دانش بیماری‌شناسی گیاهی (شاپای ا: ۶۲۹۰-۲۵۸۸، شاپای چ: ۲۲۵۱-۹۲۷۰)
سال یازدهم، جلد ۲، بهار و تابستان ۱۴۰۱
Plant Pathology Science (eISSN:2588-6290, pISSN:2251-9270)
Vol. 11(2), 2022

Research Article

Phytophthora citricola as the causal agent of persimmon root rot in Fars province of Iran

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Received: 05.21.2022 Accepted: 09.03.2022
Ghaderi F, Hasehemi SAA (2022) *Phytophthora citricola* as the causal agent of persimmon root rot in Fars province of Iran. Plant Pathology Science 11(2): 1-10. Doi: 10.2982/PPS.11.2.1

Abstract

Introduction: *Phytophthora* species are a serious threat to plant products worldwide. Therefore, identifying them is the first step in finding a way to treat the disease. The aim of this study was to identify Phytophthora species causing root and crown rot of persimmon trees in Fars province. **Materials and methods:** Samples were taken from the crowns and roots of diseased persimmon trees, in the summer of 2018-2019. Infected root and crown tissues were cultured in CMA-PARPH medium. Isolates of *Phytophthora* species were purified by single spore method and morphological and molecular characteristics were used to identify them. **Results:** Six isolates were obtained from the roots of diseased persimmon trees and identified as *Phytophthora citricola* based on their morphological characteristics. Phylogenetic studies based on beta-tubulin (*βtub*) and 28S rDNA genes showed that all isolates (Iran-Pc1 to Iran-Pc6) were grouped into clade 2 with a validation scale of 100 and confirmed the identification of *P .citricola*. **Conclusion:** This is a new report of persimmon root and crown rot caused by *Phytophthora citricola* in Fars Province.

Keywords: Beta-tubulin, Gene, Persimmon, *Phytophthora*, 28S rDNA

مقاله پژوهشی

Phytophthora citricola عامل پوسیدگی ریشه خرمالو در استان فارس ایران

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قادری ف، هاشمی س.ع.ا (۱۴۰۱) *Phytophthora citricola* عامل پوسیدگی ریشه خرمالو در استان فارس ایران. دانش بیماری‌شناسی گیاهی ۱۱(۲):۱-۱۰. Doi: 10.2982/PPS.11.2.1

چکیده

مقدمه: گونه‌های *Phytophthora* یک تهدید جدی برای محصولهای گیاهی در دنیا هستند. بنابراین شناسایی آنها اولین قدم برای یافتن روش مدیریت بیماری است. هدف از این مطالعه شناسایی گونه *Phytophthora* عامل پوسیدگی ریشه و طوقه درختان خرمالو در استان فارس بود. **مواد و روش‌ها:**

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طوقه و ریشه درختان خرمالوی بیمار در تابستان ۱۳۹۹-۱۳۹۸، نمونه‌برداری شد. بافت‌های آلوده ریشه و طوقه در محیط CMA-PARPH کشت داده شدند. جدایه‌های گونه‌ی *Phytophthora* به روش تک اسپور خالص‌سازی شدند و برای شناسایی آنها، از ویژگی‌های ریخت‌شناسی و مولکولی استفاده گردید. **یافته‌ها:** شش جدایه از ریشه درختان خرمالوی بیمار به دست آمدند، که بر اساس ویژگی‌های ریخت‌شناسی *Phytophthora citricola* شناخته شدند. بررسی فیلوژنتیکی بر اساس ژن‌های بتاتوبولین و 28S rDNA، هر شش ایزوله (Iran-Pc1 to Iran-Pc6) در کلاد ۲ با مقیاس اعتبارسنجی ۱۰۰ گروه‌بندی گردیدند و گونه *P. citricola* را تأیید نمود. **نتیجه‌گیری:** این یک گزارش جدید از پوسیدگی ریشه و طوقه خرمالو توسط *Phytophthora citricola* در استان فارس می‌باشد.

واژگان کلیدی: بتاتوبولین، خرمالو، ژن، *Phytophthora*، 28S rDNA

Introduction مقدمه

Oriental persimmon (*Diospyros kaki* L.) is one of the traditional fruits and popular in many parts of the world for its dessert quality fruit. It is popular in Japan, Asia and South America (Lyon et al. 1995). Persimmon is as an export crop in Iran that produced about 7,320 tons (Ramin and Tabatabaie 2003).

