not pathogenic to peppers. Hong *et al.* (2010) assumed the two latter isolates to represent new species.

An isolate of the pathogen from pistachio in Harat (PH21-30-10) in Yazd province morphologically similar to *P. parsiana* with high temperature tolerance, was different from *P. parsiana*; based on ITS sequencing, it belongs to clade 6 close to *P.* taxon Walnut (Figure 1) (Anvari, 2014; Anvari *et al.*, 2014). This is the first report of *P.* taxon Walnut in pistachio in Iran.

Clade 6 comprised five taxa, among them found in riparian ecosystem such as ponds, river and flooded situation with high temperature growth requirement. Among them few are pathogenic on plants such as *Salix* and *Olea* (Brasier *et al.*, 2003). Most of these species require high temperature for growth, and are sexually sterile. Some of the clade 6 isolates still need to be described. Taxon Walnut isolated from walnut in California belongs to clade 6, grows at 38°C, and is sexually sterile (Brasier *et al.*, 2003). *Phytophthora* isolates from walnut with dieback symptoms in Italy were high temperature-tolerant and, based on ITS sequencing, are also identified as taxon Walnut with 94% similarity. The pathogenic behavior of taxon Walnut in Italy is considered to be due to recent global warming for a taxon that otherwise behaved saprophytically (Ginett *et al.*, 2014).

The present study shows that the Harat isolate (PH21-30-10), although morphologically similar to *P. parsiana*, belonged to clade six close to *P.* taxon Walnut (Figure 1). With the limited number of isolates available from pistachio orchards in Iran it appears that under *P. parsiana* some different species are hidden. More detailed phylogenetic studies using more isolates are required to describe new species.

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