Significant losses on persimmon recorded in worldwide caused by many factors including bacterial blast (*Pseudomonas syringae* pv. *syringae*), grey moulds (*Botrytis cinerea*), post-harvest rots (*Penicillium* spp.) (Kitagawa and Glucina 1984), *Colletotrichum acutatum* causing fruit rots, *Colletotrichum horiifrom* anthracnose lesions on persimmon fruit and twigs and basal root rot caused by *Cylindrocladium floridanum* (Weir and Johnston 2010).

There are reports of *Phytophthora* species causing root, crown and fruit rots on persimmon such as *Phytophthora citrophthora* on persimmon fruits in Argentina (Frezzi 1950). *P. diospyri* was recorded on persimmon (Mkervali 1990). *Phytophthora capsici* was identified as the causal agent of persimmon root and fruit rot in Italy (Farr and Rossman 2020). *Phytophthora cinnamomi*, *Phytophthora citricola* Sawada *and Phytophthora cactorum* (Lebert and Cohn) were caused root and fruit rot on persimmon in New Zealand (Tyson et al. 2014). In Iran, there are reports of isolating *Phytophthora citrophthora* from persimmon fruits in the north of Iran (Taheri et al. 2012, Ershad 2009).

Morphological characters cannot always provide all the necessary data to define a species. Today, molecular approaches have been solved many deficiencies of morphological and provided reliable methods for pathogen identification and disease diagnosis (Martin et al. 2014). The taxonomy of *Phytophthora* is almost complicated and unclear. Customarily the identification of the species of this genus was based on morphological and physiological criteria (Yang et al. 2017). So, Different parts of *Phytophthora* genome is used in molecular phylogeny-based taxonomy for morphological confirmation such as cytochrome oxidase gene (Jung et al. 2016, Ruano-Rosa et al. 2018), ITS-rDNA regions (Blair et al. 2008), 28S rDNA region (Yang et al. 2017), *βtub* gene (Maseko et al. 2007), the mitochondrial *cox1* and *NADH1* genes (Jung

et al. 2017), part of the nuclear heat shock protein 90 (HSP90) (Jung et al. 2017) and the cytochrome oxidase subunit 1 (*CO1*) gene (Puglisi et al. 2017).
We detected disease symptoms on persimmon trees, which were root and crown rot along with yellowing and wilting in Fars province of Iran, during the summer of 2019-2020. Nevertheless, no comprehensive survey on causal agents of root and crown rot of persimmon has been performed in Fars province. This research was conducted to determine the fungal pathogens causing root and crown rot on persimmon based on morphological characteristics and molecular confirmation using phylogenetic analyses of the DNA sequence data from *βtub* gene and 28S rDNA region.

Materials and Methods مواد و روشها

Fungal isolation and morphological identification

In summer 2019-2020, infected tissues from root and crown were collected and taken to the laboratory in polyethylene bags in coolers. Then, samples were washed under running tap water to remove soil particles, blotted dry with sterile paper towels, cut from margins of infected tissues (2–5 mm segments) without extra treatments and were transferred to corn meal agar-PARPH culture media [CMA: 40 mg/L ground corn extract and 15 g/L agar] amended with 10 µg/ml pimaricin, 200 µg/ ml ampicillin, 10 µg/ml rifampicin, and 25 µg/ml PCNB (Jeffers and Martin 1986). The plates were incubated at 25 °C and examined daily until the mycelial growth was observed.
Purification of *Phytophthora* isolates was carried out using the single-spore (zoospore) method on water agar (WA) medium (Ghaderi and Habibi 2021). The pure culture of isolates was used for morphological and molecular identification. Morphological studies included colony morphology on a variety of media such as CMA, malt extract agar (MEA), potato carrot agar (PCA), potato dextrose agar (PDA), and hemp seed agar (HSA), growth rate on different culture media, and growth temperatures (5, 8, 10, 15, 20, 25, 30, 35, 40), morphology of sporangium (elliptical, egg-shaped, inverted pear-shaped, lime-shaped, spheroid, filamentous), oogonium surface decorations (flat or decorated), the space between the oogonium and oospore walls (plerotic or aplerotic), the origin of antheridium (diclinous and monoclinous), connection type of antheridium to oogonium (paragynous or hypogynous), diameter of the hyphae, formation of hyphal swelling, and chlamydospore production, according to the taxonomic keys of the genus *Phytophthora* (Erwin and Ribeiro 1996, Stamps et al. 1990). Isolates were cultured on CMA slopes at 15 °C for long-term storage (Jeffers and Martin 1986).

Pathogenicity on detached branches

Three isolate, Iran-Pc1, Iran-Pc2 and Iran-Pc3, were randomly selected and tested for their ability to cause symptoms on detached persimmon branches. The detached stems from healthy and actively growing persimmon trees were collected from orchards in Fars province. The branches were trimmed to remove leaves and lateral shoots, and were cut to segments 20-25 cm long and 1–1.5 cm in diameter. The branches were washed and bark surfaces were disinfected by spraying with 70% ethanol. The detached branches were sealed at both ends with warm melted paraffin wax to reduce dehydration during the incubation period. Three T-shaped cuts were made in the bark with a scalpel. A 6 mm CMA plug from actively growing hyphae of isolate Iran-Pc1 was placed into the cut section, the bark replaced, and the inoculated part was wrapped with parafilm. A noninoculated CMA disk was used for inoculation of control detached branches. Inoculated and control branches were incubated in humid chambers (plastic containers,

with 100% relative humidity obtained by adding 250–300 ml water) at room temperature (Banihashemi and Ghaderi 2006, Ghaderi and Habibi 2021).

جدول ۱. جدایه‌های گونه‌های *Phytophthora* استفاده شده در آنالیز فیلوژنتیکی در این مطالعه.

Table 1. Isolates of *Phytophthora* species used in phylogenetic analyses in this study.

Species name	strain	Gen Bank accession no.	
		βtub	28S rDNA
Phytophthora citricola	**Iran-Pc1**	ON400508 a	MZ269504 a
P. citricola	**Iran-Pc2**	ON400509 a	MZ269505 a
P. citricola	**Iran-Pc3**	ON400510 a	MZ269506 a
P. citricola	**Iran-Pc4**	ON400511 a	MZ269507 a
P. citricola	**Iran-Pc5**	ON400512 a	MZ269508 a
P. citricola	**Iran-Pc6**	ON400513 a	MZ269509 a
P. himalsilva	61G3	KX250580	KX250584
P. occultans	65B9	KX250601	KX250605
P. terminalis	65B8	KX250608	KX250612
P. botryosa	46C2	KX250531	KX250535
P.colocasiae	35D3	KX250566	KX250570
P.meadii	61J9	KX250594	KX250598
P.inflate	P10341	EU080385	EU080389
P. citrophthora	03E5	KX250545	KX250549
P.mengei	42B2	KX250657	KX250661
P. siskiyouensis	41B7	KX250678	KX250682
P.tropcalis	22H5	KX250692	KX250696
P.mexicana	45G4	KX250671	KX250675
P.glovera	62B4	KX250650	KX250654
P.capsici	22F4	KX250636	KX250640
P.multivora	55C5	KX250776	KX250780
P.capensis	62C1	KX250727	KX250731
P.plurivora	61H1	KX250713	KX250717
P.pini	22F1	KX250804	KX250808
P.acerina	61H1	KX250713	KX250717
P. multivesiculata	30D4	KX250923	KX250927
P.elongata	33J4	KX250888	KX250892
P.bisheria	P10117	EU080742	EU080746
P.frigida	47G7	KX250909	KX250913
P.infestans	27A8	KX250475	KX250479
P. citricola	33H8	KX250748	KX250752

a Sequences were used in this study

DNA extraction, PCR, sequencing and phylogenetic analysis

For molecular confirmation of identification, genomic DNA was extracted using the CTAB method according to Murray and Thompson (1980). The primer pairs were used for DNA amplification including 28S rDNA region indicated in Blair et al. (2008). Protocol for DNA sequencing was described in detail in Kroon et al. 2004. PCR products were purified using a PCR purification kit (Fermentas, UK) and sequenced in both directions at Macrogen (Macrogen Inc., South Korea).

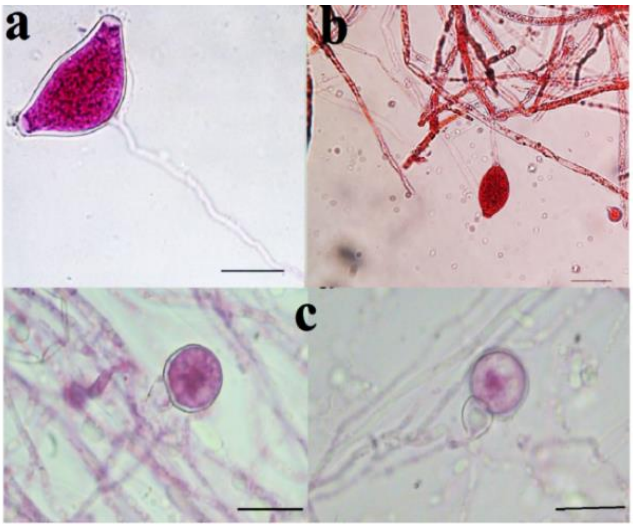
βtub and ITS-rDNA sequences generated in this study, compared to sequences of representative taxa of Clades 2 from GenBank (Table 1), were used in the phylogenetic analyses to determine the taxonomic status of the persimmon isolates. Sequences were edited manually wherever necessary and aligned by MAFFT v. 7 (Katoh and Standley 2013). The most appropriate model of sequence evolution was evaluated for each dataset with JModeltest v.2.1.4 using the Bayesian information criterion (BIC) for the following phylogenetic analyses. Phylogenetic Bayesian Inference (BI) analyses were performed using MrBayes v3.2.2 (Ronquist et al. 2012). *βtub* and 28S rDNA sequences were run for 10,000,000 generations, with a burn-in fraction set to 0.25. Maximum parsimony (MP) phylogenies were estimated using heuristic searches in PAUP v. 4.0a133 (Swofford 2002) with Bootstrap analysis of 1000 replicates to test the support of the branches. An isolate of *P. infestans* was used as an outgroup to root phylogenetic trees for members of *Phytophthora* clade 2. The resulting trees were viewed and edited in FigTree v. 1.4.0.

Results یافته‌ها

Morphological identification

The main disease symptoms were usually decline, foliar yellowing, branch dieback, and eventually tree mortality. In total, six isolates of *Phytophthora* spp. were obtained from diseased trees. Based on morphological characters, isolates belonged to *Phytophthora citricola* that were found on persimmon in Fars province. Phytophthora species were efficiently recovered in during summer.

Phytophthora citricola isolates formed sporangia abundantly on liquid medium, absent or rare on solid media, terminal, ovoid to pyriform, semipapillate, some with two papillae, wide and flat, non-caducous, 21–44 × 30–75 µm. Sporangiophores were long and slender with swelling, mainly at the point of branching, branching was irregular, not regularly sympodial, up to 3 µm wide. All *Phytophthora citricola* isolates were



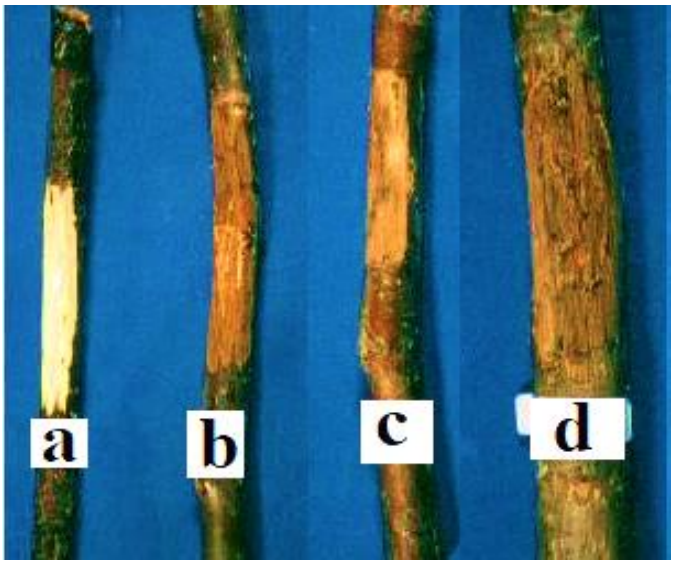
شکل ۱. *Phytophthora citricola*: (a-b) اسپورانجیوم پاپیل دار، (c) ااگونیوم کروی با آنتریدیدیوم پاراژینوس.

Figure 1. *Phytophthora citricola*: (a-b) papillate sporangia, (c) globose oogonium with paragynous antheridium. Scale bar = 10 µm

homothallic and were able to produced oospores abundantly in single culture. Oospores were almost plerotic, spherical, 16–30 µm in diameter. Oogonia were globose, smooth, 18–35 µm in diameter with paragynous antheridia (Fig. 1a-c). No chlamydospores were seen for any of the isolates. Hyphae were hyaline without hyphal swellings with 6µm wide. The maximum, optimum, and minimum temperatures for colony growth on CMA were 30, 25, and 8 °C, respectively.

Pathogenicity

Three isolate, Iran-Pc1, Iran-Pc2 and Iran-Pc3 were pathogenic on detached branches of persimmon and caused necrotic lesions 3–4 weeks after inoculation. In general, infected branches showed necrotic lesions on the bark that extended into the wood. No lesions were observed in noninoculated control branches. The causal agents were reisolated from the lesions but not from controls. Koch's postulates were completed and confirmed that *Phytophthora citricola* was responsible for persimmon root and crown rots (Fig. 2).

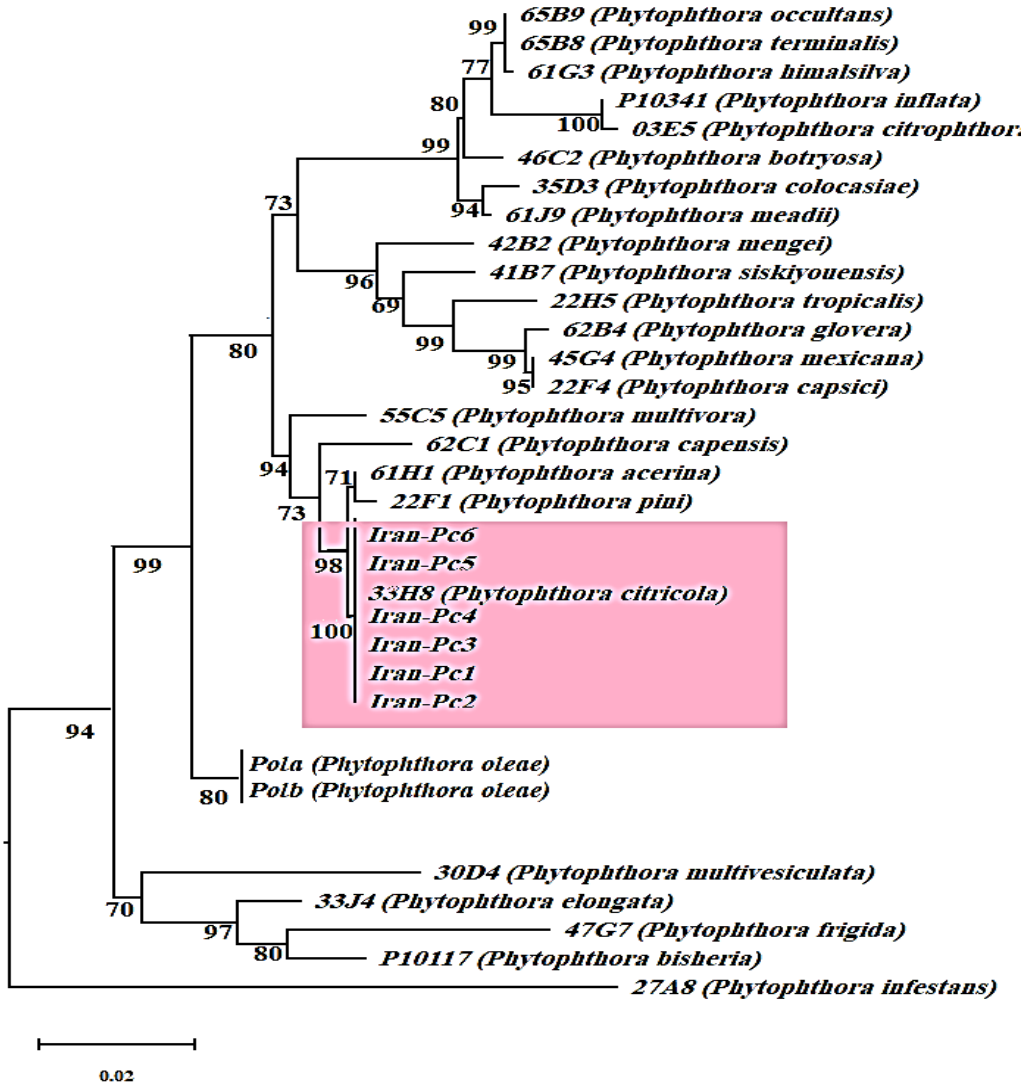


شکل ۲. شاخه‌های بریده از درخت خرمالو، ۳ تا ۴ هفته بعد از مایه‌زنی با *Phytophthora citricola*، لکه‌های نکروتیک را نشان می‌دهند: a) شاهد، b,c,d) به ترتیب سه ایزوله Iran-pc1، Iran-Pc2 و Iran-Pc3 از گونه *Phytophthora citricola*

Figure 2. Detached branches of persimmon trees showing necrotic lesions of the inner bark after 3-4 weeks after inoculation with *Phytophthora citricola*: (a) control; (b,c,d) Three isolates, Iran-Pc1, Iran-Pc2 and Iran-Pc3 of *Phytophthora citricola* respectively.

Phylogenetic analysis

PCR amplification and sequencing were successful for six isolates. The obtained sequences of *Phytophthora* isolates were submitted to GenBank under the following accession numbers: MZ269504 to MZ269509 for 28S rDNA, ON400508 to ON400513 for β-tubulin (Table 1). Phylogenetic analysis displays the phylogenetic position of six isolates including Iran-Pc1 to Iran-Pc6 using a Bayesian analysis of combined data set of 28S rDNA and *βtub* sequences. These isolates resided in clade 2 of *Phytophthora* and grouped with *P. cirtricola* in a well-supported clade (posterior probability = 100) (Fig. 3).



شکل ۳. درخت فیلوژنیکی (Bayesian inference) کلاد ۲ از فیتوفتورا بر اساس دو ژن بتاتوبولین و 28S rDNA. مقدار اعتبارسنجی در زیر شاخه‌های درخت نمایش داده شده است. از گونه *Phytophthora infestans* به عنوان خارج گروه استفاده شد.

Figure 3. Phylogram derived from Bayesian inference analysis of *βtub* and 28S rDNA data set of clade 2 of *Phytophthora*. Bayesian posterior probabilities (in%) are showed under the branches. *P. infestans* is used as outgroup.

Discussion بحث

Phytophthora species represent an important threat to agricultural crops, forestry, and ecosystems in the world; thus, the detection and distinction of *Phytophthora* species is essential in disease management (Martin et al. 2014). The main goal of this study was to identify *Phytophthora* species causing persimmon root rots in Fars province of Iran. In this survey, *P. citricola* are reported as a causal agent of root rot on persimmon trees. To the best of our knowledge, this is the first report of these species causing persimmon root rot in Iran.

Until now, species of *Phytophthora* reported to infect persimmon root and crown were *P. citrophthora* from clade 2a (Frezzi, 1950, Taheri et al. 2012, Ershad 2009), *P. diospyri* (Mkervali 1990), *P. capsici* from clade 2 (Farr and Rossman 2020). *P. cinnamomi* from clade 7c and *P. cactorum* from clade 1a (Tyson et al. 2014).
Morphological and phylogenetic analyses of the DNA sequence data of *βtub* and 28S rDNA allowed identification of one species from clade 2 of *Phytophthora*, *P. ciricola* which this species is an important pathogenic species with wide host ranges in worldwide (Kroon et al. 2012).
During morphological analyses, isolates Iran-Pc1 to Iran-Pc6 were identified as *P. citricola*. This species morphologically resembles other species such as *P. oleae*; however, it can be clearly separated from other *Phytophthora* species on the basis of 28S rDNA and β-tubulin sequences (Ruano-Rosa et al. 2018). Our results are consistent with Hansen and Maxwell (1991) who reported that some *Phytophthora* species are not distinguishable with morphological characteristics, it is better to use a combination of both molecular and morphological features.
In reality, phylogenetic analysis of 28S rDNA region and *βtub* gene enabled the clustering of the *Phytophthora* taxon isolated from persimmon in Fars province within clade 2. This clade is one of the largest clades in the *Phytophthora* phylogeny and includes important pathogenic species such as *Phytophthora capsici, P. citricola, P. citrophthora, P. multivora, P. plurivora* and *P. tropicalis* (Kroon et al. 2012).

Conclusion نتیجه‌گیری

In general, morphological characterization and phylogenetic analyses (both nuclear and mitochondrial DNA regions) are necessary to identify *Phytophthora* species (Martin et al. 2014, Puglisi et al. 2017). The ITS-rDNA region may in some cases not be sufficient to differentiate closely related species characterized by identical or very similar ITS sequences (Schena and Cooke 2006) that corresponding to this context. Because, the combination of 28S rDNA with β-tubulin enables a precise and reliable identification.

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